Contraction-by-contraction \( \dot{V}_{O_2} \) and computer-controlled pump perfusion as novel techniques to study skeletal muscle metabolism in situ

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The study of metabolism in isolated, perfused muscles has a long history. Verzár (29), who employed the isolated cat gastrocnemius in metabolic investigations, cited such studies from the 1800s including those by Chauveau and Kaufmann on the levator labii superioris and masseter muscles of the horse. In 1926, Burn and Dale (1) performed experiments utilizing perfused hindlimbs of cats, dogs, and monkeys. Modern day use of the perfused rat hindquarter received its impetus from the work of Ruderman et al. (20) in 1971, while Spriet et al. (22) introduced the use of washed bovine erythrocytes in this preparation in 1985. Of particular interest in the present context is the isolated canine gastrocnemius muscle complex (gastrocnemius plus superficial digital flexor; GS) in situ. Use of this model has its modern origins in the classic studies of \( O_2 \) delivery and lactate exchange by Stainsby and colleagues (24, 25) in the 1960s. Recently (5–10), this model has been used to study the control of \( O_2 \) uptake at the onset of exercise (\( \dot{V}_{O_2} \) on-kinetics). Currently, investigators debate whether \( \dot{V}_{O_2} \) on-kinetics is primarily controlled by \( O_2 \) delivery, factors intrinsic to the muscle itself such as the build-up of respiratory stimuli, or some combination of the two (4, 26).

The isolated canine GS has many distinct advantages over other models for elucidating muscle metabolic regulatory mechanisms. It is of sufficient size for vascular isolation, which allows for one arterial inflow and one venous outflow. This vascular isolation enables the precise control of blood flow (and thus \( O_2 \) delivery), administration of various metabolic stimuli and inhibitors directly to the contracting muscle, and measurement of \( \dot{V}_{O_2} \) directly across the contracting muscle. This is particularly useful since experiments that measure pulmonary \( \dot{V}_{O_2} \) are confounded by factors such as motor unit recruitment (31), distribution of blood flow to working vs. nonworking muscles (21), and fiber type heterogeneities within multiple exercising muscles (31). On the opposite extreme, isolated myocytes present a homogeneous, contracting preparation (12), but \( \dot{O}_2 \) delivery kinetics are absent due to the lack of a blood supply.

\( \dot{V}_{O_2} \) across the GS is calculated via Fick’s principle:

\[
\dot{V}_{O_2} = Q \cdot C(a - v)O_2
\]

where \( Q \) is the blood flow and \( C(a-v)O_2 \) is the difference in \([O_2]\) between the arterial and venous blood. During experiments, arterial \([O_2]\) is typically maintained constant while blood flow is continuously measured via an inline venous flow probe. Originally, measurement of venous \([O_2]\) required manual sampling (e.g., Ref. 5). This method allowed for approximately five venous samples to be obtained during the first 30–45 s of contractions. This period of time is the key transitional period for assessment of \( \dot{V}_{O_2} \) on-kinetics in this model. Accordingly, a more rapid sampling technique should enhance the estimates of the parameter values of mathematical models and the assessment of \( \dot{V}_{O_2} \) at the onset of contractions.

Another key advantage of vascular isolation of the canine GS contracting in situ is the ability to control blood flow (and thus \( O_2 \) delivery) to the muscle via pump perfusion. In the past, controlled perfusion has been limited to simple manual changes from one constant-flow rate to another (5, 6, 8) based on maintaining a constant blood perfusion pressure. A method to precisely control and thus alter blood flow kinetics at the onset of contractions without altering resting or steady-state blood flow would not only increase our understanding of the role of \( O_2 \) delivery in \( \dot{V}_{O_2} \) on-kinetics but also expand the model to mimic...
Innovative Methodology

SKELETAL MUSCLE CONTRACTION-BY-CONTRACTION VO₂

Disease conditions that display slowed blood flow on-kinetics. Here, we present significant technical advances to the isolated canine GS preparation (in situ to 1) to rapidly sample venous O₂ saturation (SvO₂) to determine contraction-by-contraction VO₂, and 2) precisely control the rate and pattern of blood flow adjustment from one chosen steady-state blood flow (e.g., resting baseline) to another (e.g., contracting steady state).

METHODS

Animals. Adult mongrel hounds (Canis lupus familiaris) of both sexes were used. All procedures were approved by the Auburn University Institutional Care and Use Committee (PRN 2007-1185). Dogs had access to food and water ad libitum until 24 h before experimental use. At this time, food was removed from the animal to be used.

Anesthesia. Briefly, dogs were anesthetized with an intravenous injection of pentobarbital sodium (30 mg/kg) with maintenance doses as required to maintain a deep, surgical plane of anesthesia. Following anesthetization, animals were intubated with an endotracheal tube. A heating pad was placed under the animal and adjusted as needed to maintain the rectal temperature near 37°C.

Experimental Use. At this time, food was removed from the animal to be used.

Determination of contraction-by-contraction VO₂. Blood [O₂] (ml O₂/100 ml blood) was calculated as follows:

\[
\left\{ \left( \frac{1.39 \text{ ml O}_2}{\text{g Hb}} \right) \times (\text{g Hb/100 ml blood}) \right\} \times (\text{fractional SO}_2)\]

Due to its minor contribution and the inability to measure dissolved O₂ continuously on the venous side, dissolved O₂ was not included in the calculation of C(a-VO₂). SvO₂ was obtained continuously via an indwelling inline oximeter probe connected to an Oximetrix 3 meter. The Oximetrix 3 sampled SvO₂ at a rate of 244 samples/s, averaged the samples each second, and then gave an output of a 5-s rolling average each second. This output has a 90% response time of 5 s. Noise, high time resolution and time delay (TD), and a response specific to the Oximetrix 3 accompanied the recorded signal. For this reason, the SvO₂ recorded data were converted by sequential signal processing: filtering, deconvolution, and moving average to approximate the original raw data input signal. In particular, SvO₂ data were filtered by Butterworth’s method to attenuate the noise and reduce the high sampling time. After the filtering process, SvO₂ data were deconvolved (SVO₂) by a transfer function of the first-order system to account for the characteristic TD and time constant (τ) of the oximeter. Finally, the \( \frac{1}{\tau} \) signal was processed by a moving average second by second. Determination of the TD and τ values of the oximeter for deconvolution involved detecting a step decrease of SvO₂ using an experimental protocol whereby SvO₂ was imposed to decrease according to a square-wave function. The characteristic values of TD and τ of the oximeter were used in the transfer function of the first-order system to convert the recorded data by a deconvolution process.

Methods. Outputs from the pressure transducer and load cell (first through strain-gauge couplers), ultrasonic flowmeter (T206, Transonic Systems, Ithaca, NY), and indwelling inline oximeter probe (Opticath model no. U425C, size 4 F, Hospira, Lake Forest, IL) connected to an oximeter (Oximetrix 3, Abbott Laboratories, North Chicago, IL) were fed into a computerized data-acquisition system (Oxymon MKIII, Artinis Medical Systems BV, Zetten, The Netherlands). All signals were sampled at a rate of 125 Hz. The load cell reaches 90% of full response within 1 ms while the flowmeter was set at its highest pulsatile cutoff frequency (100 Hz). The load cell was calibrated with known weights before each experiment. The flowmeter was manually calibrated with a graduated cylinder and clock during and after each experiment.

Samples of arterial and venous blood were drawn anaerobically into 3-ml plastic syringes before and after each experimental trial. Venous blood samples were collected from the catheter draining the muscle and were used to calibrate the Oximetrix 3 signal. Blood samples were immediately capped, stored in ice water, and analyzed within 30 min of collection. Both arterial and venous blood samples were analyzed at 37°C for PO₂, PCO₂, and pH by a blood gas pH analyzer (GEM Premier 3000, Instrumentation Laboratory, Lexington, MA) and for [Hb] and SO₂ with a CO-Oximeter (682 CO-Oximeter, Instrumentation Laboratory, Lexington, MA) set for dog blood. These instruments were calibrated before and during each experiment.

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sampling rate of 125 Hz; \( \approx 188 \) samples per contraction cycle. To alleviate this issue, a Microsoft Office Excel macro was written in-house to determine the onset of each contraction cycle based on the rate of increase in force from one sample to the next. In most cases, a threshold increase of \( 80 \) N/s appropriately indicated the beginning of each contraction cycle. To verify the output from this method, force was plotted against time for every contraction cycle within a data set, and then visually inspected.

**Perfusion pump-controlled blood flow kinetics.** Blood flow from the right carotid or right femoral artery was directed to the left popliteal artery via tubing. This tubing was passed through a peristaltic pump (Minipuls 3 MP2/HF, Gilson) to allow for control of blood flow to the contracting GS. Computer software (706 developer’s kit, Gilson) and a program developed in-house were used to control the pump via an interface box (RS-232 to RS-485 converter; 508 box, Gilson). This program enabled full control of blood flow; not only with regard to the level of blood flow, but also for the rate and pattern of blood flow adjustment between flow levels, e.g., a monoexponential change in blood flow (with a user-chosen \( \tau \)) from a user-chosen baseline to a user-chosen steady-state blood flow level for contractions. In this manner, if desired, only the rate of blood flow adjustment between metabolic rates can be altered while maintaining both resting and steady-state blood flows the same between different trials.

**Contraction protocols.** For determination of contraction-by-contraction \( \dot{V}O_2 \), the GS was stimulated to contract tetanically (supramaximal voltage, 8 V; 50 Hz, 0.2-ms pulse width; train duration 200 ms) at a rate of either 1 contraction/2 s, or 2 contractions/3 s. User-chosen perfusion pump-controlled blood flow kinetics were also examined under this contraction protocol.

**Analysis of \( \dot{V}O_2 \) and blood flow on-kinetics.** \( \dot{V}O_2 \) and blood flow on-kine-tics data were fitted with a monoexponential function of the type (7):

\[
y(t) = y_{\text{Bas}} + A \left[ 1 - e^{-(t-TD)\tau} \right]
\]

In this equation, \( y_{\text{Bas}} \) indicates the baseline value obtained at rest before contractions onset (typically the average of the previous 5 s before the onset of contractions), \( A \) indicates the amplitude between \( y_{\text{Bas}} \) and the asymptote of the primary component at the end of the contraction period, \( t \) is time, TD the time delay, and \( \tau \) the time constant of the function (time to achieve \( \approx 63\% \) of the monoexponential response between \( y_{\text{Bas}} \) and the primary asymptote). Mean response time (MRT) of the overall response was calculated by summing \( \tau \) and TD. The \( \dot{V}O_2 \) on-response includes an early divergence from the monoexponential rise, potentially due to 1) the muscle pump elevating blood flow, 2) some transit delay from the mitochondrial response to the corresponding signal in the venous blood, and/or 3) some other delay yet to be fully explained. Accordingly, the response was fit with an equation that included a TD.

Fitting was done via nonlinear least-squares procedures with OriginPro 7.5 (OriginLab, One Roundhouse Plaza, Northampton, MA). To determine the best fit, several criteria were used (19, 30). First, our principal concern was with the “primary response,” and our fitting window was varied accordingly. Second, because some “slow component” was apparent for the \( \dot{V}O_2 \), we allowed three variables (A, TD, and \( \tau \)) to “float” while fixing the baseline \( \dot{V}O_2 \) that was directly measured. However, for the blood flow fits, only two variables (TD and \( \tau \)) were allowed to “float” while baseline and steady-state flow were fixed (i.e., there was no slow component to the blood flow response). Third, several “windows” were examined to minimize the 95% confidence interval, residuals, and chi-square of the fit. To evaluate residuals, we aimed for both “flatness” during the transition as well as minimization of the sum of squared errors. The window that allowed for all of the above in concert with a stable \( \tau \) value was considered the “best fit.”

**RESULTS**

**Mathematical deconvolution of SO2.** Figure 1A illustrates a typical response of the Oximetrix 3 to a square wave decrease in \( \text{SO}_2 \) and the results of deconvolving the signal. The oximeter probe was plunged from a vial of blood with a greater \( \text{SO}_2 \) to a vial with a lesser \( \text{SO}_2 \). Additional repetitions of this experimental test were performed to estimate TD and \( \tau \) of the oximeter before deconvolution. The characteristic mean (\( \pm SD \)) Oximetrix 3 output values of TD and \( \tau \) were \( 2.1 \pm 2 \) s and \( 2.5 \pm 0.08 \) s, respectively. Once the characteristic response of the device (TD and \( \tau \)) in measuring \( \text{SV}_O_2 \) was obtained, the original \( \text{SV}_O_2 \) input to the Oximetrix was recovered by signal processing and deconvolution. An example of the application of this data processing to an actual experimental trial is shown in Fig. 1B.

**Contraction-by-contraction \( \dot{V}O_2 \).** Figure 2 depicts force (Fig. 2A), blood flow (Fig. 2B), venous \([O_2]\) (Fig. 2C), and the...
resulting \( \dot{V}O_2 \) (Fig. 2D) during the first four contractions following contractions onset. Blood flow and venous \([O_2]\) were averaged for each contraction cycle (as illustrated by the shaded boxes) and used to calculate \( \dot{V}O_2 \) via the Fick principle. D: the calculated contraction-by-contraction \( \dot{V}O_2 \) for each contraction.

Software-controlled pump perfusion. Figure 5 illustrates a contraction trial in which the blood flow was pump-controlled with a \( \tau \) of \( \approx 14 \text{s} \) (Fig. 5A); the corresponding contraction-by-contraction \( \dot{V}O_2 \) response is shown in Fig. 5B. Calculation of contraction-by-contraction \( \dot{V}O_2 \) during the on-transition also allowed the precise separation of primary and slow component \( \dot{V}O_2 \) responses (e.g., Figs. 4 and 5B). Figure 6A displays the ability of our pump-control method to rapidly adjust blood flow (\( \tau = 2.0 \text{s} \)) at the onset of contractions, and the corresponding contraction-by-contraction \( \dot{V}O_2 \) on-response is depicted in Fig. 6B. In contrast to rapid adjustment of muscle perfusion, Fig. 7A demonstrates blood flow on-kinetics with a \( \tau \) of over 60 s; the concomitant contraction-by-contraction \( \dot{V}O_2 \) on-response during this period is depicted in Fig. 7B. In these exemplary examples from separate animals, only the \( \tau \) of the blood flow on-kinetics was manipulated, while resting and steady-state blood flow were the same across trials.

Fig. 2. Graphic representation of the method for calculating contraction-by-contraction oxygen uptake (\( \dot{V}O_2 \)). A–D: data for the first 4 contraction cycles for force, blood flow, venous oxygen concentration ([O_2]), and \( \dot{V}O_2 \), respectively. With arterial [O_2] (not shown) maintained constant, blood flow (B) and venous [O_2] (C) were averaged for each contraction cycle (exemplified by the shaded boxes) and used to calculate \( \dot{V}O_2 \) via the Fick principle. D: the calculated contraction-by-contraction \( \dot{V}O_2 \) for each contraction.

Fig. 3. Comparison of the number of data samples obtained during the key transition period using manual sampling (A) vs. the new contraction-by-contraction method (B). Note that 5–8 times more data points are obtained with the new method. Data are normalized to the end of the depicted 50-s transition period.

Fig. 4. Contraction-by-contraction \( \dot{V}O_2 \) on-response for 120 s of contractions (2 contractions/3 s) during spontaneous adjustment of self-perfused blood flow.
DISCUSSION

Many metabolic studies do not directly assess blood flow or $\dot{V}O_2$ across a working muscle, instead relying on pulmonary $\dot{V}O_2$ measured at the mouth as a proxy for muscle $\dot{V}O_2$ and limb blood flow or even whole body cardiac output as a proxy for muscle blood flow. For mechanistic investigations, a more direct approach across a single muscle is preferred. However, sampling speed and the ability to manipulate the rate of blood flow responses without altering the resting or steady-state blood flows have limited previous investigations with this model and other similar models. These two limitations have been removed with the present techniques.

First, the use of an inline oximeter probe directly outside of the muscle enabled continuous measurement of $SvO_2$. When this probe was plunged instantly from a vial of blood at one $SvO_2$ to a vial containing blood at a lower $SvO_2$, the output had an average TD of $2.1 \pm 2$ s and an average $\tau$ of $2.5 \pm 0.08$ s. These data were used to devise a mathematical deconvolution routine that essentially eliminated the TD and improved the $\tau$ so that the oximeter signal could reliably follow extremely rapid changes in input as illustrated in Fig. 1A. In its present configuration, the oximeter and its probe should be able to easily track physiological changes in $SvO_2$ that have $\tau$ values within the range of about 4–6 s (roughly 3 times the oximeter/probe $\tau$). Since arterial [O$_2$] was maintained constant and muscle blood flow was continuously measured via an ultrasonic flow probe, $\dot{V}O_2$ was calculated on a contraction-by-contraction basis. To be clear, we are not the first group to devise rapid measures across contracting canine muscle. Mohrman and colleagues (16, 17) performed this type of sampling over 35 years ago but specific contraction-by-contraction data for a series of contractions were not presented or analyzed.

Second, in the present study, a computer program was developed to drive a peristaltic pump and thus offer considerable variability in the control of perfusion to the contracting muscle. As stated in the introduction, the isolated canine GS is ideally suited to investigate the role of blood flow and thus $O_2$ delivery in $\dot{V}O_2$ on-kinetics as well as other metabolic settings. However, until now, pump perfusion-controlled blood flow was essentially limited to changes in constant flow levels (5, 6, 8) and thus blood flow kinetics could not be used as an independent perturbation. Our computer-controlled perfusion pump allows for full control of blood flow levels, speed of transition kinetics between levels, and although not illustrated here, pattern of transition kinetics.
It should be noted that manual sampling of venous blood via syringe suction causes artifact to occur in the signal from the oximeter. The reason for the manifestation of this artifact is not clear. Accordingly, if the experiment of interest is designed to examine VO$_2$ responses and concentrations of substances in the venous blood (e.g., lactate, pyruvate, H$^+$) during the transition period, multiple bouts would need to be performed to acquire the necessary data. Alternatively, sampling will have to be arranged in a manner to allow collection via passive draining rather than suction. As currently configured, however, this method requires minimal manual sampling to maintain stability of the SvO$_2$ signal.

**Advantages to new technique.** Simply stated, this new technique provides many times the number of data points (typically 5–8 times as many) usually obtained with manual blood sampling. While we do not have simultaneous data for manual sampling and the new contraction-by-contraction technique to allow side by side fitting comparisons, we posit that more points obligatorily improve the confidence of the fits. When fitting data to our exponential equation, the 95% confidence interval for $\tau$ is equal to the prediction error multiplied by the appropriate $t$-statistic as determined by the degrees of freedom (DOF). The DOF is equal to the number of data values minus the number of “floating” variables (e.g., A, TD, and $\tau$). As an example, with manual sampling, there might be 8 data points within the first minute compared with 20–60 points with contraction-by-contraction measurement depending on the stimulation rate. After subtraction of “three” for the floating variables, this leaves 5 DOF for manual sampling compared with 17–57 for the new technique. In terms of the $t$-statistic to be multiplied by the error of prediction, this amounts to 2.571 vs. 2.023–2.086. This means that even if the errors of prediction were identical, there would be a 20% improvement in the confidence interval due to the greater number of points alone. Aside from any improvement in fitting, the contraction-by-contraction method also allows the possibility of different experimental protocols such as 1) only 1 min of contractions to focus on the primary $\tau$ and minimize fatigue effects, or 2) single contractions to study the muscle VO$_2$ response to a pulse of activity.

The method proposed here offers a new perspective to investigate muscle bioenergetics using an electrically induced contraction-by-contraction model. The more rapid sampling time of this technique may help to elucidate differences in the metabolic responses of voluntary vs. electrically induced muscle contractions (28). A better understanding of the regulation of the muscle metabolic response to different patterns of muscle contraction is relevant in clinical rehabilitation where combinations of electrical and voluntary stimulation are used to treat muscle atrophy related to immobilization (2, 23). Moreover, our new method allows muscle fatigue investigation at various contraction frequencies and durations combining measurement of the force and estimates of the O$_2$ cost of each contraction.

**Further, our computer-controlled perfusion pump enables the investigation of altered blood flow responses during contractions.** We are able to precisely control baseline and steady-state blood flows, as well as the rate and pattern of blood flow adjustment. For example, the software program allows any combination of changes in baseline blood flow, asymptotic (or steady state) blood flow, and the monoexponential $\tau$. There is also no limitation that would prohibit manipulation of the

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**Methodological limitations.** These new techniques require the use of a muscle/hindlimb/organ of sufficient size for vascular isolation. Additionally, vascular isolation needs to be done in a manner that allows the indwelling oximeter to be placed as close to the preparation as possible. Without proximate positioning, the transit time will be increased so much that the kinetics response will not be reliably followed. Sufficient blood volume is also a necessity as the indwelling oximeter must be enveloped in the preparation’s blood effluent to render a valid response. The vessel of choice for insertion of the oximeter must also be of adequate size such that spasm will not be induced, nor will the probe obstruct too much of the vessel lumen.

Dissolved O$_2$ is not measured in this method. Continuous measurement of the contribution of this component to the VO$_2$ would require a rapidly responding, indwelling PO$_2$ electrode. Alternatively, dissolved [O$_2$] might be estimated by continual information about pH, PCO$_2$, and temperature to be used in combination with a standard canine oxyhemoglobin dissociation curve to approximate the PO$_2$. Our calculations indicate that ignoring dissolved O$_2$ in the blood permits potential errors in VO$_2$ in the range of 3–5% maximum.

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**Fig. 7.** An example of our computer-controlled pump’s ability to slowly adjust blood flow at the onset of contractions (A). Blood flow was set to a user-defined baseline, steady state, and $\tau$. The VO$_2$ on-kinetics response to this flow adjustment is shown in B.
blood flow according to some other mathematical pattern, e.g.,
double exponential or sigmoidal. This pump versatility signifi-
cantly broadens the use of this model. For example, aging (3, 11)
and various disease states (13, 15, 18) alter the blood flow
response during exercise. Now, the effect of altering only the
kinetics of blood flow/O2 delivery (independently of resting
and steady-state levels) can be specifically investigated.

Certainly, these flow alterations have an effect on perfusion
pressure to the muscle. For example, the rapid increase in flow
\((\tau = 2 \, s)\) illustrated in Fig. 6A resulted in an increase in
average perfusion pressure from \(\sim 110 \, \text{mmHg}\) at rest to \(\sim 200 \, \text{mmHg}\)
during contractions. On the other hand, the slow in-
crease in flow \((\tau = 63.3 \, s)\) illustrated in Fig. 7A induced a
decline in average perfusion pressure from \(\sim 120 \, \text{mmHg}\)
pre-contractions to \(\sim 85 \, \text{mmHg}\) at 20 s of contractions before
pressure returned to 120 mmHg. These pressure changes do not
negate the primary effect of altered O2 delivery due to flow
changes elicited by the pump manipulation. Investigators also
have the option of administering pharmacological agents, e.g.,
adenosine, to maintain perfusion pressure near normal levels.
With pump control, there is also a pulsatility factor that is not
identical to the ordinary pulsatility of the cardiac cycle. How-
ever, the major pulsatile agent during contractions is the
contraction force itself, and this effect is largely the same with
either spontaneous or pump-controlled perfusion.

Future improvement. In the present study, we are calculating
whole muscle \(\dot{V}O_2\), which should be clearly distinguished from
instantaneous end-capillary \(\dot{V}O_2\) and actual mitochondrial \(\dot{V}O_2\)
in the process of oxidative phosphorylation \((14, 16, 17)\).
Previous studies in isolated hearts (e.g., Ref. 27), the afore-
mentioned reports from the Sparks laboratory \((16, 17)\) on
canine muscle, and our work with the GS \((14)\) have attempted to
estimate the time course of mitochondrial \(\dot{V}O_2\) on the basis of
estimates of vascular transit times, tissue diffusion times,
and mathematical modeling. In the future, data of the type
reported in the present trials should be combined with the
approaches just noted to estimate more closely the mitochon-
drial metabolic activation time in vivo.

Conclusion. We have developed a new method to measure
\(\dot{V}O_2\) on a contraction-by-contraction basis. This method re-
quires that blood flow and \(SV_{\dot{O}_2}\) are rapidly and continuously
sampled while arterial \([O_2]\) is maintained constant. The recent
addition of an inline oximeter and mathematical deconvolution
of its signal allows for rapid \(SV_{\dot{O}_2}\) sampling. Acquiring
contraction-by-contraction \(\dot{V}O_2\) dramatically increases the amount
of data available for curve fitting to estimate the kinetics of the
underlying physiological processes and therefore the ability to
find differences in the effects of interventions. The addition of
a computer-controlled perfusion pump expands our model’s
ability to address questions related to blood flow. Overall, these
new techniques provide an enhanced means by which the
control of oxidative metabolism during metabolic transitions
can be mechanistically explored.

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