Oxygen consumption rate *v.* rate of energy utilization of fishes: a comparison and brief history of the two measurements

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Accounting for energy use by fishes has been taking place for over 200 years. The original, and continuing gold standard for measuring energy use in terrestrial animals, is to account for the waste heat produced by all reactions of metabolism, a process referred to as direct calorimetry. Direct calorimetry is not easy or convenient in terrestrial animals and is extremely difficult in aquatic animals. Thus, the original and most subsequent measurements of metabolic activity in fishes have been measured *via* indirect calorimetry. Indirect calorimetry takes advantage of the fact that oxygen is consumed and carbon dioxide is produced during the catabolic conversion of foodstuffs or energy reserves to useful ATP energy. As measuring $[CO_2]$ in water is more challenging than measuring $[O_2]$, most indirect calorimetric studies on fishes have used the rate of O_2 consumption. To relate measurements of O_2 consumption back to actual energy usage requires knowledge of the substrate being oxidized. Many contemporary studies of O_2 consumption by fishes do not attempt to relate this measurement back to actual energy usage. Thus, the rate of oxygen consumption ($\dot{M}O_2$) has become a measurement in its own right that is not necessarily synonymous with metabolic rate. Because all extant fishes are obligate aerobes (many fishes engage in substantial net anaerobiosis, but all require oxygen to complete their life cycle), this discrepancy does not appear to be of great concern to the fish biology community, and reports of fish oxygen consumption, without being related to energy, have proliferated. Unfortunately, under some circumstances, these measures can be quite different from one another. A review of the methodological history of the two measurements and a look towards the future are included.

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WHAT IS METABOLISM?

Metabolism is the word used to describe the totality of energy consuming, manipulative and storage chemical reactions by organisms. The second law of thermodynamics dictates that all processes increase the amount of entropy (disorder) in the universe. Thus, a highly ordered entity like a fish can only exist with a constant input of energy that allows it to remain ordered. In the process of obtaining and assimilating that energy, the fish randomizes the rest of the universe more than the ordering of itself. This results in a net increase in the disorder of the universe and the possibility of fish life, but only with this constant energy input. Therefore, the initial requirement of fish survival is to obtain sufficient energy to offset this universal randomization process by, *e.g.* maintaining ion

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gradients and renewing proteins (Chabot *et al.*, 2016). This energy needs to be of two types, chemical bond energy and high-energy electrons (reducing power) that, along with some minerals and various macromolecules that fishes are unable to synthesize, constitute the minimum daily nutrient requirement for the fishes. Any excess energy assimilated can then be used to fuel activity, process meals and stored as polymers to fuel future periods without dietary intake or be used to do other work in an environment that will ensure the individual's continued ecological success. Continued excess acquisition of energy can then be variously channelled into growth and reproduction according to the species' life-history trajectory in a manner that maximizes Darwinian fitness. Thus, energy is the currency of life at all levels of biological organization, and accounting for it has been a major scientific endeavour for over 200 years.

A BRIEF HISTORY OF CALORIMETRY, FOCUSING ON FISHES

The study of metabolism began with human efforts to understand the nature of life itself. Antoine-Laurent Lavoisier, along with his student Pierre-Simon de Laplace, was the first to demonstrate with an ice calorimeter that animal respiration is essentially the controlled combustion of foodstuffs. They were able to build upon Scheele's & Priestley's recent discovery of the yet-to-be-named oxygen component of air, to deduce the true nature of animal respiration. Their conclusion at the dawn of animal respirometry can hardly be improved upon today: "Respiration is nothing but a slow combustion of carbon and hydrogen, similar in all respects to that of a lamp or a lighted candle, and from this point of view, animals which breathe are really combustible substances burning and consuming themselves" (Lavoisier & Laplace, 1783; translation from West, 2013). This was a radical departure from the prevailing phlogiston theory of combustion and required a stroke of true experimental genius. Lavoisier can also be credited with the discovery of indirect calorimetry or the tracking of respiration through the production or consumption of metabolic reaction components. He combined his understanding of the conservation of mass with chemical knowledge of mineral hydroxides that quantitatively absorb CO_2 gas to volumetrically measure oxygen consumption in animals, including humans. Unfortunately, the French revolutionary court executed him in 1794 before he could contribute further to the understanding of animal metabolism (Holmes, 1985). A less well-known fact is that Lavoisier can also be credited with the first quantification of the energy required for meal processing and assimilation [specific dynamic action (SDA) or thermal effect of food (TEF)], the elevation of metabolic rate by endotherms for thermoregulation in the cold and the increase in metabolic activity needed to support exercise (West, 2013). Lavoisier's exploits are well documented by historians of science, but too many metabolic physiologists are unaware of the depth of his contributions. Lavoisier was truly the Newton of animal metabolic studies.

The discovery of calorimetry, both direct and indirect, by Lavoisier in the late 18th century did not produce a surge of animal metabolic studies. In fact, it took almost a century until substantial numbers of animals, primarily domestic animals and livestock, began having their rates of energy use measured (Krogh, 1916). There were, however, some trickles of knowledge concerning fish metabolism in the early 19th century. Not long after Lavoisier's seminal work, Spallanzani (1803) observed that a fish out of water consumed oxygen and produced carbon dioxide. He also observed that

both living and dead fishes depleted the oxygen in the air in the headspace above the water they were in and that this depletion was more rapid in distilled than 'common' water; possibly the first measurement of the 'cost of osmoregulation' but, more likely, the first quantification of the metabolic cost of the fish stress response. Provençal & de Humboldt (1808) can be credited with the first quantification of oxygen consumption rate by fishes. They used eudiometry (the quantitation of oxygen in a gas volume through combustion with hydrogen) to measure the oxygen consumption of groups of tench *Tinca tinca* (L. 1758) over substantial periods of time (5–17 h). Unfortunately, Provençal & de Humboldt (1808) do not report the sizes of their animals, so there is no way to check the veracity of their measurements, and, as their respirometers were sealed, the water oxygen concentration fell quite low (as low as 3% saturation) and to different levels in each experimental trial. This would have initiated different metabolic responses to hypoxia in each trial and thus lowered the quality of the data. Provençal & de Humboldt (1808) recognized that body mass should be an important variable in determining metabolic rate, and they write that they will begin to account for it in their measurements; they even express a desire to relate oxygen consumption of *T. tinca* to heart mass and rate. Unfortunately, these latter experiments appear never to have been published. Interestingly, about this same time (1807), Biot (1807) used eudiometry to measure the oxygen concentration of fish swimbladders and obtained values consistent with modern measurements; he also demonstrated for the first time that fishes use oxygen for buoyancy regulation as well as for respiration. Unfortunately, the focus of most early-mid 19th century respiratory physiologists was to explain animal heat or to design better calorimeters and respirometers, primarily for use of humans (Frankenfield, 2010), so that studies of the metabolic physiology of ectotherms appear to have languished for a number of years.

By the late 19th century, a majority of physiologists were accepting the true nature of animal respiration that had been so poetically articulated by Lavoisier a hundred years earlier (Pflüger, 1873). The respiratory physiology of the late 19th century concentrated on two main issues: (1) understanding how metabolic rate changed with body size and (2) characterizing the nature of oxygen transport across epithelia. There was, in addition, some interest in how environmental factors influenced the respiratory exchange of gases (Krogh, 1916). The manometric techniques of the day were cumbersome, and working with aquatic organisms required extra steps of boiling or evacuating the water to analyse gases. Some investigators even used techniques that required gas analysis of both the water and air above it (Gréhant, 1886). These challenges may explain why most of the work from this period focused on terrestrial organisms, primarily endotherms. Direct calorimetry advanced steadily during the late 19th century with improvements in thermometer quality and increasingly sophisticated animal chambers. Indirect calorimetry still relied on manometric or gravimetric techniques (*e.g.* weighing the CO_2 absorbed by alkaline hydroxides) so, as yet, presented little advantage to direct calorimetry. Comparisons between direct and indirect calorimetry in terrestrial organisms also began in earnest at the turn of the 20th century (Rubner, 1894; Benedict & Milner, 1907).

Understanding how metabolism varied between species of different size and between different-sized animals of the same species was a leading motivation for metabolic studies at the turn of the 20th century, as it continues to be today (Krogh, 1916; Glazier, 2014). Lavoisier had already alluded to the potential for size to influence metabolic rate (West, 2013), and Sarrus & Rameaux (1837–1838) theoretically predicted the

non-proportional scaling of metabolic rate in endotherms, but it was not until 1883 that the first empirical test of metabolic scaling was concluded using different-sized domestic dogs *Canis lupus familiaris* and direct calorimetry (Rubner, 1883). Rubner's (1883) finding that metabolic rate scaled with body mass to the 2/3 power, or the same as the surface area to volume ratio of three dimensional objects, confirmed for many the 'surface rule' proposed by Sarrus & Rameaux (1837–1838) and led subsequent investigators to often express their metabolic rate measurements per unit surface area. This practice lingers today despite controversy over its validity almost from the outset (Benedict, 1915; Krogh, 1916). These late 19th to early 20th century debates on metabolic scaling took place mostly amongst physiologist studying endothermic homeotherms; fishes figured only tangentially into the discussions. Late 19th century measurements on different-sized gilthead sea bream *Sparus aurata* L. 1758 by Jolyet & Regnard (1877) and common carp *Cyprinus carpio* L. 1758 by Knauthe (1898) were not considered by Krogh (1916) to be of sufficient breadth or quality to form a conclusion concerning metabolic scaling in fishes. Similarly, measurements by Montuori (1913) (data reported in Krogh, 1916) on three different species of fishes of varied size yielded scaling coefficients of 0.928 for eight flathead grey mullet *Mugil cephalus* L. 1758, 1.15 for six common torpedo *Torpedo torpedo* (L. 1758) and 0.663 for five stargazers *Uranoscopus scaber* L. 1758. It is interesting to note that almost 100 years after Krogh (1916) summarized the metabolic scaling knowledge of the day with this statement: "It should be borne in mind that there is at present no valid reason for assuming a priori that the same rule should hold with regard to all cold-blooded animals", the same can be said today for different-sized fishes or even different-sized fish of the same species (Killen *et al.*, 2007, 2010; Yagi & Oikawa, 2014).

Fishes also factored into the other main respiratory controversy of the late 19th and early 20th centuries, namely epithelial oxygen transport. Bohr (1894) studied the gas composition of Atlantic cod *Gadus morhua* L. 1758 swimbladders and confirmed the earlier findings of Biot (1807) that oxygen concentrations in this organ could be exceedingly high, and, as they exceeded those of the blood and ambient water, Bohr (1894) concluded that they were in violation of Fick's law of diffusion and required an active oxygen secretory mechanism to be explained. Because methods for measuring blood oxygen content were imprecise in the late 19th century, Bohr and other physiologists (most notably Haldane) used this observation and their blood oxygen measurements in mammals to deduce that the mammalian lung was an active oxygen secretory organ. This view persisted for decades, even after Bohr's own student, August Krogh, and his wife Marie Krogh showed in a series of papers, that mammalian oxygen uptake could be explained entirely by diffusion alone (Schmidt-Nielsen, 1984). Although these papers by the Kroghs were seminal contributions to medical and mammalian physiology, Krogh's earlier experiments on the European eel *Anguilla anguilla* (L. 1758) (Krogh, 1904) possibly laid the foundation for their later refutation of the oxygen secretory hypothesis. Krogh (1904) began this paper by paying homage to his mentor Bohr and reaffirming the dogma of the day that the mammalian lung is an oxygen secretory organ, but then proceeded to demonstrate that *A. anguilla* could meet its entire respiratory needs *via* cutaneous diffusion (at low temperature). Krogh (1904) also measured the surface area of *A. anguilla* to calculate oxygen consumption and CO_2 release per unit surface area. From these, he concluded: "That the cutaneous respiration is governed solely by the difference of tension between the blood and the atmosphere and the permeability of the skin, in short, by purely physical features" (Krogh, 1904).

Interest in the influence of environmental variables such as gas concentration, temperature and water chemistry on the metabolism and gas exchange of aquatic organisms also began to arise at the end of the 19th century (Krogh, 1916). A number of investigators examined how changing temperature influenced the exchange of respiratory gases in fishes. Most of these measurements were sporadic and made under non-standard conditions, so that few generalizations could be made (Krogh, 1916). Lindstedt (1914), however, measured the metabolic rate of several fish species over a wide range of temperatures, and Ege & Krogh (1914) made a series of careful measurements on a single goldfish *Carassius auratus* (L. 1758), both resting and narcotized, at different temperatures. These measurements began to make it apparent that fish metabolic rate increased exponentially with temperature. Krogh (1916) deduced from the available measurements that metabolism in fishes was largely being governed by the rate of molecular motion and conformed to a Van't Hoff (Arrhenius) relationship. Krogh (1916) was also able to calculate from his and others' data that the Arrhenius coefficient (Q_{10}) was not constant across the temperature ranges that fishes live at. Interestingly, at this time, Krogh (1916) also proposed the concept of metabolic cold adaptation or the elevation of metabolic activity at low temperatures to compensate for reduced thermal molecular motion. This concept has not held up to experimental scrutiny (Steffensen, 2002; Nelson & Chabot, 2011) but was appealing enough to be held as true by many throughout the 20th century and is still motivating experiments (Guderley, 2011). The initial measurements of how aquatic hypoxia influences oxygen consumption of marine (Henze, 1910) and freshwater (Winterstein, 1908) fishes were also made around this time.

An event at the end of the 19th century that had a great effect on the future of fish respiratory physiology was the development of the Winkler titration (Winkler, 1888). The Winkler method converts manganese(II) hydroxide into manganese(III) hydroxide by quantitatively consuming dissolved oxygen. The manganese(III) hydroxide is then used to oxidize iodide ion, which can then be accurately measured by titration with thiosulphate. The simplicity and accuracy of this titration allowed indirect calorimetry to be performed without sophisticated gas analysis equipment. Thus, indirect calorimetry using Winkler titrations began to supplant manometric techniques and direct calorimetry when investigators measured aerobic metabolism of intact, aquatic animals. Krogh (1916) immediately recognized the utility of the Winkler titration and advocated its use for assessing the aerobic metabolic activity of aquatic ectotherms. Manometric techniques such as the Warburg (1908) manometric technique would continue to be used for measuring tissue respiration well into the late 20th century and Van Slyke's manometric technique for measuring total blood CO_2 (Van Slyke & Neill, 1924) would be adopted for measuring CO_2 output of aquatic animals and used widely. Manometric techniques also enjoyed a brief mid-20th century resurgence with the development of Cartesian diver technology for accurately measuring O_2 uptake in small animals and cells (Linderstrøm-Lang, 1937), but the Winkler titration would remain the preferred method for assessing whole-animal aquatic oxygen consumption well into the second half of the 20th century.

A further advance in the measurement of aquatic oxygen consumption came with the invention of the polarographic O_2 electrode (Clark, 1956). The Clark electrode has a silver–silver chloride anode half-cell separated from the solution of interest by an oxygen permeable membrane and a noble-metal cathode that requires an applied voltage. This invention allowed an investigator to measure pO_2 continuously in real time, obviated the need to take water samples for titration and made continuous-flow

respirometry more tractable. Coupled with advances in chart recorder technology and eventually computerization, these electrodes greatly expanded the duration and scope of possible aquatic oxygen consumption measurements (Clark *et al.*, 2013; Chabot *et al.*, 2016; Svendsen *et al.*, 2016). Commercialization of the Clark electrode enabled a dramatic increase in the number and types of laboratories engaged in the measurement of water pO_2 for the purpose of calculating oxygen consumption rate by aquatic organisms. These electrodes were relatively accurate and stable but required regular calibration and careful maintenance. Clark-type electrodes had some limitations because the membranes were sensitive to pressure and flow, and they could be compromised by bio-fouling or stretching. Clark-type electrodes also consume oxygen during the measuring process in proportion to their size, so improvements included miniaturization and pulsed circuitry that limited this. Galvanic oxygen-consuming probes were also developed shortly after the Clark-type electrodes that avoided the external polarization required for the Clark cell (Mackereth, 1964) and have found some use in aquatic respirometry. While the development of both the Winkler titration and pO_2 sensing electrodes facilitated indirect calorimetry of aquatic organisms and increased the number of laboratories engaged in this activity, another sea change in aquatic oxygen consumption measurement is underway. The development and commercialization of fluorescent optical oxygen sensors (optodes; Demas *et al.*, 1999) allow investigators unprecedented flexibility in the volume, location and dimensions of waters and chambers that can have their pO_2 determined (Clark *et al.*, 2013). These optodes consume no oxygen and are able to assess pO_2 in samples of micron dimensions (Chu *et al.*, 2011) making them a useful tool for measuring oxygen uptake of everything from isolated mitochondria to tunas.

Meanwhile, whatever happened to CO_2 in fish respirometry? As indirect calorimetry came under increased usage in the early 20th century, practitioners were well aware that having a measure of CO_2 production as well as O_2 consumption allowed the respiratory quotient to be calculated that, in turn, would indicate the nature of the organic substrate being oxidized and, thus, allow a more accurate assessment of the actual energy transformation taking place as well as providing information on diet. Investigators that use indirect calorimetry to gauge metabolic activity in terrestrial organisms routinely measure both O_2 consumption and CO_2 production, as do most metabolic biochemists. Why then have respiratory physiologists interested in the metabolism of water-breathing organisms virtually stopped measuring CO_2? The problem is that CO_2 is very soluble in water and the total CO_2 content can vary substantially with water chemistry. This results in a small signal-to-noise ratio that can change during the course of an experiment as the organism under study absorbs and releases molecules from the water. Krogh (1904, 1916) was well aware of this issue and used it to advocate for the preferential use of O_2 consumption with water-breathing animals. A number of technical advances have been made over the years, but the quantification of CO_2 in water remains challenging today, especially in seawater (Dickson *et al.*, 2007). Total CO_2 in the water can change due to factors besides respiratory gas exchange [*e.g.* acid–base imbalance (Heisler, 1984)], and methods for quantification can fall prey to water pH changes or organic molecules released by the organism (Pfeiffer *et al.*, 2011). Electrodes for measuring pCO_2 in solution were developed (Stow *et al.*, 1957) and steadily improved upon (Severinghaus, 1968), so that they became as accurate as pO_2 electrodes (Flenley *et al.*, 1967). The development of these electrodes did not offer the same efficiency advance over the manometric techniques of Van Slyke & Neill (1924) that pO_2 electrodes and

Winkler titrations had offered for the quantification of oxygen levels. Water $p\text{CO}_2$ could now be continuously and accurately assessed, but to measure total CO_2 in the water still required additional processing. This usually involved acidification of the water sample to titrate dissolved HCO_3^- and CO_3^{2-} with subsequent quantification of the evolved CO_2 gas. Investigators developed a variety of ways to do this, including measuring the evolved CO_2 with a $p\text{CO}_2$ electrode (Severinghaus, 1960; Cameron, 1971), by mass spectrometry (Bridges & Scheid, 1982), by gas chromatography (Boutilier *et al.*, 1985) including infrared detection (McKenzie & Randall, 1990) and *via* electrical conductivity changes after conversion to CO_3^{2-} (Maffly, 1968). None of these techniques were widely adopted by scientists trying to measure metabolic rates of aquatic organisms but were used extensively by investigators trying to assess acid–base relevant transfers of molecules between aquatic organisms and their medium (Heisler, 1984). Quantification of total CO_2 in the water was also by those trying to ascertain substrate utilization by aquatic organisms (Lauff & Wood, 1996; McKenzie *et al.*, 2007), but was not generally used as the variable of indirect calorimetry. Thus, the difficulty and uncertainty of measuring CO_2 in water has caused this measurement to virtually disappear from the landscape of metabolic rate studies on aquatic animals, despite calls for this not to happen (Fry, 1971). Perhaps the development of optical fibre sensors for CO_2 in water (Wolfbeis *et al.*, 1998) and their subsequent refinement (Chu *et al.*, 2011) will enable future generations of physiologists to incorporate more total CO_2 measurements into their indirect calorimetric studies of aquatic organisms.

MEASUREMENT OF METABOLISM IN FISHES

There are basically two energy currencies that fishes use to accommodate the two types of energy that they extract from their food. Chemical bond energy is generally transferred and stored on a short-term basis as phosphate bond energy, primarily ATP, while reducing power is primarily shuttled as electrons on nicotinamide adenine dinucleotide and its phosphorylated form (NAD and NADP). Long-term storage of both types of energy is as organic polymers, primarily tri-acyl glycerols (lipids) and glycogen in fishes. Just as modern economic activity is tracked as money spent, money earned and money invested, the ideal measure of metabolic rate would be to have a measure of this organismal ATP and NAD (P)/NAD (P) H cycling, while also accounting for any net macromolecular biosynthesis (storage). While there have been some advances in measuring ATP cycling *in vivo* with nuclear magnetic resonance imaging (Du *et al.*, 2008) and NADH–NAD cycling *in vivo via* fluorescence microscopy (Mayevsky & Rogatsky, 2007), these are still very specialized techniques that are unlikely to find their ways into routine aquatic metabolism measurements anytime soon.

Perhaps the simplest way to measure metabolic energy expenditure is to measure the total energy content of an animal before and after a time interval and use the total energy differential as the measure of metabolic energy expenditure during that time. Under certain specialized conditions such as non-feeding yolk-sac fry or animals that volitionally starve as part of their life history (*e.g.* migrating salmonids and aestivating fishes), this can be a very effective method. This technique is, however, unsuitable for the vast majority of experimental questions and comes with the following provisos: (1) obtaining an accurate measure of a fish's total energy requires sacrificing the fish; this, coupled with large intraspecific variability in energy content between individual

fish requires a large sample size; (2) the animals must be unfed, or the investigator needs to account for the energy content of all food consumed, excreta and tissue growth; (3) behavioural costs that vary on an individual level are generally unaccounted; depending upon experimental design, these can include costs of foraging, differences in personality and interactions with conspecifics and predators; (4) costs of food processing also vary on an individual level, including capturing or handling food and costs of digestion and assimilation. Points (3) and (4) are also common to other types of long-term or group metabolic rate measurements. The energy content technique is most attractive for unfed fishes, a non-standard physiological state for most. Measuring the metabolism of unfed fishes also might not be indicative of normal metabolism and there is the potential for individual variation in the response to fasting (*i.e.* a significant metabolic rate by fasting interaction). Despite these limitations, this technique can be used when others are inappropriate and produce useful results (Brett, 1973; Boggs & Kitchell, 1991). The energy content technique is not acceptable for estimating standard metabolic rate (SMR) (Chabot *et al.*, 2016).

A variant of the energy content technique is to account for all of the energy taken in by a fish and to measure the energy in all of the fish excreta. This method requires an accurate measure of food consumption, accurate collection of all excreta and assumes that there is no growth or change in storage metabolites of the fish and so is also impractical for most contingencies. Both of these techniques rely on the accurate bomb calorimetry of fish tissue and food that must then be related back to energetic expenditure by the fish, which can also be problematic (Gnaiger, 1983*a*).

Organismal energy transduction is thermodynamically inefficient. Thus, organisms are unable to extract work from some of the potential energy of food molecules that is then dissipated as heat energy and flows from the animal according to the physical laws of heat transfer. Therefore, metabolic activity can be tracked by measuring the sum heat dissipated from each metabolic transformation. Indeed, the first measurements of animal respiration by Lavoisier & Laplace (1783) were of heat production. Accounting for the heat produced by the aggregate enthalpy changes of all metabolic reactions is still the most thorough way to assess metabolism. Therefore, it is called direct calorimetry, despite the fact that the heat being measured is actually a by-product of metabolic reactions. An important distinction between direct and indirect calorimetry is that direct calorimetry is tracking the inefficiencies of all metabolic transformations, not just those consuming O_2 or producing CO_2. Direct calorimetry is still considered the most rigorous measure of metabolic activity (Kaiyala & Ramsay, 2011), but, if the animal under consideration is storing heat or synthesizing energy-rich compounds, it can still underestimate immediate metabolic activity. Likewise, if an animal is cooling or catabolizing long-term energy stores, immediate metabolic rate will be overestimated. Heat storage is generally not a concern for most aquatic ectotherms and experimental designs, and, if animals are measured post-prandially and followed for a sufficient period of time, net biosynthetic activity will produce minimal error (Hammarsten, 1915). Because reaction enthalpies are unique to each reaction, heat production can vary according to which metabolic pathways are active (Gnaiger, 1989). Direct calorimetry can also deliver erroneous results, if the net Gibbs-free energy change of the reactions being monitored (what one is interested in) is substantially different from the enthalpy change (what one is measuring; Gnaiger, 1989). Fortunately, these two state functions are usually closely matched in animal metabolism (Gnaiger, 1989). Of far greater concern to fish biologists is the high heat capacity of water. Coupled with the relatively low metabolic

activity of mostly ectothermic fishes results in a small signal-to-noise ratio for aquatic animals. Thus, few direct calorimetric measurements of fish metabolism are found over the years, starting at the very beginning. Indeed, the first measurements of fish metabolism by Provençal & de Humboldt (1808) were indirect. Throughout the years, some investigators have advocated for preferential use of direct calorimetric measurements for fishes (Smith *et al.*, 1978). Most likely because of the expense, the technical challenges and possibly also because some notable scientists in the field advised against it (Fry, 1971; Brett & Groves, 1979), there are few published studies of metabolic rate determined by direct calorimetry for fishes (Cech, 1990). Direct calorimetric studies of aquatic organisms surged slightly in the 1980s, fuelled by advances in calorimeter technology that allowed investigators to conduct sensitive measurements on small aquatic samples (van Waversveld *et al.*, 1988; Gnaiger *et al.*, 1989). These were used primarily for tissues, small and immobile animals, and to measure metabolism under anoxic conditions or other conditions where substantial anaerobic metabolism was suspected (Gnaiger, 1983*b*; van Waversveld *et al.*, 1988; Gnaiger *et al.*, 1989). Possibly because of the expense of this equipment, and because calorimeter construction is more challenging than respirometer construction, direct calorimetry still has not caught on for studies of intact fishes (Regan *et al.*, 2013). Direct calorimetry has, however, been extensively used for cellular and mitochondrial studies (Buck *et al.*, 1993; Min *et al.*, 1996). Direct calorimetry has also found use amongst those with an interest in larval fish energetics (Mäenpää *et al.*, 2004; McCollum *et al.*, 2006). Perhaps, as global climate change and anthropogenic activity increase the global extent of oxygen-deprived waters (Keeling *et al.*, 2010), more investigators will be interested in measuring the totality of fish metabolism, not just aerobic metabolism *via* indirect calorimetry (Regan *et al.*, 2013). As technology advances, there will inevitably be a resurgence of direct calorimetric studies of fishes, most likely coupled with indirect calorimetry and optodes to gain experimental inference. For the moment, when it comes to measuring metabolism of aquatic animals, indirect calorimetry, almost exclusively oxygen consumption, rules the day.

Indirect calorimetry takes advantage of the fact that substances are consumed or produced during the catabolic conversion of foodstuffs to organismal chemical bond energy and reducing power. Equation (1) depicts the complete aerobic respiration of an example foodstuff molecule, glucose with standard vertebrate stoichiometry:

$$C_6H_{12}O_6 + 36ADP + 36P_i + 36H^+ + 6O_2 \Rightarrow 6CO_2 + 36ATP + 42H_2O + Heat \quad (1)$$
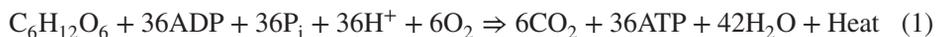
Both oxygen (O_2) and carbon dioxide (CO_2) are gases at the range of temperatures and pressures encountered by fishes on earth, and their stoichiometric consumption (O_2) or release (CO_2) can be monitored to gauge the rate of this reaction. Recall that the original indirect calorimetry of Lavoisier was actually volumetric; by quantitatively transforming the emanated CO_2 into a solid, he could estimate the oxygen consumed by air-breathing organisms through measuring the loss of respirometer volume. Because each major foodstuff (protein, fat or carbohydrate) produces different amounts of energy per amount of O_2 consumed or CO_2 emanated, accurate use of indirect calorimetry for bioenergetics requires a strict accounting of the substrate being oxidized and the energy lost through excretion of waste nitrogen. The relationship between the enthalpy change and the amount of oxygen consumed (oxycaloric

coefficient) is less dependent upon the foodstuff being oxidized than is the caloric coefficient for CO_2 release (Hammarsten, 1915) which, in addition to the reasons outlined above, makes oxygen a much better gas to use if the investigator is only going to use one gas. The measurement of CO_2 released by an organism can only be used to calculate metabolic heat production if the composition of the substrate oxidized by the animal is precisely known (Hammarsten, 1915). This situation is reversed if the metabolic rate of freely ranging animals is of interest. Carbon dioxide production can be estimated with the doubly labelled water technique (DLW), whereas O_2 consumption can only be estimated from telemetered proxies that are then related back to oxygen consumption measurements under controlled laboratory conditions (*e.g.* tri-axial acceleration, heart rate, electromyogram, tail displacement and ventilation; Briggs & Post, 1997; Webber *et al.*, 2001; Butler *et al.*, 2004; Millidine *et al.*, 2008; Gleiss *et al.*, 2011). The DLW technique has been useful in estimating CO_2 output and relating it to energetic expenditure in terrestrial animals (Nagy, 2005), but because this technique relies on assessing the dilution of isotopically labelled body water by metabolically produced water (Lifson & McClintock, 1966), a quantity that is small when compared with osmotic water flux in water-breathing ectothermic fishes, it is considered inappropriate for fishes (Butler *et al.*, 2004).

By the end of the 19th century, there was a substantial interest in using oxygen consumption to gauge animal metabolic rate and substantial effort had gone into determining oxycaloric coefficients of different diets (Krogh, 1916). If both oxygen uptake and CO_2 are measured and protein metabolism is determined from nitrogen excretion during the course of a calorimetry experiment, the enthalpy loss from oxidizing various diets can be accurately determined (Gnaiger, 1983*a*). These types of experiments had settled on pretty standard values for oxycaloric coefficients of the major food groups by the early 1900s (Hammarsten, 1915; Krogh, 1916). The biochemistry and calorimetry were also sophisticated enough to have generated oxycaloric coefficients for specialized situations such as animals depositing fat on a carbohydrate diet or metabolism of hibernating animals (Krogh, 1916). Therefore, armed with a fairly modest knowledge of the diet being catabolized, an investigator could measure oxygen consumption and know the total heat production of a completely aerobic organism with considerable accuracy. Thus, from the early 20th century onwards, measurements of oxygen consumption have dominated studies of metabolism, especially for fishes. Many of these early oxycaloric coefficients were calculated from bomb calorimetry and did not account for enthalpies of solution, dilution and protonation resulting in some error (Gnaiger, 1983*a*). An investigator can obtain a precise oxycaloric coefficient for a fish if they simultaneously apply direct calorimetry and oxygen consumption measurements to each species on each diet (Brett, 1973; Gnaiger, 1983*a*), but this is rarely done. Most investigators use oxycaloric coefficients garnered from the literature and assume an average protein composition and mode of nitrogenous excretion. The maximum error in doing so is on the order of 10% for extreme diets (Gnaiger *et al.*, 1989). If the animal is oxidizing a mixture of foodstuffs, the error from using a generalized oxycaloric coefficient will generally be in the range of $\pm2\%$ (Gnaiger *et al.*, 1989). Gnaiger (1983*a*) provides a detailed accounting of which oxycaloric coefficients to use and when. Many investigators today do not even attempt to relate their oxygen consumption measurements back to energy. As such, oxygen consumption rate ($\dot{M}O_2$) has actually become a measurement in its own right. Without accounting for the substrate being oxidized, mode of nitrogen excretion or accounting

for any anaerobic metabolism that occurs, it may be quite different from the actual metabolic rate.

OXYGEN CONSUMPTION *V*. DIRECT CALORIMETRY

The advantages of using $\dot{M}O_2$ as a surrogate for metabolic rate or as its own measurement were apparent early on Krogh (1916) and have already been discussed. The major liability of using $\dot{M}O_2$ as a surrogate for metabolic rate is that use of any metabolic pathways that do not eventually result in oxygen uptake will be untracked. Therefore, any net anaerobic metabolic activity will be missed, making $\dot{M}O_2$ a poor experimental choice under some conditions, for example, in hypoxic or anoxic waters (Gnaiger, 1983b). Fishes also use anaerobic metabolism to fuel powerful muscle contractions; investigators have attempted to quantify the metabolic expense of this activity with $\dot{M}O_2$ measurements after the activity [*e.g.* excess post-exercise oxygen consumption (EPOC)]. This practice assumes that the cost of returning to the resting state, *i.e.* fuelling metabolism with the by-products of anaerobic metabolism and the re-synthesis of normal storage metabolites with these by-products is equal in magnitude to the component of metabolism fuelled by the actual anaerobic ATP production. This assumption appears to be unsubstantiated in fishes. Likewise, the extent of anaerobic metabolism under hypoxia or anoxia can be approximated by assuming that the excess oxygen consumption upon return to normoxia is of the same magnitude, also unproven as far as is known.

How accurate indirect calorimetry is at assessing total metabolic activity is a question that is as old as the science of calorimetry itself. Indeed, Lavoisier was the first to compare the two techniques; critics used his 20% difference between the two to discount his theory of animal respiration for years (Holmes, 1985). More than 100 years later, Rubner (1894) was the first to report reasonable agreement between heat production measured in a calorimeter with estimates made from gas exchange. Comparisons between direct and indirect calorimetry in terrestrial organisms continued in earnest at the turn of the 20th century (Benedict & Milner, 1907), with the general conclusion that they are quite close when standard conditions were used (Krogh, 1916). Krogh (1916) provided a thoughtful and comprehensive analysis of the two techniques, when each was to be most profitably used and under what metabolic conditions indirect calorimetry was likely to lead an investigator astray. By 1989, a review of direct calorimetry studies in intact fishes (van Waversveld *et al.*, 1989) could only report on five studies. This is a testimony to the complete dominance of indirect calorimetry as metabolic studies of fishes expanded throughout the 20th century. van Waversveld *et al.* (1989) concluded from the few studies that were not tainted by human handling, that the two methods gave similar estimates of metabolic rate (*c*. 12% difference). Most studies that have used simultaneous oxygen consumption and heat production measurements in fishes were doing so to calculate an oxycaloric coefficient and discern substrate being oxidized, not to compare the two methods. Recent comparisons of metabolic rate in endotherms from diverse taxa using both direct and indirect calorimetry on the same individual animal suggest that the use of indirect calorimetry incurs errors on the order of 20%, with isolated cases as high as 35% (Walsberg & Hoffman, 2005). Fortunately for fish biologists, a similar comparison from the same investigators found much less discrepancy in a terrestrial ectotherm (Walsberg & Hoffman, 2006). Perhaps more

disturbing than differences between the two techniques are that careful measurements in laboratory mice *Mus musculus* came up with differences between the two that are not a fixed value, but vary with the physiological condition of the animal (Burnett & Grobe, 2013). An interesting idea is that discrepancies between the two techniques might lie in the metabolism of the micro biome that generally accounts for *c.* 90% of the cells in an organism, many of which will be metabolizing anaerobically (Kaiyala, 2014). Certainly one major assumption of indirect calorimetry, that of net aerobiosis, does not apply to the gut microbiome of many fishes. Nelson (2014), however, mentions the untested idea that the gut microbial community of fishes that breathe air with their digestive tract would have to be aerobic or at least microaerophiles. Considering the inevitable proliferation of oxygen consumption studies following the advent of optodes, it may be time to start considering $\dot{M}O_2$ as its own measurement and not a surrogate for metabolic rate. It is predicted that even fewer of the next generation of fish metabolic physiologists will relate their $\dot{M}O_2$ measurements back to actual energy utilization by the animal, so why not call it what it is, and not 'metabolic rate'? As the price and availability of better technology for direct calorimetry of aquatic organisms improves (Regan *et al.*, 2013), more laboratories will want to use it and the term metabolic rate should be reserved for their results. As these direct calorimetric results on fish accumulate, more informed comparisons between the two techniques will be made and the understanding of energy flow through fishes improved. Energy, after all, is the currency of fish life and its accurate measurement has implications across the entirety of fish biology.

References

Benedict, F. G. (1915). Factors affecting basal metabolism. *Journal of Biological Chemistry* **20**, 263–299.

Benedict, F. G. & Milner, R. D. (1907). Experiments on the metabolism of matter and energy in the human body, 1903-1904. *United States Department of Agriculture Office of Experimental Studies Bulletin* **175**.

Biot, J.-B. (1807). Mémoire sur la nature de l'air contenu dans la vessie natatoire des poissons. *Mémoires de Physique et de Chimie de la Société d'Arcueil* **1**, 252–281. Available at https://fr.wikisource.org/wiki/M%C3%A9moire_sur_la_nature_de_l%E2%80%99air_contenu_dans_la_vessie_natatoire_des_poissons

Boggs, C. H. & Kitchell, J. F. (1991). Tuna metabolic rates estimated from losses during starvation. *Physiological Zoology* **64**, 502–524.

Bohr, C. (1894). The influence of section of the vagus nerve on the disengagement of gases in the air-bladder of fishes. *Journal of Physiology* **15**, 494.

Boutilier, R. G., Iwama, G. K., Heming, T. A. & Randall, D. J. (1985). The apparent pK of carbonic acid in rainbow trout blood plasma between 5 and 15°C. *Respiration Physiology* **61**, 237–254.

Brett, J. R. (1973). Energy expenditure of sockeye salmon, *Oncorhyncus nerka*, during sustained performance. *Journal of the Fisheries Research Board of Canada* **30**, 1799–1809.

Brett, J. R. & Groves, T. D. D. (1979). Physiological energetics. In *Fish Physiology*, Vol. VIII (Hoar, W. S., Randall, D. J. & Brett, J. R., eds), pp. 280–352. New York, NY: Academic Press.

Bridges, C. R. & Scheid, P. (1982). Buffering and CO_2 dissociation of body fluids in the pupa of the silkworm moth, *Hyalophora cecropia*. *Respiration Physiology* **48**, 183–197.

Briggs, C. T. & Post, J. R. (1997). *In situ* activity metabolism of rainbow trout (*Oncorhynchus mykiss*): estimates obtained from telemetry of axial muscle electromyograms. *Canadian Journal of Fisheries and Aquatic Sciences* **54,** 859–866.

Buck, L. T., Hochachka, P. W., Schon, A. & Gnaiger, E. (1993). Microcalorimetric measurement of reversible metabolic suppression induced by anoxia in isolated hepatocytes. *American Journal of Physiology* **265,** R1014–R1019.

Burnett, C. M. L. & Grobe, J. L. (2013). Direct calorimetry identifies deficiencies in respirometry for the determination of resting metabolic rate in C57Bl/6 and FVB mice. *American Journal of Physiology* **305,** E916–E924.

Butler, P. J., Green, J. A., Boyd, I. L. & Speakman, J. R. (2004). Measuring metabolic rate in the field: the pros and cons of the doubly labelled water and heart rate methods. *Functional Ecology* **18,** 168–183.

Cameron, J. N. (1971). Rapid method for determination of total carbon dioxide in small blood samples. *Journal of Applied Physiology* **31,** 632–634.

Cech, J. J. Jr. (1990). Respirometry. In *Methods for Fish Biology* (Shreck, C. B. & Moyle, P. B., eds), pp. 335–362. Bethesda, MD: American Fisheries Society.

Chabot, D., Steffensen, J. F. & Farrell, A. P. (2016). The determination of standard metabolic rate in fishes. *Journal of Fish Biology* (in press, this issue).

Chu, C. S., Lo, Y. L. & Sung, T. W. (2011). Review on recent developments of fluorescent oxygen and carbon dioxide optical fiber sensors. *Photonic Sensors* **1,** 234–250. doi: 10.1007/s13320-011-0025-4

Clark, L. C. Jr. (1956). Monitor and control of blood and tissue O_2 tensions. *Transactions of the American Society for Artificial Internal Organs* **2,** 41–48.

Clark, T. D., Sandblom, E. & Jutfelt, F. (2013). Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. *Journal of Experimental Biology* **216,** 2771–2782.

Demas, J. N., Degraff, B. A. & Coleman, P. B. (1999). Oxygen sensors based on luminescence quenching. *Analytical Chemistry* **71,** 793A–800A.

Dickson, A. G., Sabine, C. L. & Christian, J. R.. 2007. Guide to best practices for ocean CO_2 measurements. *PICES Special Publication* **3**.

Du, F., Zhu, X. H., Zhang, Y., Friedman, M., Zhang, N., Uğurbil, K. & Chen, W. (2008). Tightly coupled brain activity and cerebral ATP metabolic rate. *Proceedings of the National Academy of Sciences of the United States of America* **105,** 6409–6414.

Ege, R. & Krogh, A. (1914). On the relation between the temperature and the respiratory exchange in fishes. *Internationale Revue der gesamten Hydrobiologie und Hydrographie* **7,** 48–55.

Flenley, D. C., Millari, J. S. & Rees, H. A. (1967). Accuracy of oxygen and carbon dioxide electrodes. *British Medical Journal* **2,** 349–352.

Frankenfield, D. C. (2010). On heat, respiration, and calorimetry. *Nutrition* **26,** 939–950.

Fry, F. E. J. (1971). The effect of environmental factors on the physiology of fish. In *Fish Physiology*, Vol. 6 (Hoar, W. S. & Randall, D. J., eds), pp. 1–98. New York, NY: Academic Press.

Glazier, D. S. (2014). Metabolic scaling in complex living systems. *Systems* **2,** 451–540. doi: 10.3390/systems2040451

Gleiss, A. C., Wilson, R. P. & Shepard, E. L. C. (2011). Making overall dynamic body acceleration work: on the theory of acceleration as a proxy for energy expenditure. *Methods in Ecology and Evolution* **2,** 23–33. doi: 10.1111/j.2041-210X.2010.00057.x

Gnaiger, E. (1983a). Calculation of energetic and biochemical equivalents of respiratory oxygen consumption. In *Polarographic Oxygen Sensors. Aquatic and Physiological Applications* (Gnaiger, E. & Forstner, H., eds), pp. 337–345. Berlin: Springer.

Gnaiger, E. (1983b). The twin–flow microrespirometer and simultaneous calorimetry. In *Polarographic Oxygen Sensors. Aquatic and Physiological Applications* (Gnaiger, E. & Forstner, H., eds), pp. 337–345. Berlin: Springer.

Gnaiger, E. (1989). Physiological calorimetry: heat flux, metabolic flux, entropy and power. *Thermochimica Acta* **151,** 23–34.

Gnaiger, E., Shick, J. M. & Widdows, J. (1989). Metabolic microcalorimetry and respirometry of aquatic animals. In *Techniques in Comparative Respiratory Physiology* (Bridges, C. R. & Butler, P. J., eds), pp. 113–135. Cambridge: Cambridge University Press.

Gréhant, N. (1886). Nouvel appareil pour l'etude de la respiration des animaux et des vegetaux aquatiques. *Comptes Rendus des Séances et Mémoires de la Société de Biologie* **38,** 421.

Guderley, H. (2011). Mitochondria and temperature. In *Encyclopedia of Fish Physiology: From Genome to Environment*, Vol. 3 (Farrell, A. P., ed.), pp. 1709–1716. San Diego, CA: Academic Press.

Hammarsten, O. (1915). *A Text-Book of Physiological Chemistry*, 7th edn. New York, NY: John Wiley & sons.

Heisler, N. (1984). Acid-base regulation in fishes. In *Fish Physiology*, Vol. X, Part A (Hoar, W. S. & Randall, D. J., eds), pp. 315–401. Orlando, FL: Academic Press.

Henze, M. (1910). Über den Einfluss des Sauerstoffdrucks auf den Gaswechsel einiger Meerestiere. *Biochemische Zeitschrift* **26,** 255–278.

Holmes, F. L. (1985). *Lavoisier and the Chemistry of Life*. Madison, WI: University of Wisconsin Press.

Jolyet, F. & Regnard, P. (1877). Recherches physiologiques sur la respiration des animaux aquatiques. *Archives de Physiolgie. Series II* **44–62**, 584–633.

Kaiyala, K. J. (2014). What does indirect calorimetry really tell us? *Molecular Metabolism* **3,** 340–341.

Kaiyala, K. J. & Ramsay, D. S. (2011). Direct animal calorimetry, the underused gold standard for quantifying the fire of life. *Comparative Biochemistry and Physiology A* **158,** 252–264.

Keeling, R. F., Kortzinger, A. & Gruber, N. (2010). Ocean deoxygenation in a warming world. *Annual Review of Marine Science* **2,** 199–229.

Killen, S. S., Costa, I., Brown, J. A. & Gamperl, A. K. (2007). Little left in the tank: metabolic scaling in marine teleosts and its implications for aerobic scope. *Proceedings of the Royal Society B* **274,** 431–438.

Killen, S. S., Atkinson, D. & Glazier, D. S. (2010). The scaling of metabolic rate with body mass in fishes depends on lifestyle and temperature. *Ecology Letters* **13,** 184–193.

Knauthe, K. (1898). Zur Kenntnis der Stoffwechsel der Fische. *Archiv für die gesammte Physiologie des Menschen und der Thiere* **73,** 490–500.

Krogh, A. (1904). Some experiments on the cutaneous respiration of vertebrate animals. *Skandinavisches Archiv fur Physiologie* **16,** 348–357.

Krogh, A. (1916). *The Respiratory Exchange of Animals and Man*. London: Longmans, Green and Co.

Lauff, R. F. & Wood, C. M. (1996). Respiratory gas exchange, nitrogenous waste excretion and fuel usage during starvation in juvenile rainbow trout, *Oncorhynchus mykiss*. *Journal of Comparative Physiology B* **165,** 542–551.

Lavoisier, A. L. & Laplace, P. S. (1783). Mémoire sur la chaleur 1783. In *Oeuvres de Lavoisier 1862–1893*, Vol. 2, pp. 283–333. Paris: Imprimerie Impériale.

Lifson, N. & McClintock, R. (1966). Theory of use of the turnover rates of body water for measuring energy and material balance. *Journal of Theoretical Biology* **12,** 46–74.

Linderstrøm-Lang, K. (1937). The principle of the Cartesian diver applied to gasometric technique. *Nature* **140,** 108.

Lindstedt, P. (1914). Untersuchungen über Respiration und Stoffwechsel von Kaltblütern. *Zeitschrift für Fischerei* **14,** 193–245.

Mackereth, F. J. H. (1964). An improved galvanic cell for determination of oxygen concentrations in fluids. *Journal of Scientific Instruments* **41,** 38. doi: 10.1088/0950-7671/41/1/311

Mäenpää, K. A., Penttinen, O. P., Jussi, V. K. & Kukkonen, C. (2004). Pentachlorophenol (PCP) bioaccumulation and effect on heat production on salmon eggs at different stages of development. *Aquatic Toxicology* **68,** 75–85. doi: 10.1016/j.aquatox.2004.02.004c

Maffly, R. H. (1968). A conductometric method for measuring micromolar quantities of carbon dioxide. *Analytical Biochemistry* **23,** 252–262.

Mayevsky, A. & Rogatsky, G. G. (2007). Mitochondrial function *in vivo* evaluated by NADH fluorescence: from animal models to human studies. *American Journal of Physiology* **292,** C615–C640. doi: 10.1152/ajpcell.00249.2006

McCollum, A., Geubtner, J. & Hunt Von Herbing, I. (2006). Metabolic cost of feeding in Atlantic cod (*Gadus morhua*) larvae using microcalorimetry. *ICES Journal of Marine Science* **63,** 335–339.

McKenzie, D. J. & Randall, D. J. (1990). Does *Amia calva* aestivate? *Fish Physiology and Biochemistry* **8,** 147–158.

McKenzie, D. J., Pedersen, P. B. & Jokumsen, A. (2007). Aspects of respiratory physiology and energetics in rainbow trout (*Oncorhynchus mykiss*) families with different size-at-age and condition factor. *Aquaculture* **263,** 280–294.

Millidine, K. J., Metcalfe, N. B. & Armstrong, J. D. (2008). The use of ventilation frequency as an accurate indicator of metabolic rate in juvenile Atlantic salmon (*Salmo salar*). *Canadian Journal of Fisheries and Aquatic Sciences* **65,** 2081–2087.

Min, T. A., Li, X. C., Sheng, Q. C., Ping, K. & Yu, G. (1996). Microcalorimetric study of mitochondria isolated from fish liver tissue. *Journal of Biochemical and Biophysical Methods* **31,** 189–193.

Montuori, A. (1913). Les processus oxydatifs chez les animaux marins par rapport à la température. *Archives Italiennes de Biologie* **59,** 213–234.

Nagy, K. A. (2005). Field metabolic rate and body size. *Journal of Experimental Biology* **208,** 1621–1625.

Nelson, J. A. (2014). Breaking wind to survive: fish that breath air with their gastrointestinal tract. *Journal of Fish Biology* **84,** 554–576.

Nelson, J. A. & Chabot, D. (2011). General energy metabolism. In *Encyclopedia of Fish Physiology: From Genome to Environment*, Vol. 3 (Farrell, A. P., ed.), pp. 1566–1572. San Diego, CA: Academic Press.

Pfeiffer, T. J., Summerfelt, S. T. & Watten, B. J. (2011). Comparative performance of CO_2 measuring methods: marine aquaculture recirculation system application. *Aquacultural Engineering* **44,** 1–9.

Pflüger, E. (1873). Uber die Diffusion des Sauerstoffs, den Ort und die Gesetze der Oxydationsprozesse im thierischen Organismus. *Archiv für die gesammte Physiologie des Menschen und der Thiere* **6,** 43–64.

Provençal, J.-M. & de Humboldt, A. (1808). Recherches sur la respiration des poissons. *Mémoires de physique et de chimie de la Société d'Arcueil* **2,** 359–404.

Regan, M. D., Gosline, J. M. & Richards, J. G. (2013). A simple and affordable calorespirometer for assessing the metabolic rates of fishes. *Journal of Experimental Biology* **216,** 4507–4513. doi: 10.1242/jeb.093500

Rubner, M. (1883). Über den Einfluss der Korpergrosse auf Staff- und Kraftwechsel. *Zeitschrift für Biologie* **19,** 536–562.

Rubner, M. (1894). Die Quelle der tierischen Wärme. *Zeitschrift für Biologie* **30,** 73.

Sarrus, F. & Rameaux, J. (1837–1838). Rapport sur une mémoire addressé à l'Académie royale de Médecine. Commissaire Robiquet et Thillaye, rapporteurs. *Bulletin Académie Royale Médecine (Paris)* **2,** 538.

Schmidt-Nielsen, B. (1984). August and Marie Krogh and respiratory physiology. *Journal of Applied Physiology: Respiratory, Environmental and Exercise Physiology* **57,** 293–303.

Severinghaus, J. W. (1960). Methods of measurement of blood and gas carbon dioxide during anaesthesia. *Anesthesiology* **21,** 717–726.

Severinghaus, J. W. (1968). Measurements of blood gases: PO_2, and PCO_2. *Annals of the New York Academy of Sciences* **148,** 115–132.

Smith, R. R., Rumsey, G. L. & Scott, M. L. (1978). Net energy maintenance requirements of salmonids as measured by direct calorimetry: effect of body size and environmental temperature. *Journal of Nutrition* **108,** 1017–1024.

Spallanzani, L. (1803). *Mémoires sur la Respiration par Lazare Spallanzani Traduits en Français, d'après son Manuscrit Indit, par Jean Senebier*. Geneva: Paschoud.

Steffensen, J. F. (2002). Metabolic cold adaptation of polar fish based on measurements of aerobic oxygen consumption: fact or artefact? Artefact!. *Comparative Biochemistry and Physiology A* **132,** 789–795.

Stow, R. W., Baer, R. F. & Randell, B. F. (1957). Rapid measurement of the tension of carbon dioxide in blood. *Archives of Physical Medicine and Rehabilitation* **38,** 646–650.

Svendsen, M. B. S., Bushnell, P. G. & Steffensen, J. F. (2016). Design and setup of an intermittent-flow respirometry system for aquatic organisms. *Journal of Fish Biology* (in press, this issue).

Van Slyke, D. D. & Neill, J. M. (1924). The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. I. *Journal of Biological Chemistry* **61,** 523–543.

Walsberg, G. E. & Hoffman, T. C. M. (2005). Direct calorimetry reveals large errors in respirometric estimates of energy expenditure. *Journal of Experimental Biology* **208,** 1035–1043.

Walsberg, G. E. & Hoffman, T. C. M. (2006). Using direct calorimetry to test the accuracy of indirect calorimetry in an ectotherm. *Physiological and Biochemical Zoology* **79,** 830–835.

Warburg, O. (1908). Über die Oxydationen in lebenden Zellen nach Versuchen am Seeigelei. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* **59,** 112.

van Waversveld, J., Addink, A., van den Thillart, G. & Smit, H. (1988). Direct calorimetry on free swimming goldfish at different oxygen levels. *Journal of Thermal Analysis* **33,** 1019–1026.

van Waversveld, J., Addink, A., van den Thillart, G. & Smit, H. (1989). Heat production of fish: a literature review. *Comparative Biochemistry and Physiology A* **92,** 159–162.

Webber, D. M., Boutilier, R. G., Kerr, S. R. & Smale, M. J. (2001). Caudal differential pressure as a predictor of swimming speed of cod (*Gadus morhua*). *Journal of Experimental Biology* **204,** 3561–3570.

West, J. B. (2013). The collaboration of Antoine and Marie-Anne Lavoisier and the first measurements of human oxygen consumption. *American Journal of Physiology. Lung Cellular and Molecular Physiology* **305,** L775–L785.

Winkler, L. (1888). Die Bestimmung des in Wasser Gelösten Sauerstoffes. *Berichte der Deutschen Chemischen Gesellschaft* **21,** 2843–2855. doi: 10.1002/cber.188802102122

Winterstein, H. (1908). Beiträge zur Kenntnis der Fischatmung. *Archiv für die gesammte Physiologie des Menschen und der Thiere* **125,** 73–98.

Wolfbeis, O. S., Kovács, B., Goswami, K. & Klainer, S. M. (1998). Fiber-optic fluorescence carbon dioxide sensor for environmental monitoring. *Mikrochimica Acta* **129,** 181–188.

Yagi, M. & Oikawa, S. (2014). Ontogenetic phase shifts in metabolism in a flounder *Paralichthys olivaceus*. *Scientific Reports* **4,** 7135. doi: 10.1038/srep07135