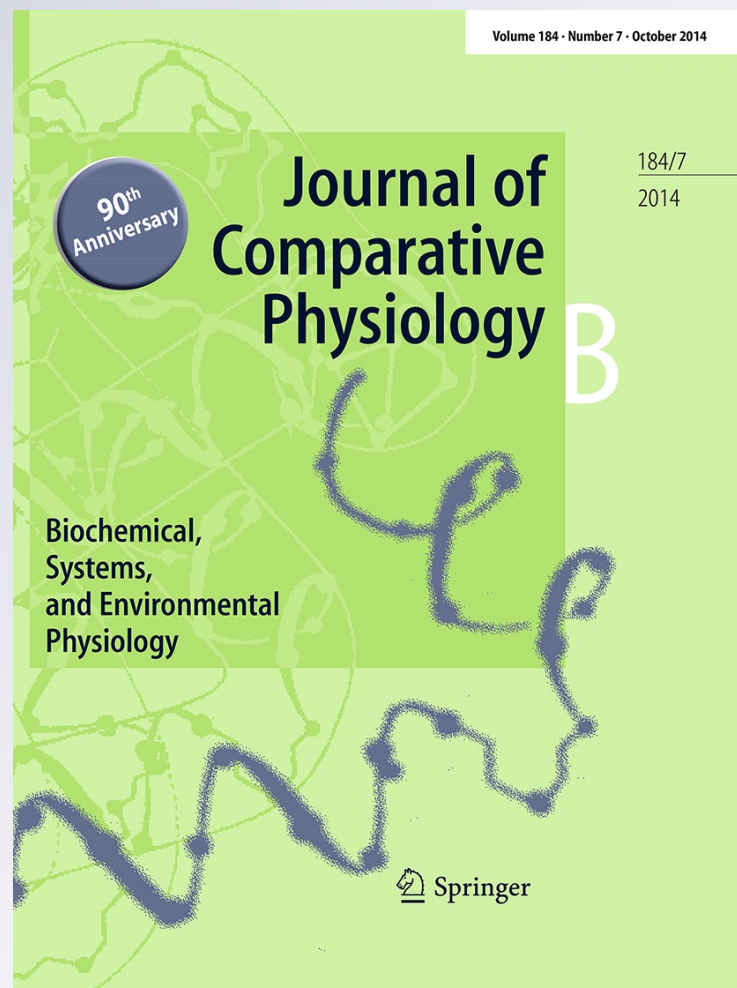# *Oxygen removal from water versus arterial oxygen delivery: calibrating the Fick equation in Pacific salmon*

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Oxygen removal from water versus arterial oxygen delivery: calibrating the Fick equation in Pacific salmon

**Anthony P. Farrell · Erika J. Eliason ·
Timothy D. Clark · Maria F. Steinhausen**

Abstract While it is well known that O_2 is directly removed from the water by skin and gill tissues of fish, the mismatch between O_2 removal from water (O_2 uptake; $\dot{V}O_2$) and the O_2 delivered to tissues by the primary circulation (O_2 consumption; $\dot{V}aO_2$) has never been measured directly. Using data from four recent studies that simultaneously measured $\dot{V}O_2$ and $\dot{V}aO_2$ in 2–5 kg Pacific salmon, our analysis revealed that sockeye salmon can remove an additional 12–48 % more O_2 from the water than the primary circulation delivers to the systemic tissues. This percentage did not change significantly during swimming activity, a result that contradicts an earlier prediction that the difference should decrease when $\dot{V}O_2$ increases during exercise. In resting Chinook salmon, a similar percentage difference in simultaneously measured $\dot{V}O_2$ and $\dot{V}O_2$ was observed, yet the difference tended to disappear during acute heat stress to a near lethal temperature. These results emphasize that caution should be exercised when using the Fick equation to estimate cardiac output because the overestimate of cardiac output that results from using the Fick equation in Pacific salmon is not small, may not be fixed and may exist in other teleosts.

Abbreviations

CaO_2	Arterial blood O_2 content
CvO_2	Venous blood O_2 content
f_H	Heart rate
T_{opt}	Optimal temperature for aerobic scope
V_s	Cardiac stroke volume
$\dot{V}b$	Cardiac output; rate of blood flow in ventral aorta $= f_H \times V_s$
$\dot{V}O_2$	Rate of O_2 removal from water by the fish
$\dot{V}aO_2$	Rate of O_2 delivery to tissues by the primary circulation $= \dot{V}b \times (CaO_2\text{-}CvO_2)$
$\dot{V}b\text{-}cO_2$	Rate of branchial-cutaneous O_2 removal from water as calculated by $\dot{V}O_2 - \dot{V}aO_2$
U_{crit}	Critical swimming speed

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Introduction

The Fick equation (Eq. 1) is used to relate O_2 uptake of an animal ($\dot{V}O_2$) to tissue O_2 delivery by the circulatory system:

$$\dot{V}O_2 = \dot{V}b \times (CaO_2 - CvO_2) \tag{1}$$

where $\dot{V}b$ is cardiac output and $(CaO_2 - CvO_2)$ is the tissue O_2 extraction. Thus, simultaneous measurements of $\dot{V}O_2$ with arterial and venous blood O_2 concentrations (CaO_2 and CvO_2, respectively) can indirectly estimate $\dot{V}b$. Application of the Fick equation to estimate $\dot{V}b$ is an extremely valuable technique for very large and small animals

whenever direct blood flow measurements are either difficult or impossible. In fact, pioneer cardiorespiratory studies that predate today's blood flow measurement technologies had to estimate $\dot{V}b$ in fishes using the Fick equation. These $\dot{V}b$ values remain highly cited for exercising and hypoxic rainbow trout (*Oncorhynchus mykiss*) (Kiceniuk and Jones 1977; Randall et al. 1967), for hypoxic common carp (*Cyprinus carpio*) (Garey 1970), and for temperature effects on sockeye salmon (Davis 1968; Brett 1971) and winter flounder (*Pseudopleuronectes flesus*) (Cech et al. 1976).

However, because all of the O_2 removed from water by a fish does not necessarily get transported to tissues by arterial blood contained in the primary circulation, it is well known that the application of the Fick equation to fish may overestimate $\dot{V}b$. In fact, all larval fishes use their skin as the primary mode of gas exchange until their gills become fully developed (Rombough 1988; Rombough and Ure 1991). Moreover, even for fish as large as 0.5 kg, O_2 uptake from water into or across the skin of the posterior body is known to account for 6–35 % of resting $\dot{V}O_2$ (Kirsch and Nonnotte 1977; Nonnette and Kirsch 1978; Nonnette 1981; Steffensen et al. 1981; Steffensen and Lomholt 1985; reviewed in Glover et al. 2013). Interestingly, while absolute skin O_2 consumption rates were similar among three fish species, the relative contribution of skin O_2 consumption to routine $\dot{V}O_2$ varied considerably among species, accounting for 35 % of $\dot{V}O_2$ in the eel (*Anguilla anguilla* L.), 23 % in tench (*Tinca tinca* L.) and 14 % in rainbow trout (Kirsch and Nonnotte 1977). Gill tissues also consume O_2 directly from the water. In rainbow trout, for example, an average of 27 % of O_2 removed from water passing over the gills did not enter the arterial blood, varying widely (19–75 %) among individual fish (Daxboeck et al. 1982).

The traditional measurement of $\dot{V}O_2$ in a fish, quantified as O_2 removal from water, is actually the sum of the O_2 delivered to tissues by the fish's primary circulation ($\dot{V}aO_2$, which is $\dot{V}b \times (CaO_2 - CvO_2)$, as in the Fick equation), plus any O_2 removed directly from water by skin and gill epithelial tissues, which we collectively term here the total branchial-cutaneous O_2 uptake ($\dot{V}b\text{-}cO_2$). Thus, as previously pointed out, a modified Fick equation (Eq. 2) is needed for fishes (Thorarensen et al. 1996):

$$\dot{V}O_2 = \dot{V}aO_2 + \dot{V}b\text{-}cO_2 \qquad (2)$$

Consequently, without using the modified Fick equation, routine $\dot{V}b$ in rainbow trout might be overestimated by as much as 41 % (27 % for gills plus 14 % for skin; Daxboeck et al. 1982; Kirsch and Nonnotte 1977). Even so, it has been predicted that during aerobic swimming and when $\dot{V}O_2$ increases, relative $\dot{V}b\text{-}cO_2$ ($\dot{V}b\text{-}cO_2$ expressed as a percentage of $\dot{V}O_2$) may decrease because $\dot{V}b\text{-}cO_2$

might not increase to match the increase in $\dot{V}aO_2$. Thus, any overestimate of $\dot{V}b$ would be reduced by swimming activity (Thorarensen et al. 1996). Another prediction is that plasma skimming at the gills (plasma removal into the secondary circulation from the efferent filamental arteries of the primary circulation, which would reduce the amount of $\dot{V}b$ reaching systemic tissues and elevate the red blood cell concentration in dorsal aortic blood; Olson 1984) might negate some of the error in estimating $\dot{V}b$ with a non-modified Fick equation (Randall 1985). However, recent work has shown that red blood cell entry into the secondary circulation of glass catfish varies considerably according to the fish's metabolic state (Rummer et al. 2014). To date, neither prediction has been empirically tested because $\dot{V}b\text{-}cO_2$ has not been derived from direct, simultaneous measurements of $\dot{V}O_2$ and $\dot{V}aO_2$.

In view of this important information gap, the present study quantified the mismatch between $\dot{V}O_2$ and $\dot{V}aO_2$ by analyzing published data for two species of large (>2 kg) Pacific salmon (sockeye and Chinook; Steinhausen et al. 2008; Clark et al. 2008; Eliason et al. 2013a, b). Analytical errors were minimized because each study directly measured $\dot{V}O_2$ using classic respirometry and simultaneously combined a direct measurement of $\dot{V}b$ with blood samples taken pre-gill (ductus Cuvier or sinus venosus) and post-gill (dorsal aorta) to directly measure $\dot{V}aO_2$. Furthermore, because these studies examined resting and swimming states, as well as the effect of heat stress at supra-optimal temperatures, it was possible to explore for the first time the influence of non-resting states on relative $\dot{V}b\text{-}cO_2$.

Materials and methods

The Animal Care Committee of the University of British Columbia in accordance with the Canadian Council on Animal Care approved the studies used here. Two studies examined resting and exercising adult sockeye salmon (*Oncorhynchus nerka*) at an optimum temperature for aerobic scope (termed T_{opt} herein) (Steinhausen et al. 2008; Eliason et al. 2013b) and another study with sockeye salmon examined exercise at a supra-optimal temperature (Eliason et al. 2013a). The fourth study examined the effect of acute warming of resting adult Chinook salmon (*Oncorhynchus tshawytscha*) to a supra-optimal temperature (Clark et al. 2008). Because the original works contain all the detailed methodological information, only brief descriptions of the experimental protocols are provided here. The potential effect of different methodologies was minimized because the four studies used a similar and standard intermittent-closed respirometry methodology to measure $\dot{V}O_2$ and used the same Brett-type swim tunnel respirometers (Steffensen 1985; Farrell et al. 2003; Lee et al. 2003). Each

study checked and confirmed that the background oxygen removal from an empty respirometer filled with water was negligible. In addition, all four studies directly measured $\dot{V}b$ with a flow probe (Transonic Systems Inc., Ithaca, New York, USA) surgically placed on the ventral aorta. The arterial and venous blood samples were taken from the same sites (pre-gill = ductus Cuvier or sinus venosus and post-gill = dorsal aorta) to determine their O_2 content. All paired arterial and venous blood samples (0.3–0.8 mL per sample) were removed anerobically into heparinized syringes from each fish and replaced by fish saline (estimated to remove <10 % of the total blood volume in each study reported here). All blood samples were refrigerated immediately and analyzed within 1 h. Blood oxygen content was measured using a Tucker chamber (Tucker 1967) maintained at 37 °C. All measurements for all four studies reported here ($\dot{V}O_2$, $\dot{V}b$, CaO_2 and CvO_2) were taken simultaneously and, to maximize statistical power, were limited to individual fish for which these simultaneous measurements were made at all sampling points for a given test protocol (the original studies may have reported values for more fish, but these two criteria were not met).

Sockeye salmon resting and exercising up to 75 % of maximum at T_{opt}

Experiments were performed on five lower Adams River sockeye salmon (2.2–2.9 kg) that had been intercepted before they entered the Fraser River and held at the Centre for Aquaculture and Environmental Research, West Vancouver, British Columbia in freshwater (initially 14.5 °C but declining seasonally to 12.0 °C; see Steinhausen et al. 2008 for details). Each fish recovered from surgery overnight (~10–12 h) in a swim tunnel, which was warmed to 15 °C (~T_{opt} for aerobic scope for this population) at a rate of 1 °C h^{-1} with a nominal water velocity of 0.20 m s^{-1} [~0.4 body lengths s^{-1} (BL s^{-1})]. At this modest water velocity, salmon maintained station into the water flow without any tail beats. Routine $\dot{V}O_2$ and $\dot{V}b$ were measured over a 30–45 min period, at the end of which arterial and venous blood samples were taken to measure CaO_2 and CvO_2. The water velocity in the swim tunnel was first increased by 0.1 m s^{-1} every 2 min up to a final velocity of 0.85 m s^{-1} (~1.35 BL s^{-1}), a velocity that could be sustained for at least 4 h and approximated 75 % of the critical swimming velocity (U_{crit}). After 30 min at the sustained swimming velocity, the cardiorespiratory variables were re-measured as in resting fish. The transonic flow probes were factory calibrated to 15 °C.

Sockeye salmon resting and exercising at and above T_{opt}

Experiments were performed on adult Fraser River sockeye salmon (1.8–3.2 kg) that had been intercepted 2–4 days after they had entered the Fraser River and were held in freshwater at 11–12 °C at the Cultus Lake Salmon Research Laboratory, Fisheries and Oceans Canada, British Columbia. The data analyzed here [7 fish tested at their T_{opt} for aerobic scope (15–20 °C), plus 8 fish tested at a supra-optimal temperature (22–24 °C)] are a subset from >100 fish that had been tested over a larger temperature range (Eliason et al. 2011, 2013a, b). Each fish recovered from surgery overnight (~10–12 h) in a swim tunnel at a nominal water velocity of 0.20 m s^{-1} (~0.4 BL s^{-1}). Routine $\dot{V}O_2$ and $\dot{V}b$ were measured over a 30–45 min period, at the end of which arterial and venous blood samples were taken to measure CaO_2 and CvO_2. Each fish then performed a ramped-U_{crit} test to fatigue (Jain et al. 1997, 1998) before being recovered at ~0.4 BL s^{-1} for a 45-min period. Cardiorespiratory variables were measured over a period of 5–10 min at strategic times: during steady state swimming (~0.7 m s^{-1} or ~1.2 BL s^{-1}), during burst swimming (~1.1 m s^{-1} or ~1.8 BL s^{-1}), within 5 min of fatigue (fatigue), and after recovery (45-min recovery). A total of five paired arterial and venous blood samples were removed from each T_{opt} fish to measure O_2 content (estimated to remove <10 % of the total blood volume) and replaced by fish saline. Only two time points (rest and 1.2 BL s^{-1}) are reported for the fish tested at a supra-optimal temperature.

Resting Chinook salmon during acute warming

Four adult male Chinook salmon (2.1–5.4 kg body mass) were captured near their spawning site at the Chilliwack River Hatchery, British Columbia, Canada and held outdoors at a temperature of 13–14 °C for 1.5 weeks (see Clark et al. 2008 for details). The fish recovered from surgery overnight (>10 h) in one of two respirometer tubes at a nominal water velocity 0.2–0.3 m s^{-1} (0.3–0.4 BL s^{-1}) that ensured good water exchange without forcing the fish to swim. In the morning, routine cardiovascular variables were recorded for 1 h at 13 °C, with $\dot{V}O_2$ measurements and blood samples being taken during the final 20-min period. Afterwards, water temperature was increased by 4 °C over 1 h to 17 °C, which was maintained for a 1-h period while cardiorespiratory variables were re-measured. This protocol was repeated for 21 and 25 °C, with 25 °C being considered an extreme acute heat stress because it was near the acute lethal limit for Chinook salmon (Richter and Kolmes 2005). Following the three temperature treatments, water temperature was returned to 13 °C over a 1-h period before the cardiorespiratory variables were re-measured after recovery periods of 1- and 2-h at 13 °C. The flow probes were factory calibrated to 15 °C and corrected to the experimental temperature by calibrating each flow probe (see Fig. 2a).

Data analysis

$\dot{V}$b-cO_2 was calculated as the difference between $\dot{V}O_2$ and $\dot{V}aO_2$ and expressed as a relative value (relative $\dot{V}$b-cO_2) using $\left(1 - \left(\dot{V}aO_2/\dot{V}O_2\right) \times 100\right)$. All mean (±SEM) values were calculated from individual fish data. Each fish acted as its own control in determining significant effects. Eliason et al. (2013a, b) used two-way repeated measures ANOVA with Holm–Sidak post-hoc test to test whether $\dot{V}aO_2$ and $\dot{V}O_2$ differed and whether values differed from rest. A one-way repeated measures ANOVA with Holm–Sidak post-hoc test was used for multiple comparisons to test whether values differed from rest (for $\dot{V}$bcO_2 and relative $\dot{V}$bcO_2). The same statistical approaches were used to assess raw data for Chinook salmon taken from Clark et al. (2008). Steinhausen et al. (2008) used a paired t test. A two-way repeated measures ANOVA with Holm–Sidak post-hoc test was used to test for differences between fish swum at T_{opt} for aerobic scope and supra-optimal temperatures. Statistical significance was based on $P < 0.05$ except where critical values are indicated for multiple comparisons.

Results

Sockeye salmon resting and exercising up to 75 % of maximum at T_{opt}

At 15 °C, routine $\dot{V}O_2$ was 1.30 ± 0.22 mL O_2 min^{-1} kg^{-1} and $\dot{V}aO_2$ was 0.95 ± 0.24 mL O_2 min^{-1} kg^{-1}, which resulted in $\dot{V}$b-cO_2 being 0.35 ± 0.28 mL O_2 min^{-1} kg^{-1} and relative $\dot{V}$b-cO_2 being 22.6 ± 18.3 % (Table 1). $\dot{V}O_2$ increased over sixfold to 8.72 ± 1.10 mL O_2 min^{-1} kg^{-1} when sockeye salmon were swimming at a sustained velocity of ~75 % of U_{crit} (Table 1). In addition, $\dot{V}aO_2$ increased fivefold to 4.33 ± 0.49 mL O_2 min^{-1} kg^{-1}, which resulted in $\dot{V}$b-cO_2 increasing to 4.40 ± 1.00 mL O_2 min^{-1} kg^{-1}. The upwards trend in relative $\dot{V}$b-cO_2 did not reach statistical significance (Table 1).

Sockeye salmon resting and exercising at T_{opt}

Routine $\dot{V}O_2$ in sockeye salmon at T_{opt} was 1.89 ± 0.16 mL O_2 min^{-1} kg^{-1} and $\dot{V}aO_2$ was 1.61 ± 0.33 mL O_2 min^{-1} kg^{-1}, which resulted in $\dot{V}$b-cO_2 being 0.29 ± 0.28 mL O_2 min^{-1} kg^{-1} and relative $\dot{V}$b-cO_2 being 15 ± 15 % (Fig. 1a–c). These routine values were quite similar to those presented above. $\dot{V}O_2$ and $\dot{V}aO_2$ increased during burst swimming by approximately fourfold to 8.79 ± 0.73 mL O_2 min^{-1} kg^{-1} and 6.18 ± 1.06 mL O_2 min^{-1} kg^{-1}, respectively, while $\dot{V}$b-cO_2 increased approximately ninefold to 2.60 ± 0.61 mL O_2 min^{-1} kg^{-1} (Fig. 1). After a 45-min

Table 1 Oxygen uptake ($\dot{V}O_2$), oxygen consumed from the primary circulation by tissues ($\dot{V}aO_2$) and the difference between these two values ($\dot{V}$b-cO_2) for resting and swimming sockeye salmon at 15 °C ($N = 5$)

Measured rates	Rest at 15 °C ($N = 5$)	~70 % U_{crit} at 15 °C ($N = 5$)
$\dot{V}O_2$ (mL min^{-1} kg^{-1})	1.30 ± 0.22	8.72 ± 1.10*
$\dot{V}aO_2$ (mL min^{-1} kg^{-1})	0.95 ± 0.24	4.33 ± 0.49*
$\dot{V}$b-cO_2 (mL min^{-1} kg^{-1})	0.35 ± 0.28	4.40 ± 1.00*
Relative $\dot{V}$b-cO_2 (%)	22.6 ± 18.3	48.2 ± 6.1

$\dot{V}$b-cO_2 expressed as a percentage of $\dot{V}O_2$ is called relative $\dot{V}$b-cO_2. (Original data from Steinhausen et al. 2008)

Significant differences from rest are indicated by an asterisk * ($P < 0.05$; paired t test)

recovery period following exhaustive swimming, $\dot{V}O_2$ remained significantly elevated above the routine value, while $\dot{V}aO_2$ returned to the routine value (Fig. 1a).

Sockeye salmon resting and exercising above T_{opt}

As expected, sockeye salmon did not swim well at supra-optimal temperatures compared with the sockeye salmon tested at T_{opt}, but could still reach 1.2 BL s^{-1} before fatigue. $\dot{V}O_2$ and $\dot{V}aO_2$ approximately doubled, but $\dot{V}$b-cO_2 and relative $\dot{V}$b-cO_2 did not change significantly from routine levels. $\dot{V}O_2$ and $\dot{V}aO_2$ in fish at supra-optimal temperatures were significantly higher at rest and at 1.2 BL s^{-1} compared with values at T_{opt} (Fig. 1). In contrast, $\dot{V}$b-cO_2 and relative $\dot{V}$b-cO_2 were not significantly different at T_{opt} and at a supra-optimal temperature for fish performing at 1.2 BL s^{-1}.

Resting Chinook salmon during acute warming

At 13 °C, routine $\dot{V}O_2$ was 2.41 ± 0.72 mL O_2 min^{-1} kg^{-1} and $\dot{V}aO_2$ was 1.69 ± 0.97 mL O_2 min^{-1} kg^{-1}, which resulted in $\dot{V}$b-cO_2 being 0.72 ± 0.85 mL O_2 min^{-1} kg^{-1} and relative $\dot{V}$b-cO_2 being 33 ± 24 % (Fig. 2). With acute warming to 25 °C, $\dot{V}O_2$ increased 2.5-fold ($t = 3.711$; $P = 0.001$ with critical $P = 0.004$) and $\dot{V}aO_2$ increased 3.8-fold ($t = 4.193$; $P < 0.001$ with critical $P = 0.004$), while mean relative $\dot{V}$b-cO_2 tended to decrease to zero (Fig. 2c) because $\dot{V}$b-cO_2 remained unchanged or tended to decrease (Fig. 2b). Following a 2-h recovery from the acute heat stress at 13 °C, both $\dot{V}O_2$ and $\dot{V}aO_2$ were restored to levels similar to their initial 13 °C values and individual variability was reduced considerably (Fig. 2a). Similarly, $\dot{V}$b-cO_2 and relative $\dot{V}$b-cO_2 tended to increase to their initial values at 13 °C and with a much-reduced individual variability (Fig. 2b, c).
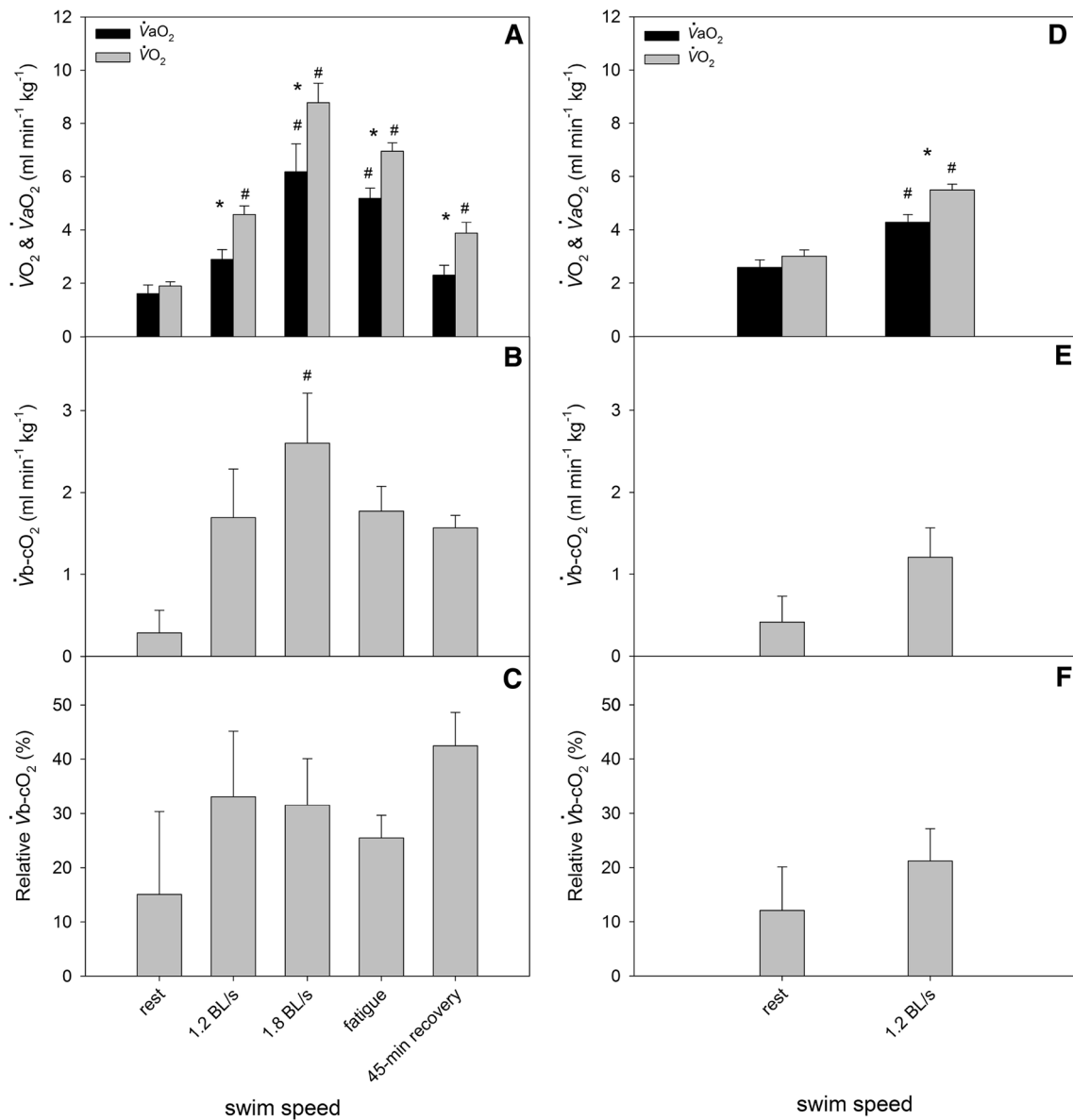
Fig. 1 a, d Rate of oxygen uptake from the water ($\dot{V}O_2$) and rate of oxygen consumption from the primary circulation ($\dot{V}aO_2$). **b, e** Rate of cutaneous oxygen consumption ($\dot{V}b\text{-}cO_2$) and **c, f** relative $\dot{V}b\text{-}cO_2$ $\left(1-\left(\dot{V}aO_2/\dot{V}O_2\right)\times 100\right)$ in adult sockeye salmon swimming to U_{crit} at their optimal temperature for aerobic scope (15−20 °C) in panels (**a, b, c**) and at their supra-optimal temperatures for aerobic scope (22–24 °C) in panels (**d, e, f**). Mean ± SEM are shown, original data from Eliason et al. 2013a, b. Significant differences from rest are indicated by the symbol *hash* and significant differences between $\dot{V}O_2$ and $\dot{V}aO_2$ are indicated by an *asterisk*

Discussion

The present study is the first to directly quantify the difference between the rate of the O_2 removal from water by a fish and the rate of the O_2 delivered to tissues by arterial blood contained in its primary circulation. In doing so, we revealed the extent and variability in relative $\dot{V}b\text{-}cO_2$ and in the Fick equation overestimation of $\dot{V}b$ (the percentage is the same for both). The overestimate of $\dot{V}b$ arises, of course, because the primary circulatory system delivers less O_2 to tissues than the fish removes from the water.

The four separate estimates made here for relative $\dot{V}b\text{-}cO_2$ in resting adult Pacific salmon show good agreement with each other (23 and 15 % for T_{opt}, and 12 % for $>T_{opt}$ with sockeye salmon and 29 % with Chinook salmon) and they concur with an earlier estimate of 27 % for just branchial O_2 consumption from water in rainbow trout (Daxboeck et al. 1982). In addition, $\dot{V}b\text{-}cO_2$ values (0.29, 0.35, 0.42 and 0.72 mL O_2 min^{-1} kg^{-1}) similarly concur
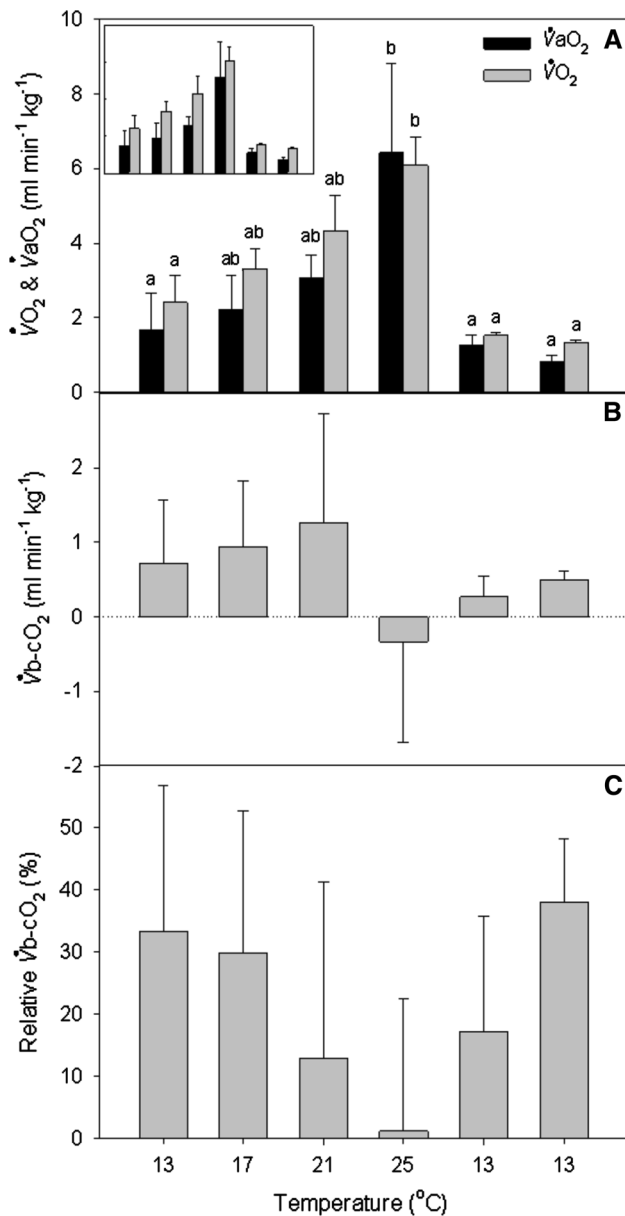
Fig. 2 Effects of an acute temperature challenge on **a** $\dot{V}O_2$ and $\dot{V}aO_2$, **b** $\dot{V}b$-cO_2 and **c** relative $\dot{V}b$-cO_2 in adult Chinook salmon (original data from Clark et al. 2008). *Inset* in **a** illustrates raw $\dot{V}O_2$ and $\dot{V}aO_2$ data prior to temperature-correcting the output from the Transonic blood flow probes. Significant differences within panel **a** are indicated by *dissimilar lowercase letters*, where critical *P* for post-hoc tests ranged from 0.003 to 0.004

with those reported for a wide variety of teleosts (0.75–1.50 mL O$_2$ min^{-1} kg^{-1}) (Kirsch and Nonnotte 1977; Nonnette and Kirsch 1978; Nonnette 1981). While these earlier studies suggested species with a low routine $\dot{V}O_2$ had a high relative skin $\dot{V}O_2$, relative $\dot{V}b$-cO_2 in resting Pacific salmon was consistently >15 %. Therefore, these findings invalidate an earlier prediction (Randall 1985) that plasma

skimming from efferent gill blood vessels might offset the error associated with estimating routine $\dot{V}b$ using the Fick equation.

Absolute $\dot{V}b$-cO_2 increased significantly during swimming, which was an unexpected discovery given the earlier prediction that relative $\dot{V}b$-cO_2 should decrease during swimming (Thorarensen et al. 1996). In fact, $\dot{V}b$-cO_2 increased in proportion with $\dot{V}O_2$ such that relative $\dot{V}b$-cO_2 was unchanged during swimming. Thus, any overestimate of routine $\dot{V}b$ using the Fick equation remains an overestimate when salmon swim. A potential explanation for this result could be that the unstirred boundary water layer next to the skin could limit O$_2$ diffusion into the skin in resting fish and this limitation becomes reduced or is removed as water moves at a faster velocity over the skin during swimming. Alternatively, the metabolic requirements of gill and skin tissues might increase during swimming.

The present study also revealed that absolute $\dot{V}b$-cO_2 was variable among individual fish and depended to some extent on the state of the fish. Such individual variability was not unexpected given that Daxboeck et al. (1982) reported that gill O$_2$ consumption from water in individual rainbow trout varied from 19 to 75 % of routine $\dot{V}O_2$. This high individual variability in $\dot{V}b$-cO_2 reduced the possibility of detecting significant changes, e.g., the tendency of $\dot{V}b$-cO_2 to decrease at 25 °C in Chinook salmon. While it is unclear whether this is true individual variability or a consequence of the summation of minor errors when measuring four variables independently, the individual variability for $\dot{V}b$-cO_2, $\dot{V}aO_2$ and $\dot{V}O_2$ all decreased following the heat stress as a resting state returned and the value of relative $\dot{V}b$-cO_2 was restored. Similarly, when Pacific salmon recover from exhaustive exercise, individual variability for $\dot{V}O_2$ becomes reduced (Wagner et al. 2006).

To the best of our knowledge, the studies used here are the only salmonid studies that allow a comparison of direct $\dot{V}b$ and indirect $\dot{V}b$ measurements. Here, we used exacting criteria to prevent bias, by only using simultaneous measurements for all fish in each study, which has rarely been the case in Fick estimates of $\dot{V}b$. As a result of the technical difficulty of meeting these criteria, we eliminated a number of fish and conditions that were tested in the original works. While this reduced the statistical power of the present analysis, we are confident in the quality of the results. For example, the swim tests in the sockeye salmon studies performed by Eliason et al. (2013b) were replicated after a 45-min recovery period and they yielded similar results to those presented here. However, the additional blood samples taken during the second swim test slightly diluted the blood because the blood samples were replaced with saline and hematocrit declined during the second swim test. Here, we adopted a criterion that hematocrit should not decrease

significantly for any of the test conditions despite the replacement of blood with saline in all four studies.

The only other fish study that we are aware of that allows a comparison of direct $\dot{V}b$ and indirect $\dot{V}b$ measurements was performed on the common eel (Hughes et al. 1982). In contrast with the present study, measured $\dot{V}b$ exceeded the Fick calculation of $\dot{V}b$ by 37 % in the eel. However, an important difference in the vascular arrangement of the eel gill compared with salmonids prevents a direct comparison of the underestimate of $\dot{V}b$ in the eel with the overestimate of $\dot{V}b$ demonstrated here for salmon. In the eel, unlike salmon, blood vessels on the afferent side of the gill filament allow a portion of $\dot{V}b$ to bypass the secondary lamellae. As a consequence, the portion of $\dot{V}b$ that bypasses the secondary lamellae and is not involved in branchial O_2 uptake will underestimate the Fick estimate of $\dot{V}b$ by a similar amount because the Fick calculation of $\dot{V}b$ assumes that the entire $\dot{V}b$ passes through the gas exchange surface.

Our results need to be closely inspected for any measurement error, particularly because $\dot{V}b$-cO_2 increased when the skeletal muscle O_2 demand was near maximal. All the measurement methodologies were standard and resulted in reasonable absolute values when compared with the literature (see original works). Calibrations were regular and thorough. In addition, we minimized any effect of individual variability and increased statistical power by limiting the analysis to data sets where the arterial and venous blood samples were taken at the same time and when all measurements were made for every fish. The logistical challenge of simultaneously sampling arterial and venous blood in swimming fish is neither trivial nor new. For example, a citation classic (Brett 1971) only estimated rather than measuring CvO_2. Likewise, Kiceniuk and Jones (1977) only reported simultaneous measurements of CaO_2 and CvO_2 from 3 to 5 fish during swimming, but compared these to routine values from 9 fish. In contrast, our experimental design was completely balanced.

To overestimate $\dot{V}b$-cO_2, we would have needed to underestimate $\dot{V}aO_2$, which would require some combination of either overestimating CvO_2 or underestimating $\dot{V}b$ and CaO_2. The $\dot{V}b$ measurements are regarded as highly reliable for several reasons. Foremost, Transonic flow probes are the gold standard for measuring absolute blood flow and each flow probe was individually calibrated at physiological flow rates over a relevant range of temperatures whenever the test temperature deviated from the factory calibration temperature. Moreover, as shown by the inset in Fig. 2a, a temperature differential of at least 10 °C would be needed to generate any appreciable error in $\dot{V}b$. Second, the temperature correction that we typically applied increased rather than decreased $\dot{V}aO_2$. Furthermore, we can discount the possibility that gill ventilation generated an underestimate of $\dot{V}b$ through flow probe movement.

If such a problem existed, it would have disappeared (and relative $\dot{V}b$-cO_2 would decrease) whenever salmon made the transition from active gill ventilation to ram ventilation during swimming. This did not occur. Conversely, a movement artifact would have been exacerbated (and relative $\dot{V}b$-cO_2 increased) during the deep gill ventilations associated with heat stress. Again, this was not the case.

Measuring CaO_2 and CvO_2 with a Tucker chamber is a well-established procedure and is not suspect. Yet, the possibility of O_2 exchange across the wall of the cannula cannot be ignored because polyethylene is not completely impermeable to gases. Also, to permit swimming as in previous cardiorespiratory studies (e.g., Davis 1968), the polyethylene cannulae used here were necessarily long (~1 m). While the possibility of O_2 being lost from the arterial cannula to ambient water (and overestimate $\dot{V}b$-cO_2) is negligible given that the ambient water O_2 partial pressure is greater than the arterial O_2 partial pressure, the possibility of O_2 gain into the venous cannula from ambient water is real. The ambient water O_2 partial pressure is considerably higher than the venous partial pressure of O_2, especially during swimming (up to 14 kPa). Of course, O_2 movement into venous cannula would result in an overestimate of $\dot{V}b$-cO_2 because of an underestimate of tissue O_2 extraction. To check for this possibility, we simulated such O_2 exchange by slowly drawing venous blood through a dummy cannula immersed in aerated water (data not shown). The small gain of O_2 during blood transit in this dummy cannula was then estimated to reduce relative $\dot{V}b$-cO_2 by about 6 % for a resting salmon. Thus, $\dot{V}b$-cO_2 and relative $\dot{V}b$-cO_2 would be increased by the same percentage during swimming. Since this error cannot be easily circumvented whenever long polyethylene venous cannula are used for studies of fish swimming, the problem should be acknowledged and investigated in future studies.

Another potential source of error in estimating $\dot{V}b$-cO_2 relates to the placement of the venous cannula, which preceded the heart. Thus, our calculation of $\dot{V}b$-cO_2 assumed myocardial O_2 consumption is negligible, which we know is not true. Myocardial O_2 consumption in fish has been estimated as about 2 % of resting $\dot{V}O_2$ and can increase to about 4 % with swimming (Farrell and Steffensen 1987; Farrell 1991). While this calculation error could be avoided by placing the venous cannula in the ventral aorta, the quality of the $\dot{V}b$ measurement in the same vessel might be degraded and introduce a new error. Therefore, we conclude that relative $\dot{V}b$-cO_2 was overestimated by about 2 % in resting fish and perhaps by as much as 10 % during swimming (6 % for the cannula and 4 % for the heart).

We propose another physiological explanation of why cutaneous tissue O_2 extraction might vary in fishes. We suggest that the secondary circulation to the skin plays an important role in altering $\dot{V}b$-cO_2. Fishes have a secondary

circulatory system with a similar blood volume compared to the primary circulation (Vogel 1985; Steffensen and Lomholt 1992). However, hematocrit (Hct) of the secondary circulation is much lower than the primary circulation probably because of the peculiar arrangement of arterial vascular connections between the primary and secondary vascular systems. These connecting vessels are typically tortuous and their openings are protected with 'hair-like' projections that preferentially allow plasma into, but exclude red blood cells from, the secondary circulation, hence the term plasma skimming. They are also vasoactive. A higher Hct and hemoglobin concentration ([Hb]) in the dorsal aorta compared with the ventral aorta of rainbow trout (Soivio et al. 1981) has provided evidence for plasma skimming in the gills, where an estimated 7 % of $\dot{V}b$ would have to have been diverted into secondary vessels to generate the Hct of 3.5 % (Ishimatsu et al. 1988). In vitro studies of saline-perfused gill have shown the capacity for very large movements of saline into the secondary circulation (e.g., 30–49 % of inflow; Olson 1984). (Note: none of the present studies detected a higher [Hb] in pregill versus post-gill blood samples.) Plasma skimming also occurs at numerous systemic connecting vessels near segmental arteries (Vogel 1985; Steffensen et al. 1986; Steffensen and Lomholt 1992). Recent work with glass catfish indicates that post-exercise recovery produced such a large movement of red blood cells into the secondary circulation that Hct was halved in the primary circulation (Rummer et al. 2014). Part of the secondary circulation is an elaborate vascular network on each scale (see schematic Fig. 3; see Satchell 1991) that normally carries 'blood' with low Hb and O_2 concentrations. Regardless, when compared with the skin tissue itself, the vascular network on scales provides a large surface area and reduced diffusion distance for gas and ion exchanges (Krogh 1904; Glover et al. 2013) with blood having a low Hct that would not appear bright red like the gills. We envisage that if O_2-depleted blood in the secondary circulation reaches the scales it could remove O_2 directly from the water and, theoretically, O_2 uptake could exceed local skin O_2 consumption, with oxygenated blood being returned into the primary circulation near the heart, which is where the secondary circulation reconnects with the primary circulation. As a result, $\dot{V}b$-cO_2 might vary whenever Hct and flow of the secondary circulation are regulated. Potentially, then, an increase in $\dot{V}b$-cO_2 (or maintaining $\dot{V}b$-cO_2 when skeletal muscle O_2 demand is elevated, as observed here during increased exercise and during recovery) could be related to an increase in the number or flow rate of red blood cells entering into the secondary circulation, perhaps in response to either elevated blood pressures or a β-adrenergic vasodilation mechanism. Conversely, $\dot{V}b$-cO_2 could decrease as a result of the secondary circulation closing down, perhaps through an α-adrenergic
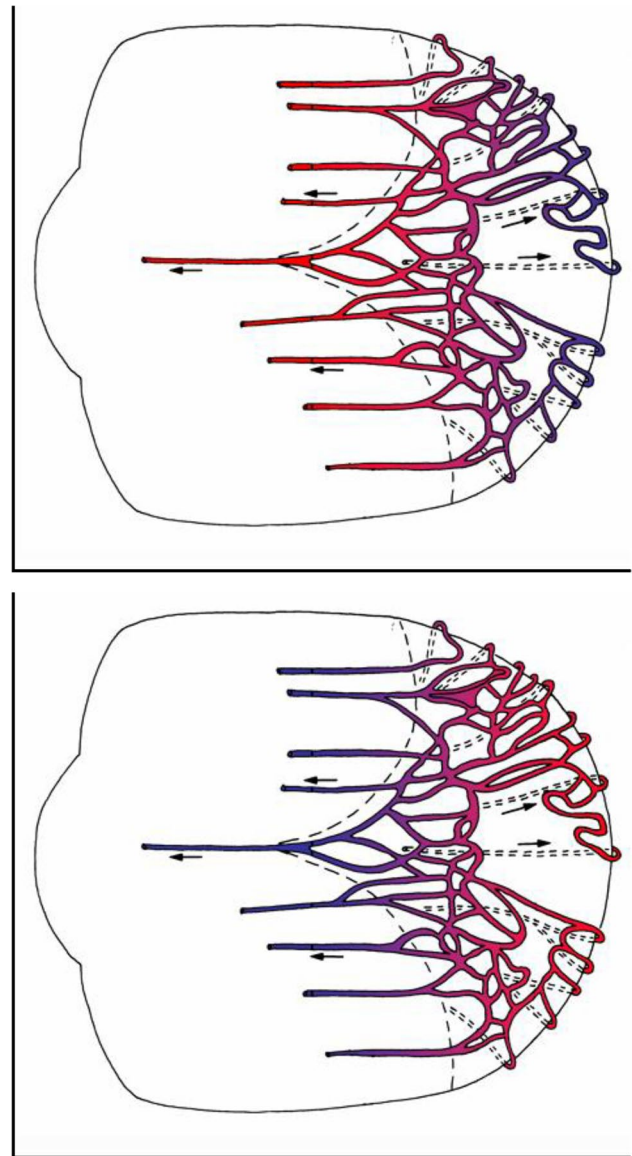

Fig. 3 A schematic diagram of the secondary circulation vessels found on the scales of fish (adapted from Fig. 23 in Satchell 1991) to illustrate the potential for oxygen uptake from (*upper*) and oxygen loss to the surrounding water. *Red color* denotes an oxygenated state and *blue color* denotes a deoxygenated state for the blood contained in secondary vessels. See text for more details

vasoconstriction mechanism and perhaps during extreme heat stress. While such mechanistic speculation requires empirical testing, a recent study with glass catfish (Rummer et al. 2014) adds weight to our suggestions. For example, both blood flow and the Hct in secondary vessels of glass catfish increased during recovery from hypoxia and exercise, so much so that Hct in the primary circulation was markedly reduced. Moreover, the β-agonist isoproterenol greatly increased red blood cell entry into secondary vessels, which supported the earlier suggestion by Rasmussen

et al. (2013) for rainbow trout that the connecting vessels can be actively regulated. How fish might lose O_2 to water (like two individual Chinook salmon did here at 25 °C) requires additional speculation. One possibility is that a high blood flow in the secondary circulation limits tissue O_2 removal before blood reaches the scales. Then, water leaving the gills during a high ventilatory effort and with a low partial pressure of O_2 might pass over scales containing blood with a higher partial pressure of O_2. Such an exchange most probably would be located just behind the operculum. Suppression of normal metabolic activities would also reduce $\dot{V}$b-cO_2, as shown previously for branchial O_2 consumption trout during hypoxia in rainbow trout (Daxboeck et al. 1982) and for skin O_2 uptake during severe hypoxia in the eel (Kirsch and Nonnotte 1977). Perhaps extreme heat stress creates severe tissue hypoxia at near lethal temperatures due to inadequate O_2 delivery by the primary circulatory system. Lactate production certainly occurs at supra-optimal temperatures (Farrell 2009; Steinhausen et al. 2008; Clark et al. 2008; Farrell et al. 2008; Farrell et al. 2009), but the sockeye salmon were not tested at such a severe temperature because they were still able to swim albeit at a moderated rate. Thus, while elevated and perhaps supra-optimal temperature can elevate local epidermal and gill epithelial O_2 consumption (as evidenced by increased activities of lysozyme and acid phosphatase in the skin of turbot; Huang et al. 2011) and increase absolute $\dot{V}$b-cO_2, hypoxic suppression of local epidermal and gill epithelial O_2 consumption at a near lethal temperature would tend to eliminate any mismatch between $\dot{V}$aO_2 and $\dot{V}O_2$. Moreover, suppression of epidermal metabolism may be a regulated response because the O_2 consumed by isolated skin patches exposed to hypoxia does not decrease by nearly as much as the skin uptake of hypoxic fish (see Figs. 5 and 7 in Kirsch and Nonnotte 1977).

In conclusion, the most important implication of the present in vivo work is the possibility that using an unmodified Fick equation overestimates resting $\dot{V}$b in Pacific salmon by about 25 %. This error, which was termed $\dot{V}$b-cO_2 here and was calculated as the difference between direct measurements of $\dot{V}O_2$ and $\dot{V}$aO_2, does not disappear when salmon swim. Therefore, caution is still needed when using previously published $\dot{V}$b data that predate direct $\dot{V}$b measurements. Clearly, more work is needed to better understand the relative and variable contributions by direct O_2 consumption of skin and gill tissues, and determine to what degree the present findings for salmon apply to other fish species. The importance of the secondary circulation in this regard certainly needs more attention.

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