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Glucose uptake as an alternative to oxygen uptake for assessing metabolic rate in *Danio rerio* larvae

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ABSTRACT

Respirometry, based on oxygen uptake, is commonly employed for measuring metabolic rate. There is a growing need for metabolic rate measurements suitable for developmental studies, particularly in *Danio rerio*, where many important developmental stages occur at < 4 mm. However, respirometry becomes more challenging as the size of the organism reduces. Additionally, respirometry can be costly and require significant experience and technical knowledge which may prohibit uptake in non-specialist/non-physiology labs. Thus, using equipment routine in most developmental/molecular biology laboratories, we measured glucose uptake in 96-h post fertilisation (hpf) zebrafish larvae and compared it to stop-flow respirometry measures of oxygen uptake to test whether glucose uptake was a suitable alternative measure of metabolic rate. A Passing-Bablok regression revealed that within a 95% limit of agreement, the rate of glucose uptake and the rate of oxygen uptake were equivalent as measures of metabolic rate in 96 hpf *Danio rerio* larvae. Thus, the methodology we outline here may be a useful alternative or a complementary method for assessing metabolic rate in small organisms.

1. Introduction

Metabolism encompasses a complex network of chemical reactions fundamental for sustaining life. Metabolic reactions convert chemicals from one form to another to release energy (e.g., respiration), generate molecules required for other processes (e.g., amino acids and nucleotides), and facilitate waste removal (e.g. carbon dioxide and nitrogen).

Due to the complicated nature of whole-organism metabolism, reductionist approaches are often used to identify and measure single, key metabolic pathways (Darden, 2016). This enables the standardisation of metabolic rate measurements and facilitates comparisons between different studies. Pathways in the metabolic network are generally targeted for measurement under the assumption that a complex system is the sum of its parts, and thus each step is carried out in proportion to any other step, and that a limiting factor will limit the entire system (e.g., the ten steps in the glycolysis pathway are ultimately limited by glucose availability (Tanner et al., 2018).

Key steps targeted in metabolic research include the rate of substrate consumption, biproduct production, and generation and excretion of metabolites. For example, respirometry systems are employed to measure the rate of oxygen consumption at the level of the whole organism (Bailey et al., 1957), the tissue (Scheiber et al., 2019), or the isolated mitochondria (Levitsky et al., 2019). Respirometry is also used to measure production of CO_2 (Tickle et al., 2018) and metabolite production at the cellular level can be detected using analysers such as the Agilent Seahorse (Agilent, Santa Clara, California, US) and Oroboros Microrespirometers (Oroboros Instruments, Innsbruck, Austria) (Müller et al., 2019). Calorimetry, which measures heat production as a biproduct of chemical reactions (Gillis et al., 2015), can be used to measure metabolic rate (Kaiyala and Ramsay, 2011) but is costly to implement and is impractical for most living systems, particularly small organisms(Speakman, 2013). Thus, respirometry (indirect calorimetry) is often used to assess metabolic rate.

Nutrient metabolism can be subdivided into three broad pathways: glucose, protein, and lipid. Of these, glucose metabolism is the most efficient (Akhurst, 2007), and begins when glucose is taken up by respiring cells via glucose transporters and immediately phosphorylated

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Abbreviations

2DG	2-Deoxyglucose
2DG6P	2-Deoxyglucose-6-Phosphate
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BLUP	Best Linear Unbiased Prediction
G6P	Glucose-6-Phosphate
G6PDH	Glucose-6-Phosphate Dehydrogenase
hpf	Hours Post Fertilisation
MO_2	Oxygen Consumption Rate
MS-222	Tricaine Methanesulfonate
ROUT	Robust Regression and Outlier Removal
SEM	Standard Error of Mean

to glucose-6-phosphate (G6P) by hexokinases. This prevents diffusion out of the cell, as G6P is membrane impermeable, accumulating G6P as a substrate for energy production. G6P is modified in multiple sequential reactions, ultimately generating two pyruvate molecules, and releasing two ATP per glucose molecule via glycolysis. Pyruvate is subsequently transported from the cytosol into the mitochondria to enter the citric acid cycle and commence aerobic respiration. NADH generated by the citric acid cycle fuels the electron transport chain, ultimately generating a theoretical maximum of 36 additional ATP (DiTullio and Dell'Angelica, 2019). Glucose and oxygen are both limiting factors in glucose metabolism (equation (1)) and can therefore act as indicators of metabolic rate.

Equation (1):

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$

While respirometry is the most commonly used method for measuring whole organism metabolism, respirometry systems are often complex and, depending on the level of biological organization under investigation, can require significant setup and validation time. They can also be costly, requiring specialist equipment, particular for micro-respirometry where mitochondrial function is directly investigated. Whole animal respirometry systems require specialist knowledge to ensure reliability of experimental design and interpretation (Clark et al., 2013; Drown et al., 2020). Common errors associated with whole animal measurements are compounded when considering embryos and larvae. Background bacterial respiration can impact target oxygen uptake measurements in whole animal respirometry systems (Clark et al., 2013; Svendsen et al., 2016) and the interference increases as the size of the organism decreases (Svendsen et al., 2016). While reducing the volume of the respirometry setup (such as by reducing the size of the chambers and associated tubing) limits the impact of bacterial respiration, the volume and capacity of the system can only be reduced a finite amount (Svendsen, Bushnell and Steffensen, 2016b). While 96 well plate systems exist for increasing throughput when measuring whole organismal metabolism of small animals, these systems are also costly. Indeed, due to the cost of professionally manufactured respirometry systems, there are publications designed to help interested parties build their own from off-the-shelf components (Baker et al., 2018a).

Glucose uptake assays require only a plate reader, a microscope, and reagents, all of which are readily available in most laboratories. These assays can be adapted to 96 well plate formats, enabling relatively high throughput and higher granularity. A glucose uptake assay measures the rate of uptake of a glucose analogue, 2-deoxyglucose (2DG), which is transported into the cell by glucose transporters. Hexokinases phosphorylate 2DG into 2-deoxyglucose-6-phosphate (2DG6P), which is membrane impermeable and resistant to further modification, subsequently accumulating in the cell proportional to the rate of glucose uptake. Glucose-6-phosphate dehydrogenase converts 2DG6P into 6-phosphodeoxygluconate, reducing $NADP^+$ into NADPH. Reductases catabolise the conversion of proluciferin to luciferin, oxidising NADPH to $NADP^+$, producing luminescence proportional to the rate of glucose uptake (see Fig. 2a). Unlike respirometry systems, glucose uptake assays do not require specialist software to acquire, interpret and process data. The raw luminescence data can be used directly from the plate reader without the need for respirometry calculations. Set-up time is minimal, and inexpensive compared to purchasing a system where oxygen is measured as the rate limiting step in metabolism.

Zebrafish (*Danio rerio*) are increasingly used in metabolic studies, and are an invaluable model organism for developmental research, featuring a rapid generation time, large spawn size, genetic tractability, and transparent embryos (Howe et al., 2013). Zebrafish are also a growing model for cardiac research (Maciag et al., 2022) and in many cases, metabolism is used as an index for cardiac performance (e.g., Nyboer and Chapman, 2018). There is also a body of literature incorporating metabolism and cardiac function into developmental studies and environmental/climate change related studies with zebrafish (Seth et al., 2013; Roy et al., 2017; Dhillon et al., 2019). As several important developmental stages occur in the zebrafish before 4 mm total body length, a method for measuring metabolism at smaller sizes would be beneficial to this community.

In zebrafish larvae, the yolk serves as a primary energy store. The developing circulatory system runs directly across the yolk, into the heart, dorsal aorta, caudal vein, and back to the yolk in one continuous circuit. Nutrients dissolve into the bloodstream when passing through the yolk, which are then absorbed by actively respiring cells. As the yolk is readily accessible, a glucose analogue can be introduced easily into the system and provide the means to assess rate of glucose uptake as an index of metabolic rate. Furthermore, as the most direct metabolic process in energy generation, glucose metabolism is likely to be the primary driver in immediate adaptation to an increase in energy demand.

Here, a glucose uptake assay in 96 hpf zebrafish larva, adapted from an existing cell-culture protocol (Glucose Uptake-Glo™ Assay, Promega, Wisconsin, USA), is compared with whole-organism oxygen uptake measured using respirometry as measures of metabolism in zebrafish treated with caffeine to elicit an acute change in metabolism.

2. Materials and methods

2.1. Zebrafish husbandry

An established line of AB Notts zebrafish from the Biological Services Unit of the University of Manchester were used in this study. Adult zebrafish were housed under standard conditions (≈28°C; 14 h light/10 h dark photoperiod; stocking density <5 fish per litre). Breeding pairs were separated and housed in breeding tanks overnight at a ratio of one male to one female. Dividers were removed at the start of the following light cycle. Embryos were collected after 1 h of free breeding and incubated in embryo water (Instant Ocean salt 60 $\mu g ml^{-1}$, 2 $\mu g ml^{-1}$ methylene blue (Dunn, 2018)) at 28°C at a stocking density of <50 embryos per Petri dish. Unfertilised embryos were removed after 24 h, and embryo water was refreshed every 24 h. All larvae used in this study were from the same spawn to minimise variation. All work adheres to UK Home Office regulations for animal welfare.

2.2. Caffeine treatment

To generate embryos with differing metabolic rates, caffeine (0 mg L^{-1}, 5 mg L^{-1}, and 25 mg L^{-1}) was dissolved in embryo water. Concentrations were selected to reliably induce a metabolic effect but be below the threshold for inducing negative side effects (e.g., 1 g L^{-1}) (Chen et al., 2008; Rana et al., 2010; Abdelkader et al., 2012; Sage et al., 2015; Santos et al., 2017). Prior to the start of the experiment, larvae were randomly assigned to petri dishes containing one of the three

concentrations of caffeine and returned to the 28°C incubator for 2 h. Larvae were subsequently assigned to either stop-flow respirometry or the glucose uptake assay. Age in hpf has a notable impact on metabolic rate (Dhillon et al., 2019), so the two methods of measuring metabolic rate could not be performed on the same individuals. To minimise variation between the groups, all larvae were taken from the same breeding pair and the same clutch of eggs and treated identically until assignment to treatment groups. The stress involved in preparing the larvae for both tests was considered to be equivalent, as both techniques required brief restraint followed by free swimming.

It was not necessary to fast the subjects prior to either method as 96 hpf is before independent feeding for zebrafish larvae.

2.3. Oxygen consumption via closed-circuit stop-flow respirometry

In order to accurately measure very small changes in oxygen saturation, a closed-circuit stop-flow respirometer was designed (Fig. 1) (Svendsen et al., 2016), with the volume of the respirometer kept to a minimum (3 ml). Acrylic Flo-Thru Probe Vessels (Fig. 1.6) (Loligo Systems, 3–6 mm probe/10 mm tube size) were adapted for use as respirometry chambers. Single oxygen spots (Fig. 1.7) (Pyroscience, Aachen, Germany) were placed at the base of each chamber, paired with optical oxygen sensors (Fig. 1.8) (Pyroscience, Aachen, Germany) fed through the top of the chamber. The oxygen sensors were calibrated according to the manufacturer's instructions and tested immediately before the start of each experiment by immersion in 100% oxygen saturated water. A stop-flow pump was attached to each chamber (Fig. 1.4), controlled by a Cleware USB programmable Switch (Fig. 1.2) (Cleware GmbH, Germany) and AquaResp v.3 software (Fig. 1.13) (AquaResp, v3, Python 3.6 (Svendsen et al., 2019)). Cycling parameters were set to 60 s, 10 s, 300 s, (flush, wait, measure). This was sufficient flush time to replace all the water within the chamber with 100% oxygenated water, and a sufficient wait time to ensure complete mixing within the chamber.

To maintain a constant temperature of 28°C ± 0.3°C (Fig. 1.1), the respirometry system was immersed in a water bath (10 L) (Fig. 1.3) under constant aeration (air stone and agitation of water surface) (Fig. 1.10). The water bath was cycled from the reservoir to a UV filter (V2ecton 120 Nano, Tropical Marine Centre, UK) and back again. During flushing, water was pumped from this water bath into the chambers. Oxygen saturation within the chambers and water temperature (Fig. 1.11) was recorded using a FireStingO_2 Fiber-optic oxygen and temperature meter (Fig. 1.12) (PyroScience GmbH, Aachen, Germany) and Pyroscience Pyro Oxygen Logger (PyroScience GmbH, Aachen, Germany, v3.312, firmware 3.07) at a rate of one measurement per second, set to automatically compensate for a temperature of 28 °C. We did not include a mixing recirculation loop in the current study. This was omitted in favour of reducing the total volume of the respirometer, improving detection of small changes in oxygen saturation and limiting bacterial respiration, as reported for other low volume respirometry studies (Folkerts et al., 2017; Baker et al., 2018b; Rodgers et al., 2016). The recirculation pump in the ambient water bath in which the chambers were placed ensured consistent water temperature and caused turbulence (i.e., agitation of the chambers) similar to the gentle shaking used by (Parker et al., 2020).

Prior to each trial, the respirometer was filled with water matching the treatment group. Individual zebrafish larvae (96 hpf) were placed into a random chamber and allowed to acclimate for 30 min prior to the first trial. Data are represented as the mean of three trials per larvae (n = 10 per treatment). Oxygen consumption was measured on empty chambers immediately before and after each set of trials to account for background bacterial respiration (1 cycle each). This was assumed to be linear with respect to the total duration of each experiment (Rodgers et al., 2016), and the average background oxygen consumption was deducted from the net oxygen consumption on an individual chamber basis. The ratio of background (noise) to total (signal) oxygen consumption can been seen in Supplemental Fig. 1. To minimise bacterial respiration, the entire system was bleached between each set of larvae (system was flushed with Chemgene HLD4H Disinfectant (Medimark Scientific Ltd, Kent, UK) diluted 1:100 with water for 5 min, followed by multiple flushes with clean water).

Regression curves were generated using AquaResp from the 300 data points collected during each trial. An R2 cut off of ≥0.85 was used (Baker et al., 2018). The slope of the regression curves represents the change in oxygen saturation (ΔO_2) of the water within each chamber. This was converted into oxygen consumption per minute for each larva according to equation (2), where 7.88 mg L^{-1} is the maximum dissolved oxygen in 28°C desalinated water. AquaResp corrects for water displacement in the chamber volume by deducting an approximate subject volume based on mass. As 96 hpf zebrafish larvae are small, mass is not easily calculable. An approximate larval volume was instead used to calculate the net respirometer volume (net volume to animal volume ratio 2999:1). As such, oxygen consumption data was calculated per larvae rather than per unit mass (Parker et al., 2020).

Equation (2):

$$Oxygen\ consumption\ \left(mg\ O_2\ m^{-1}\right) = \frac{\Delta O_2\ \times 7.88\ mg\ L^{-1}\ \times adjusted\ chamber\ volume\ (L)}{time\ (m)}$$

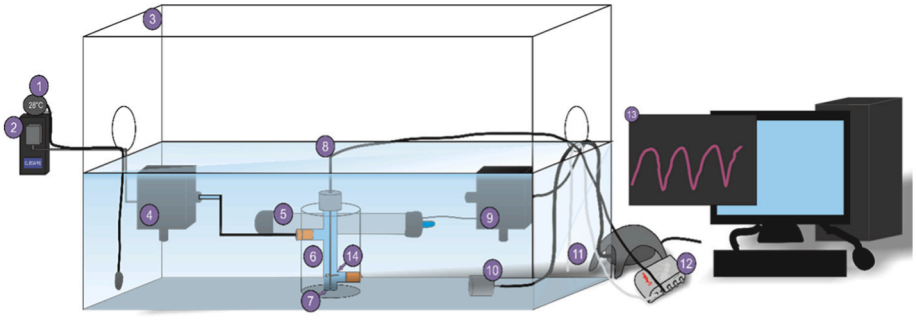

Fig. 1. Stop-flow respirometry setup for larval fish. 1. Temperature meter. 2. AquaResp-controlled USB switch. 3. Recirculation tank. 4. Stop-flow pump controlled by USB switch. 5. Water heater (28°C). 6. Respirometry chamber. 7. Oxygen spot. 8. Fibreoptic probe. 9. Recirculation pump. 10. Air stone. 11. Temperature probe. 12. FireStingO2 Oxygen and Temperature Logger. 13. AquaResp software v.3. Larvae (14.) are placed in the respirometry chamber and acclimated for 1 h before measurements begin. AquaResp controls the stop-flow pump and logger to cycle through a series of flush-wait-measure steps (60 s, 10 s, 300 s), generating oxygen saturation curves. AquaResp generates regression curves and outputs MO2 data for each cycle.

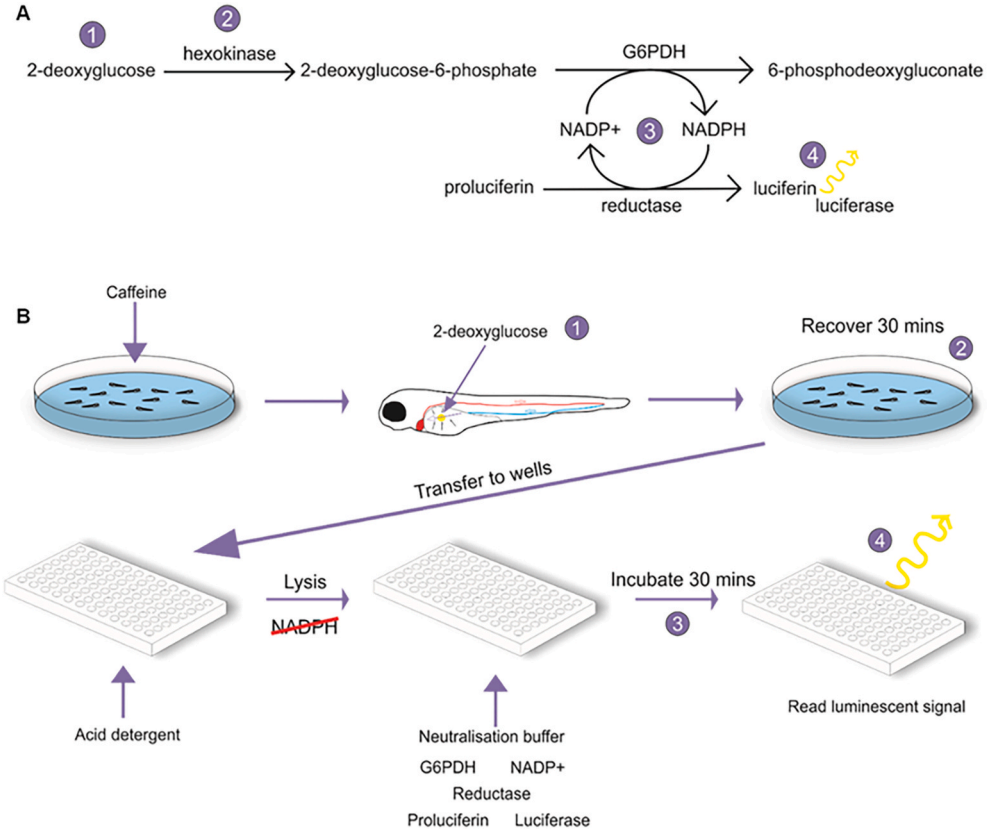

Figure 2. Glucose uptake assay protocol. 2a. Equation detailing the reactions involved in a glucose uptake assay. 2b. Experimental setup of a glucose assay performed on 96 hpf zebrafish larvae exposed to different concentrations of caffeine. Larvae are injected with 2-deoxyglucose into the yolk (yellow area) and recovered for 30 min. Larvae are transferred to individual wells and lysed with acid detergent to stop further glucose uptake, destroy any native NADPH, and homogenise the sample. Neutralisation and detection buffer are added, and samples are incubated while the luminescent signal is generated. After 30 min, luminescence is measured on a plate reader.

Total time for all the trials was 6 h, and they were carried out over the same day. Between 3 and 4 larvae were measured at any one time. The respirometer was located in a closed, single-use room with no other occupants and was not disturbed during the trials. Chambers were not shielded from each other but were staggered in the water bath to minimise disturbance. The minimum dissolved oxygen reached during the closed phases was 73.51%.

2.4. Glucose uptake assay

An adapted Glucose Uptake-Glo™ Assay (Promega, Wisconsin, USA) was performed on 96 hpf zebrafish to assess differences in the rate of glucose uptake between treatment groups. Full details on how the assay works can be found on the Promega website. All ammendments to the Promega assay for current use are detailed below. All reagents were used directly from the Promega kit. Following incubation in caffeine solution, larvae were immobilised in 0.7 μM MS-222 (neutrally buffered with Tris-HCl pH8) and methylcellulose. This concentration of MS-222 was chosen as it is sufficient to briefly immobilise the animal but is below the threshold for supressing other biological functions, such as heart rate and metabolism (RC et al., 2003; Craig et al., 2006; Nordgreen et al., 2014; Zakaria et al., 2018). Larvae were returned to the appropriate caffeine solution for recovery immediately after injection, resulting in light anaesthesia for less than 60 s. Larvae spontaneously recovered almost immediately, confirmed by observing self-righting.

Individual zebrafish larvae (n = 10 per treatment) were microinjected with 1 mM 2DG (approximately 500 pL) (Fig. 2.1) directly into the yolk sac using a fine glass needle inserted into a micromanipulator and a PLI-100A Picoliter Injector (Warner Instruments). Embryos were recovered in caffeinated water for 30 min (Fig. 2.2), allowing the 2DG to disperse into the yolk and be transported into the cells via the bloodstream. Although a microinjection system is not typical physiology lab equipment, it is routine in most zebrafish labs or zebrafish holding facilities and thus should be accessible by the target audience for this paper.

Larvae were subsequently terminated by overdose of MS-222 (500 mg L^{-1}), transferred to a 96 well plate, and homogenised in acid detergent with a fine gauge needle to terminate 2DG transportation and destroy endogenous NADPH present in the sample. Each well was neutralised with a high pH buffer and incubated with detection reagent (G6PDH, $NADP^+$, reductase, UltraGlo™ recombinant luciferase, proluciferin) for 30 min. G6PDH oxidises 2DG6P accumulated in the cells to 6-phosphodeoxygluconate, resulting in the reduction of $NADP^+$ to NADPH. The reductase enzyme catalyses the production of luciferin from proluciferin using the oxidation of NADPH to $NADP^+$ to provide the energy required (Fig. 2.3). The UltraGlo™ recombinant luciferase uses the produced luciferin as a substrate to generate luminescence (Fig. 2.4). As all native NADPH is removed from the sample during the lysis step, luminescence ↔ NADPH ↔ rate of 2DG6P accumulation ↔ rate of glucose uptake. The luminescent signal is therefore proportional to the rate of glucose uptake.

To limit the impact of native glucose interference on the signal, sham-injected larvae were included as a control to adjust for background luminescence (noise to signal data shown in Supplementary Fig. 1). Sham-injected larvae received an injection of phenol red, a common innocuous tracer used in microinjection solutions, in place of the 2DG. An empty well was included per row to further minimise background noise. Luminescence was measured on a Synergy™ H1 Microplate reader (BioTek Instruments, Inc., Winooski, VT, USA, software version 2.07.17) with 8 readings per well, adjusted against the background signal.

Other considerations included in assay design were the length of the incubation period, so as to measure luminescence during the linear portion of the reaction, and injection volume, so as to introduce an excess of 2DG into the system.

2.5. Statistical tests

Data were imported into GraphPad Prism (v7.04) for statistical analysis and converted to logarithmic values. All data were ROUT (robust regression and outlier removal) tested for outliers (Motulsky and Brown, 2006) and subject to D'Agostino and Pearson normality tests. Post-hoc power calculations were performed to confirm sample sizes were sufficient, where $\alpha = 0.05$. One-way analysis of variance (ANOVA) was used to determine if there was a significant difference between the caffeine treatments within a given metabolic index.

To compare between the two methods of assessing metabolism, Passing-Bablok regression analysis was used, where a regression line is fitted for the alternative method against the standard method (Bablok, 1983; Bilic-Zulle, 2011). Data were imported into R (version 4.1.3) with the following packages: blandr (version 0.5.1), ggplot2 (version3.3.5), mcr (version 1.2.2), MethodCompare (version 0.1.2), olsrr (version 0.5.3), segmented (version 1.5–0), and stats (version 4.3.0).

The Passing-Bablok regression requires the data to be linear, and states that if a structural relationship exists between two methods, it can be described by the linear equation $y = \alpha + \beta x$. If the 95% confidence intervals of α include 0 and β include 1, the two methods are comparable within the given range. If the confidence interval of α does not include 0, there is a systematic difference between the methods, and if the confidence interval of β does not include 1, there is a proportional difference between the methods.

The Passing-Bablok regression was generated in R, and, as the two methods use different units, the glucose uptake data (which uses arbitrary units) was scaled to minimise the proportional difference between the two methods. The 95% confidence bounds were calculated in R using the bootstrap method. The regression plot is presented together with a precision plot of the two methods and a Bland-Altman plot of the residuals, together with the bias and limits of agreement (Giavarina, 2015). The bias ($\overline{d}$) is calculated as the mean of the differences between the two methods. The limits of agreement are calculated as:

Equation (3):

$$\text{Upper limit}: \ \overline{d} + 1.96\sigma \quad \text{Lower limit}: \ \overline{d} - 1.96\sigma$$

where n is the number of individuals (30) and σ is the standard deviation. The upper and lower 95% confidence intervals are calculated as:

Equation (4):

$$LoA \pm t_{\alpha-1,\, n-1} \times \sqrt{\text{var}(LoA)}$$

Where:

Equation (5):

$$Var(LoA) = \frac{1}{n} + \sigma^2 \frac{1.96^2}{2(n-1)}$$

The precision plot comprises the standard deviation of the errors of the methods over the best linear unbiased prediction (BLUP) values, calculated in R.

The residuals were plotted as the difference (Respirometry – Glucose Uptake) vs the means, together with the bias, upper and lower limits of agreement, and upper and lower 95% confidence intervals of the limits of agreement.

To determine whether the assumptions of the Passing-Bablok are met, the linear regression model was tested for break points, and the residuals were tested for normality. The linear regression was confirmed to have no break points ($p = 0.682$) and the residuals were normally distributed (Shapiro-Wilk $p = 0.127$, Kolmogorov-Smirnov $p = 0.699$, Anderson-Darling $p = 0.0705$).

To calculate the biological power, the literature was reviewed to ascertain normal variation between individual zebrafish larvae and the expected percentage difference between groups with different metabolic rates (Barrionuevo and Burggren, 1999; Bang et al., 2004; Clark et al., 2013). PASS 2021 (Power Analysis & Sample Size, NCSS Statistical Software, Utah, USA, v21.0.2) (Bland and Altman, 2010; Lu et al., 2016) was used to calculate the power associated with the defined maximum allowable difference at a confidence level of 85%.

The limit of detection (LOD) was calculated for the two methods based on their regression lines calculated in Excel (Version 2103, Build 16.0.13901.20400). The limit of detection at a 95% confidence limit is defined as $3.3*(\sigma/s)$ where σ is the error of the y intercept of the regression line and s is the slope.

3. Results and discussion

Metabolic rate measured by whole animal oxygen uptake and by glucose uptake were elevated with increasing caffeine concentration (Fig. 3). Oxygen uptake sequentially and significantly increased between control (0 mg L^{-1}), 5 mg L^{-1}, and 25 mg L^{-1} (Fig. 3a), reflecting the increase in movement and metabolic demand induced by caffeine exposure. Oxygen is required for aerobic respiration and can be considered a limiting factor of respiratory rate. Therefore, the increase in oxygen uptake observed with caffeine exposure in 96 hpf zebrafish larva can be considered a reliable measure of increasing organismal metabolic rate. Similarly, glucose accumulation in zebrafish larvae significantly increased as caffeine concentration increased (Fig. 3b). As glucose is the initial substrate of glycolytic respiration, glucose is also a limiting factor of metabolism. Therefore, akin to changes in oxygen, glucose uptake can be considered an index of whole animal metabolic rate.

The Passing-Bablok regression was used to compare and assess the equivalence of the two methods. The Passing-Bablok regression states that if a structural relationship exists between two sets of data from a normal distribution, this relationship can be described by the line: $y = \alpha + \beta x$. D'Agostino and Pearson normality testing of each data group showed data were from a normal distribution ($p > 0.1$ so accept the null hypothesis that the data are from a normal distribution).

The parameters of the scaled data for the Passing-Bablok regression were:

Equation (6):

$$y = 1.02x - 0.18$$

95% confidence intervals: $-1.02 \leq \alpha \leq 0.89$ and. $0.97 \leq \beta \leq 1.33$

As both $\alpha = 0$ and $\beta = 1$ are true at a confidence of 95%, it can be concluded that the two methods are equivalent over this experimental range.

As shown in Fig. 3d, oxygen consumption as a measure of metabolic rate is slightly more precise at lower values. Conversely, the glucose uptake method becomes increasingly more precise as metabolic rate increases. This is likely partially due to the increase in bacterial respiration at the higher caffeine concentrations, which is not a confounding variable with the glucose uptake method.

The residuals were plotted verses the average of the two methods alongside the bias (−0.64 {-1.24, −0.05}) and the upper (2.48) and lower (−3.77) limits of agreement in a Bland-Altman plot (Fig. 3e). The 95% confidence interval of the bias included zero, indicating there is no statistically significant bias, confirmed by a two-tailed *t*-test ($p = 0.893$). As shown in Figs. 3e and 96.67% of the data fell between the upper and lower limits of agreement, suggesting the residuals are distributed around the bias line. Three statistical tests confirmed the residuals are normally distributed and the assumptions of the test have been met (Shapiro-Wilk $p = 0.127$, Kolmogorov-Smirnov $p = 0.699$, Anderson-Darling $p = 0.0705$).

The limits of detection (LOD, e.g., theoretical minimum change the technique can detect from background variation) were also calculated for the two methods based on their regression curves. The LOD of oxygen consumption measured by stop-flow respirometry was 4.40, indicating the technique can detect a difference yielded by 4.40 mg L^{-1} within a 95% confidence. The LOD for the glucose uptake method was 4.38 mg

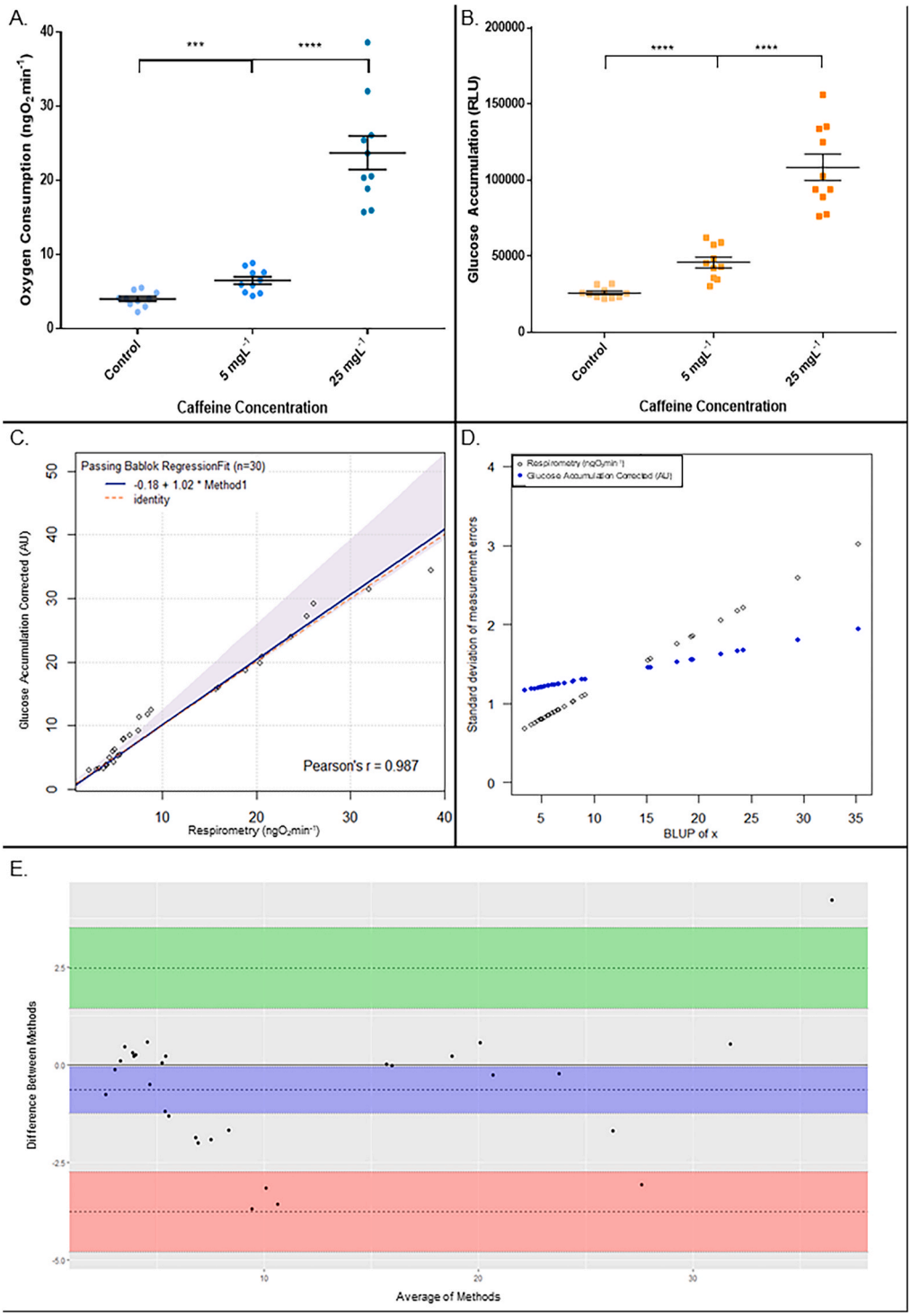

Figure 3. Comparison of metabolic rate as measured by stop-flow respirometry (3a) and glucose uptake (3b), Passing-Bablok comparison of the two methods (3c) with associated precision plot (3d) and Bland-Altman residuals plot (3e). 96 hpf zebrafish larvae were incubated in one of three caffeine concentrations (0 mg L-1, 5 mg L-1, and 25 mg L-1) to generate groups with differing metabolic rates. 3a. Oxygen uptake was measured by stop-flow respirometry in nanograms of oxygen per minute per larvae (n = 10). There was a significant increase in oxygen consumption between control and caffeine treated zebrafish larvae, with oxygen uptake increasing with increasing concentrations of caffeine (*** = 0.0006. **** <0.0001). 3b. The rate of cellular glucose accumulation was measured using a Glucose-Uptake GloTM Assay. There was a significant increase in luminescence between control and caffeine treated zebrafish larvae, with higher levels of luminescence correlating with higher concentrations of caffeine (**** <0.0001). All data are represented as individual data points with mean and SEM bars. Significance was determined by one-way ANOVA. 3c. Passing-Bablok Regression of respirometry versus scaled glucose uptake assay data, including regression line, identity line, and 95% confidence limits (grey shaded area). y = 1.02x −0.18, 95% confidence intervals $-1.018 \leq \alpha \leq 0.89$ and $0.97 \leq \beta \leq 1.33$. 3d. Precision plot comparing the two methods over the given range. Respirometry is marginally more precise at lower metabolic rates, while the Glucose Uptake Assay became increasingly more precise compared to respirometry as metabolic rate increased. 3e. Bland-Altman plot of the residuals. Bias = −0.64 (purple). Upper limit of agreement = 2.48 (green), lower limit of agreement = −3.77 (pink). Upper 95% confidence interval = 3.51, lower 95% confidence interval = −4.79.

L^{-1}. Thus, in this study, the glucose uptake method and respirometry have very similar lower limits of detection and can be considered equally sensitive over the studied range.

The aim of this study was to test whether glucose uptake and oxygen uptake can both be used to identify differences in metabolic rate in larval zebrafish. While both methods provide an index of the same overall pathway, this is not to say that the underlying processes are identical. Due to the complex nature of whole organism metabolism, both methods have their limitations, and ultimately provide an underestimation of total metabolic rate. Respirometry underestimates total metabolism as this method cannot provide an index for anaerobic respiration, and so too glucose uptake provides an underestimate as it cannot provide an index for lipid and protein metabolism. These limitations, however, should not prevent the use of either method, but should be acknowledged during the interpretation of the particular research scenario in which they are used.

In this study, protein metabolism is unlikely to contribute significantly to net metabolism, as protein stores in the yolk act primarily as a store of amino acids for the construction of new proteins (Sant and Timme-Laragy, 2018) rather than as a source of energy. Furthermore, the yolk sac is largely diminished by 96hpf, with only 9% of the lipid content representing free triglycerides involved in fatty acid metabolism (Quinlivan and Farber, 2017) and this is considered an ideal time point for glucose metabolism studies (Elo et al., 2007). The larvae are beginning to switch from lecithotrophy to free feeding, so the metabolic profile of the organism is expanding. Furthermore, glucose metabolism is far more rapid than lipid or protein metabolism and is the first to respond to acute metabolic challenges (Rui, 2014) such as the one used in this study (caffeine). Thus, changes in glucose metabolism are likely to have contributed largely to the acute changes in metabolic rate during the time frame of this study. However, the authors acknowledge the glucose uptake method will provide an underestimate of global metabolism as lipid metabolism is not represented in the index.

In this study we used caffeine as a stressor to raise metabolic rate. Individual stressors such as catecholamines, other hormones, pharmacological agents, temperature, hypoxia, or exercise impact metabolism in different ways. The exact relationship between oxygen consumption and glucose uptake may vary with the type of stress. Moreover, depending on the life stage of the organism under study, certain stressors may impact rates of carbohydrate, lipid, and protein oxidation differently. Finally, expression of GLUT receptors and hexokinase activity in the samples/animals being studied are important to consider as changes in these would impact the interpretation of the 2DG accumulation as an index of glucose metabolism. Therefore, although protocol comparison shows the two methods are equivalent, the methodology presented here cannot be extrapolated to all conditions of metabolic investigation. However, it can be usefully applied to the study of small larval fish. We do not advocate for replacing respirometry with measures of glucose uptake, but rather show that under the conditions used in many developmental studies, glucose uptake and whole animal respirometry are both suitable proxies for metabolism.

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CRediT authorship contribution statement

Bridget L. Evans: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, preparation, Writing – review & editing, and, Visualization. **Adam F.L. Hurlstone:** Conceptualization, Resources, Writing – review & editing, Supervision, Writing – review & editing, Supervision. **Peter E. Clayton:** Writing – review & editing, Supervision. **Holly A. Shiels:** Conceptualization, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crphys.2022.05.002.

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