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SHORT COMMUNICATION

Metabolic fuel use after feeding in the zebrafish (*Danio rerio*): a respirometric analysis

Marcio S. Ferreira[1,*], Chris M. Wood[1,2], Till S. Harter[2], Giorgi Dal Pont[2,3], Adalberto L. Val[1] and Philip G. D. Matthews[2]

ABSTRACT

We used respirometric theory and a new respirometry apparatus to assess, for the first time, the sequential oxidation of the major metabolic fuels during the post-prandial period (10 h) in adult zebrafish fed with commercial pellets (51% protein, 2.12% ration). Compared with a fasted group, fed fish presented peak increases of oxygen consumption (78%), and carbon dioxide (80%) and nitrogen excretion rates (338%) at 7–8 h, and rates remained elevated at 10 h. The respiratory quotient increased slightly (0.89 to 0.97) whereas the nitrogen quotient increased greatly (0.072 to 0.140), representing peak amino acid/protein usage (52%) at this time. After 48-h fasting, endogenous carbohydrate and lipid were the major fuels, but in the first few hours after feeding, carbohydrate oxidation increased greatly, fueling the first part of the post-prandial specific dynamic action, whereas increased protein/amino acid usage predominated from 6 h onwards. Excess dietary protein/amino acids were preferentially metabolized for energy production.

KEY WORDS: Stop-flow respirometry, Respiratory quotient, Nitrogen quotient, Specific dynamic action, Protein

INTRODUCTION

After ingestion of a meal, metabolic rate increases substantially in ectotherms. This is termed the specific dynamic action (SDA), representing the total elevation of energy expenditure for ingestion, digestion, absorption and metabolic processing of the diet (Jobling, 1994; McCue, 2006). In zebrafish (*Danio rerio*), O_2 consumption rate ($\dot{M}_{O_2}$) has been reported to approximately double after feeding (Lucas and Priede, 1992), but the simultaneous changes in carbon dioxide excretion rate ($\dot{M}_{CO_2}$) and nitrogenous waste excretion rate ($\dot{M}_N$=ammonia-N+urea-N) have not been studied. Thus, the metabolic fuels powering the SDA remain unknown. Indeed, this lack of knowledge appears to be true for all teleost fish. However, based on an analysis of the literature, Wood (2001) speculated that in most teleosts, lipid and carbohydrate are probably the major fuels burned during fasting (i.e. after absorption of nutrients from a meal is complete), but that protein/amino acid oxidation may increase greatly after feeding.

[1]Laboratório de Ecofisiologia e Evolução Molecular, Instituto Nacional de Pesquisas da Amazônia (INPA), 69.067-375 Manaus, Brasil. [2]Department of Zoology, University of British Columbia, Vancouver, Canada V6T 1Z4. [3]Grupo Integrado de Aquicultura e Estudos Ambientais, Universidade Federal do Paraná (UFPR), 80.060-000 Curitiba, Brasil.

*Author for correspondence (marcio.ferreira@inpa.gov.br)

M.S.F., 0000-0002-0027-5106; C.M.W., 0000-0002-9542-2219; T.S.H., 0000-0003-1712-1370; G.D.P., 0000-0002-0306-5075; A.L.V., 0000-0002-3823-3868; P.G.D.M., 0000-0003-0682-8522

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To measure the energetic substrates that are oxidized by fasting and exercising trout during aerobic metabolism, Lauff and Wood (1996a,b) developed a respirometry technique to quantify instantaneous substrate usage. In theory, the relative ratios of $\dot{M}_{O_2}$, $\dot{M}_{CO_2}$ and $\dot{M}_N$ can be used to stoichiometrically calculate the particular combination of carbohydrate, lipid and protein/amino acids being oxidized at any point in time (Kleiber, 1987, 1992). The technique was subsequently applied to other species of fish and other experimental conditions (Lauff and Wood, 1997; Alsop and Wood, 1997; Alsop et al., 1999; Kieffer et al., 1998; De Boeck et al., 2001), but since then, the approach has not been widely adopted, almost certainly because of the difficulty associated with accurately measuring $\dot{M}_{CO_2}$ in water (Nelson, 2016).

However, a recently published article has described a new apparatus which allows easy and accurate measurement of total CO_2, directly from a small volume of water. This method for $\dot{M}_{CO_2}$ determination, developed by Harter et al. (2017), was combined with $\dot{M}_{O_2}$ measurements to accurately calculate respiratory quotients in dragonfly nymphs. The apparatus, described below, employs a hollow fibre membrane for CO_2 collection into a gas phase, coupled to CO_2 measurement by an infrared analyser.

Our objective was to apply the fuel use calculations for instantaneous quantification of substrate usage of Lauff and Wood (1996a) in conjunction with the simultaneous $\dot{M}_{O_2}/\dot{M}_{CO_2}$ respirometry system of Harter et al. (2017) to calculate, for the first time, the energy substrates used during the SDA of a fish: *D. rerio*. We tested the hypothesis that lipid and carbohydrate are the major fuels used during fasting, whereas endogenous carbohydrate, which can be readily mobilized, becomes more important early in the absorptive stage when SDA is already occurring, but the nutrients from food have not yet fully reached the bloodstream. Finally, we predicted that protein/amino acids would become the preferred energy substrate later during the SDA.

MATERIALS AND METHODS

All experiments were performed according to the guidelines of the Canadian Council on Animal Care under UBC AUP-A14-025. Fifty adult zebrafish [*Danio rerio* (Hamilton 1822)] were acquired from an aquarium shop in Vancouver, Canada, and kept in a 50-liter aquarium fitted with biological and mechanical filtration for 15 days of acclimation to experimental conditions (Vancouver municipal tap water – in mmol l^{-1}: Na^+, 0.06; Cl^-, 0.05; Ca^{2+}, 0.03; Mg^{2+}, 0.007; K^+, 0.004; and in mg l^{-1} $CaCO_3$, alkalinity, 3.0; hardness 3.3; pH 7.35±0.1; temperature, 21.0±0.5°C). During this period, fish were fed twice a day with commercial pellets from a common batch (51% protein, 7% lipid, 10% ash, 1.6% fibre, 7% water, 1.8% calcium, 0.6% phosphorus; Nutrafin Max, Baby Formulae, Rolf C. Hagen Inc., Baie d'Urfé, QC, Canada) until apparent satiety. The calorie content of the diet, measured by a Parr 6100 Compensated Calorimeter (Parr Instrument Company, USA), was 5.26 kcal g^{-1}.

The experimental design compared six fish during the 10-h postprandial period with six fasted fish over the same time frame, using hourly respirometry measurements. Because the experimental setup was able to handle just one fish at a time, the procedure was performed 12 times, alternating the fish accordingly to each feeding treatment.

In preliminary trials, we found that zebrafish would not eat well alone or in pairs. Therefore, experimental fishes were fed in size-matched groups of three individuals, previously separated in a 3-liter aquarium for 48 h. Early in the morning of the experimental day, the fish were fed as a group. The food was weighed before and after feeding, and the pellets were offered *ad libitum*, in small portions until all three fish stopped feeding for at least 1 min. This approach allowed us to quantify how much food the group ate, and thus we estimated the individual feed intake by dividing the total ingested food mass (g) by three. Immediately after feeding stopped, one fish was randomly captured and transferred to a modified glass scintillation vial (22.5 ml), which was part of the novel respirometry system developed by Harter et al. (2017). Fasting fish (N=6) were treated identically to feeding fish (N=6), with the same feeding process being simulated, but with no food added to the water. Measurements started approximately 50 min after fish were placed in the respirometer (=60 min after feeding), i.e. 1 h (fed treatment) or 48 h (fasting treatment) post-feeding. The 48-h fasting period was chosen to ensure that no food was left in the digestive tract of the fish.

The respirometer (see detailed description and diagrams in Harter et al., 2017) allows simultaneous measurements of $\dot{M}_{CO_2}$ and $\dot{M}_{O_2}$, and water sampling for measurements of $\dot{M}_N$ in small aquatic organisms. The novel aspect of this apparatus is the use of a hollow fibre membrane (HFM) as the interface between the aqueous phase and a gas phase, providing a large ratio of gas exchange surface area to system volume. The water in the closed system was circulated using a peristaltic pump (Gilson MINIPULS 3, Middleton, WI, USA) that continuously perfused the vial housing the animal, the HFM and, finally, a 23-ml reservoir with an air headspace, which served as a bubble trap. Water flow rates replaced the vial volume every 1.12 min (i.e. 20 ml min^{-1}). The water flow through the HFM was counter-current to the flow of water-saturated CO_2-free air from a purge gas generator (CDA4-CO_2, Puregas, Broomfield, CO, USA). When the peristaltic pump was activated, water in the system was in equilibrium with the purge gas, which kept the partial pressure of O_2 (P_{O_2}) inside the chamber at ∼100% air saturation. In contrast, P_{CO_2} was low (mean±s.d.=36.5±4.84 ppm, N=12) and the baseline represented an equilibrium between the rate of CO_2 production by the animal and its removal through the HFM.

For each time point in the experimental protocol, a 1-ml water sample was collected from the bubble trap. This was done while the peristaltic pump was running, in order to obtain a well-mixed sample that was analysed for ammonia-N and urea-N. Owing to the repeated sampling of system water, the total water volume decreased from 47.5 ml (2 ml inside the HFM and tubing, 23 ml in the bubble trap and 22.5 ml in the vial) to ∼37.5 ml over the course of the experiment; this reduction in system volume was recorded and taken into account in the $\dot{M}_N$ calculations. Control measurements of $\dot{M}_N$ were performed without an animal in the system and all samples showed undetectable $\dot{M}_N$.

After water sampling, $\dot{M}_{O_2}$ was measured by intermittent closed-system respirometry. The peristaltic pump was stopped once an hour and $\dot{M}_{O_2}$ was measured from the decline in P_{O_2} over the stop-flow period (typically 2.4 kPa and 10 min), factored by time, respirometer volume and fish mass, using a solubility coefficient from Boutilier et al. (1984). P_{O_2} was measured with a fluorescent oxygen sensor spot (PyroScience GmbH, Aachen, Germany) that was glued to the bottom of the animal chamber, and recorded with a FireStingO_2 meter (PyroScience GmbH). Spatial profile tests showed that the animal's movements and ventilation were sufficient to create complete mixing during the period of stop-flow. Control measurements of $\dot{M}_{O_2}$ were performed without an animal in the chamber and these blanks were subtracted from every animal's measurement of $\dot{M}_{O_2}$.

After the stop-flow period, the peristaltic pump was re-started and the CO_2 that had accumulated inside the chamber was rapidly transferred into the gas phase through the HFM and measured using an LI-820 infrared CO_2 analyser (LiCOR, Lincoln, NE, USA). The recorded peak in CO_2 concentration in the purge gas was integrated to calculate $\dot{M}_{CO_2}$ over the stop-flow period. The integral under the [CO_2] curve was calculated from the time when water flow was turned on until [CO_2] returned to baseline.

Water pH inside the vial was continuously measured with a needle-style pH microelectrode (MI-414P, Microelectrodes Inc., Bedford, NH, USA) inserted through a sealed hole in the lid of the vial, that was calibrated before each run with BDH precision buffers (VWR, Radnor, PA, USA). As the CO_2 increased during the stopped flow, the pH inside the vial dropped from 7.47±0.15 (mean ±s.d., N=12) to 7.16±0.32. All $\dot{M}_{CO_2}$ measurements were corrected for blank measurements generated in the absence of an animal.

The same protocol was repeated every hour, for 10 successive hours, a time chosen based on the study of Lucas and Priede (1992). $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$ were recorded using a PowerLab 4/35 data acquisition unit (ADInstruments, Dunedin, New Zealand) and LabChart v8.1.5 software.

Calibration of the system for P_{O_2} and [CO_2] was performed exactly as described by Harter et al. (2017). Ammonia-N (total of NH_3 and NH_4^+) and urea-N were measured by the colorimetric assays described by Verdouw et al. (1978) and Rahmatullah and Boyde (1980), respectively.

The instantaneous fuel usage calculations, as well as the rationale behind them, have been detailed by Lauff and Wood (1996a,b). The calculation of the relative (%) contribution of lipid, carbohydrate and protein/amino acid fuels to the support of $\dot{M}_{O_2}$ was based on the hourly measurements of $\dot{M}_{O_2}$, $\dot{M}_{CO_2}$ and $\dot{M}_N$. The relative use of substrates was converted to absolute carbon expenditures from the three fuel types using the fuel-specific respiratory quotients (RQs) and the measured values of $\dot{M}_{CO_2}$.

The RQ (=$\dot{M}_{CO_2}/\dot{M}_{O_2}$) and nitrogen quotient (NQ=$\dot{M}_N/\dot{M}_{O_2}$) were determined for each fish at each time point. $\dot{M}_N$ was calculated from the sum of ammonia-N plus urea-N excretion. The calculations are presented in abbreviated form below.

F_P (the fraction of aerobic fuel use supplied by protein/amino acids) was calculated from the NQ as:

$$F_P = \frac{NQ}{0.27}, \quad (1)$$

where 0.27 is the theoretical maximum for NQ. Note that the value of 0.27 reflects 100% protein/amino acid use when ammonia-N and urea-N are the end products in any combination. However, the RQ reflecting 100% protein use ($RQ_{protein}$) is dependent on the particular mixture of nitrogenous wastes produced and can be calculated from biochemical theory (Kleiber, 1987, 1992; see eqn 11 of Lauff and Wood, 1996a). In our study, $RQ_{protein}$ varied slightly, from 0.96 after feeding to 0.93 in fasting animals. RQ_{lipids} and $RQ_{carbohydrate}$ are typically given as 0.71 and 1.0, respectively (Simonson and DeFronzo, 1990). Employing these fuel-specific

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RQs (with 0.96 being used for $RQ_{protein}$ in this example), it then follows:

$$RQ = F_P \times 0.96 + F_C \times 1.0 + F_L \times 0.71, \quad (2)$$

where F_P, F_C and F_L represent the fuel fractions supporting $\dot{M}_{O_2}$ from protein, carbohydrate and lipid, respectively. Because:

$$L = 1.0 - P - C, \quad (3)$$

then by substitution:

$$RQ = 0.81 \times NQ + 0.29F_C + 0.71. \quad (4)$$

The equation can be solved for F_C, and F_L determined by subtraction.

These fractional contributions to $\dot{M}_{O_2}$ can then be converted to percentages based on carbon usage via the fuel-specific RQs. The total carbon usage is provided by the $\dot{M}_{CO_2}$ data, which can be apportioned to absolute carbon expenditures of the three fuel types using these carbon-based percentages (Lauff and Wood, 1996a).

Statistical analysis was performed with a linear mixed-model (IBM® SPSS® Statistics, version 25) with a random effect of individual and fixed effects of feeding and time. Time was nested within individuals, but feeding was independent of individuals. *Post hoc* analysis was performed with a least significant difference (LSD) test.

RESULTS AND DISCUSSION

In the fasted group, $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$ did not vary significantly over time, whereas $\dot{M}_N$ exhibited small fluctuations (Fig. 1). $\dot{M}_{CO_2}$ was slightly lower than $\dot{M}_{O_2}$, yielding a 10-h average fasting RQ of 0.89, whereas the 10 h average fasting NQ was 0.072 (Fig. 2). The fed group ate a ration of 2.12±0.20% of body mass. In these fed zebrafish, $\dot{M}_{O_2}$ was significantly elevated from 1 h post-feeding onwards, throughout the 10-h experimental period. These elevated values were statistically the same from 3 h through 9 h, with the greatest absolute increase (78%) occurring at 8 h post-feeding (Fig. 1A). $\dot{M}_{CO_2}$ followed a comparable pattern, increasing significantly at all time points post-feeding, with statistically identical rates from 3 h through 8 h with an absolute peak (80% increase) at 8 h (Fig. 1B). The RQ was consistently higher in the fed fish, with a peak of 0.97 at 7 h, but it did not vary significantly over the 10-h post-prandial period, with an overall average of 0.94 (Fig. 2A). In contrast to $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$, the elevation in $\dot{M}_N$ in the fed group was slightly delayed, with no change at 1 h and a significant increase only from 4 h through 10 h, over which period the rates were statistically identical (Fig. 1C). However, the relative increase was much greater, peaking at 338% at 8 h. NQ increased approximately in parallel with $\dot{M}_N$, reaching a peak of 0.140 at 7 h (Fig. 2B). The NQ values from 4 h through 10 h were statistically identical. Notably, the changes in $\dot{M}_N$ were entirely due to increases in ammonia-N excretion; urea-N excretion did not change significantly. Thus, urea-N excretion dropped from 28.3±1.3% of $\dot{M}_N$ in fasting zebrafish to 6.9±1.2% in fed animals. These percentages did not vary significantly over the experimental periods (P=0.138), and therefore values of $RQ_{protein}$ of 0.93 for fasted animals and 0.96 for fed animals were employed in fuel use calculations. Relative to the fasting fish, the overall post-feeding increases integrated over 10 h were 56.7% in $\dot{M}_{O_2}$, 61.8% in $\dot{M}_{CO_2}$ and 187.6% in $\dot{M}_N$.

Our $\dot{M}_{O_2}$ (Fig. 1A), $\dot{M}_{CO_2}$ (Fig. 1B) and RQ values (Fig. 2A) for 48-h fasted zebrafish were similar to values reported for 12 to 24 h fasted adult zebrafish by Perry et al. (2010) and Gilmour et al.

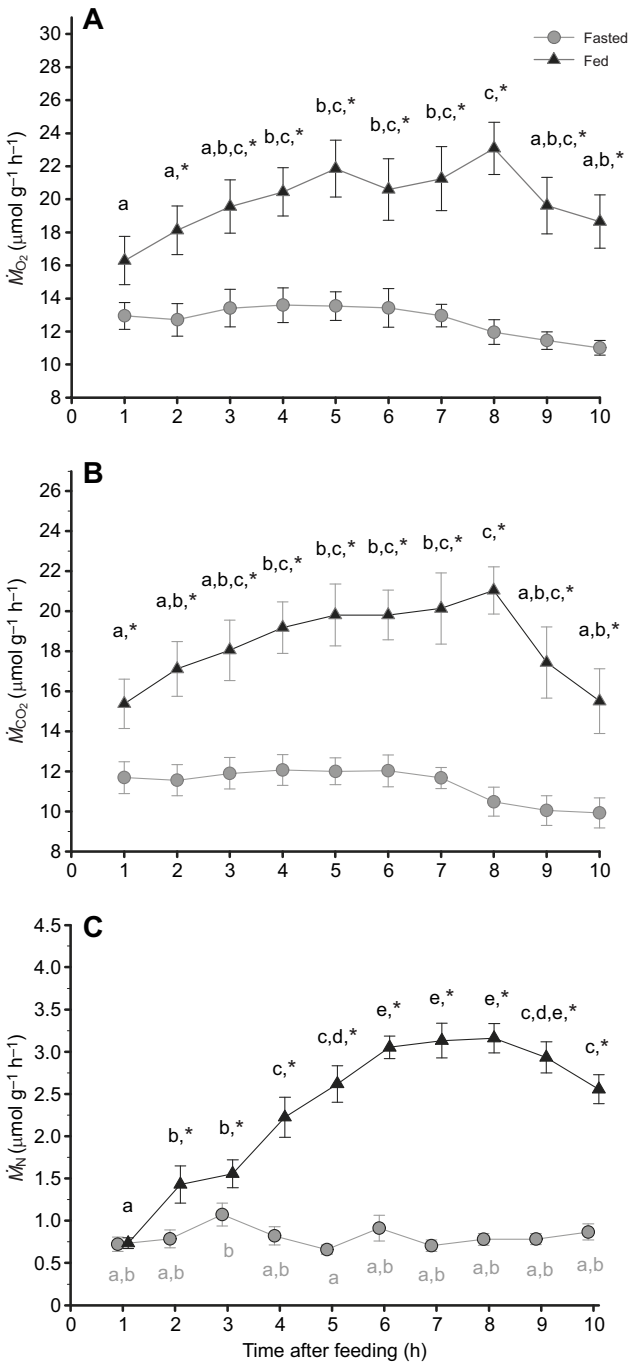

Fig. 1. Respiratory gas exchange and nitrogen excretion after feeding in fed zebrafish in comparison to fasted animals. (A) Oxygen consumption rate ($\dot{M}_{O_2}$; μmol g^{-1} h^{-1}), (B) carbon dioxide excretion rate ($\dot{M}_{CO_2}$; μmol g^{-1} h^{-1}) and (C) nitrogen excretion rate ($\dot{M}_N$; μmol g^{-1} h^{-1}) of zebrafish fed to satiation (average ration of 2.12% body mass) with a diet containing 51% protein, or fasted for 48 h. Statistical analysis employed a linear mixed-model with a random effect of individual and fixed effects of feeding and time. Main effects are represented by time, feeding and their interaction, as follows: (A) P=0.082 for time, P<0.001* for feeding, P=0.172 for time×feeding; (B) P=0.013* for time, P<0.001* for feeding, P=0.139 for time×feeding; (C) P<0.001* for time, P<0.001* for feeding, P<0.001* for time×feeding. Time was nested within individuals but feeding was independent of individuals. In C, data had to be transformed via natural log (ln) to fit a normal distribution of residuals. *Post hoc* analysis was performed with an LSD test. All data are shown as means±s.e.m. (N=6). Different superscript letters indicate significant differences (P<0.05) between sampling times within the respective feeding treatment. Asterisks (*) indicate significant differences between fasted and fed fish, within the same time period.

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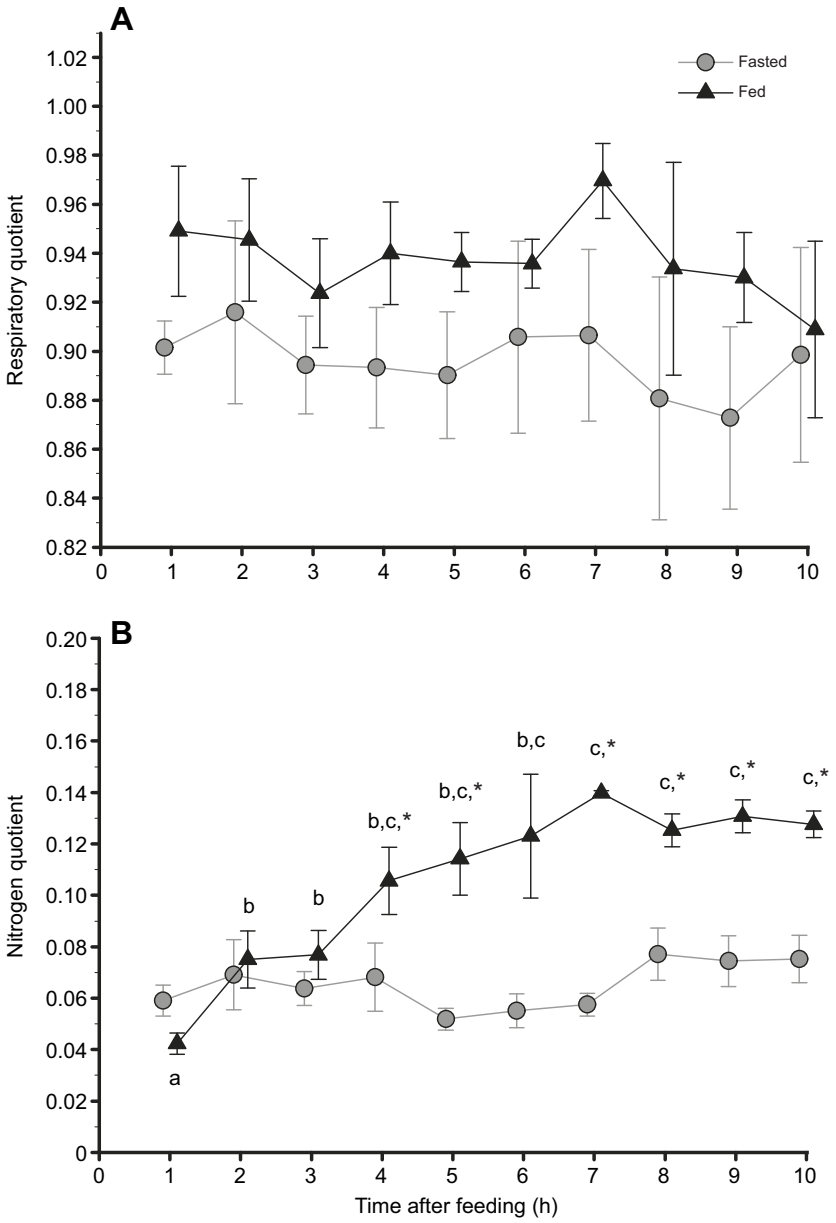

Fig. 2. Respiratory quotient and nitrogen quotient after feeding in fed zebrafish in comparison to fasted animals. (A) Respiratory quotient (RQ; $\dot{M}_{CO_2}/\dot{M}_{O_2}$) and (B) nitrogen quotient (NQ; $\dot{M}N/\dot{M}_{O2}$) of zebrafish fed to satiation (average ration of 2.12% body mass) with a diet containing 51% protein, or fasted for 48 h. Main effects are represented by time, feeding and their interaction, as follows: (A) P=0.957 for time, P=0.003* for feeding, P=0.998 for time×feeding; (B) P<0.001* for time, P<0.001* for feeding, P=0.001* for time×feeding. All other details as in Fig. 1.

(2009). However, our ammonia-N excretion rates (71.7% of the $\dot{M}_N$ values in Fig. 1C) were approximately 40% lower than those of Perry et al. (2010), perhaps reflecting the longer period of fasting; that study did not record urea-N excretion rates. Braun et al. (2009), using regularly fed adult zebrafish, reported ammonia-N excretion rates that were 2.5-fold higher than those of Perry et al. (2010) and comparable to post-prandial rates in the present study (Fig. 1C), as well as urea-N excretion rates that were approximately 13% of $\dot{M}_N$, again similar to the present data. Our $\dot{M}_N$ and $\dot{M}_{O_2}$ data on fasted adult zebrafish were also similar to, or slightly lower than, rates recorded separately in several other studies of $\dot{M}_N$ (Al-Reasi et al., 2016; Duarte et al., 2016) and $\dot{M}_{O_2}$ (Skidmore, 1967; Lucas and Priede, 1992; Plaut and Gordon, 1994; Uliano et al., 2010) in this species. Furthermore, after zebrafish were given a meal of similar size to that used in the present study, Lucas and Priede (1992) reported a clear peak in $\dot{M}_{O_2}$ that occurred far more rapidly (about 3 h post-feeding) than in the present study, where the greatest elevation more protracted (3–9 h post-feeding). This difference may have been related to activity or social interactions, as these authors assayed groups of six zebrafish in much larger respirometers. None of the previous feeding studies measured $\dot{M}_{CO_2}$.

Based on the mean RQ and NQ values (Fig. 2), we calculated the relative use of metabolic substrates in zebrafish in terms of their percentage contribution to $\dot{M}_{O_2}$ (Fig. 3A). After that, based on the total CO_2 excreted and the relative use of each metabolic substrate, the contribution of each substrate to the absolute rate of carbon use was calculated (Fig. 3B) (Lauff and Wood, 1996a). The relative use of substrates fuelling aerobic metabolism, and the absolute rate of carbon use, did not change over the experimental period in fasted fishes, so we have plotted only the average values ('F') for the 10 h period in Fig. 3. In fasted fish, the average percentages for aerobic fuel usage were 31.6% for lipids, 41.6% for carbohydrates and 26.8% for proteins (Fig. 3A); for absolute carbon usage, the values were, respectively, 33.7, 63.8, and 38.2 µg C g^{-1} h^{-1} (Fig. 3B). Thus, during fasting, zebrafish are oxidizing a mixture of fuels, but proteins/amino acids tend to be used to a lesser degree, perhaps to conserve these fuels as building blocks for protein synthesis. These observations are in line

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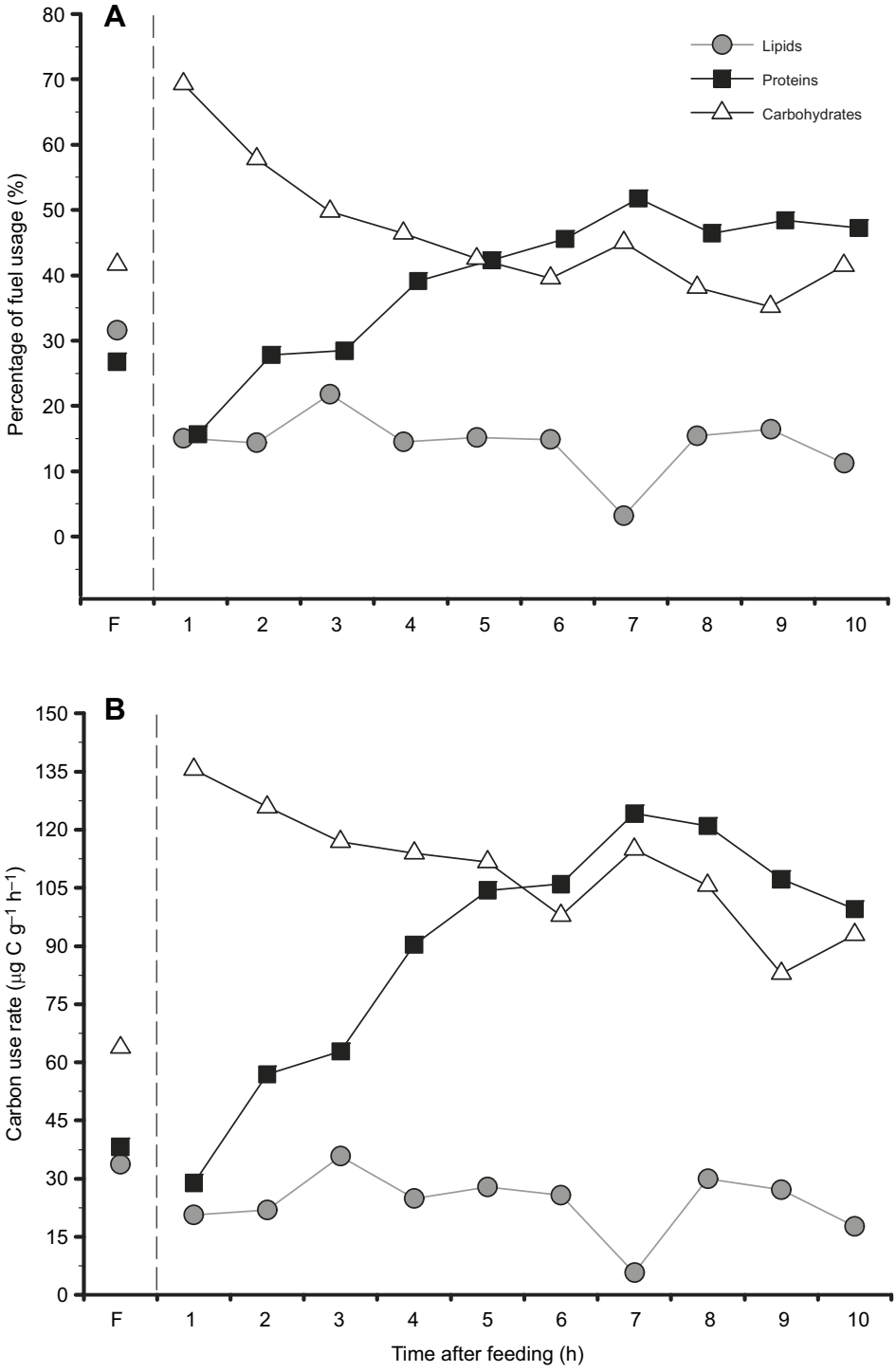

Fig. 3. Contribution of the main substrates to energy production after feeding in fed zebrafish in comparison to fasted animals. (A) Percentages of total aerobic fuel usage and (B) absolute carbon usage rates (C; μg C g^{-1} h^{-1}) from lipids, carbohydrates, and protein/amino acids of fasted or zebrafish fed to satiation (average ration of 2.12% body mass ration; 51% protein food). F represents 10-h average of fasted fish. Calculations were performed according to Lauff and Wood (1996a,b).

with our hypothesis, specifically that the usage of endogenous lipids and carbohydrates would predominate during fasting.

The higher RQ in the fed group (Fig. 2A) is the first evidence of increased oxidation of carbohydrates and/or proteins/amino acids after feeding, because these substrates have higher specific RQs than lipids (1.0, 0.96, and 0.70, respectively). Although the increase in RQ was immediate, the increase in NQ was slower, following the progressive rise in $\dot{M}_N$. Within 1 h after feeding, $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$ (Fig. 1A,B) had both increased, and this was accompanied by a marked elevation in the usage of carbohydrate to close to 70% total, while lipid and protein use dropped to approximately 15% each (Fig. 3B). As the experiment progressed, the contribution of carbohydrate fell progressively, stabilizing at its fasting level of approximately 40% after 5 h post-feeding onwards. However, right through 10 h, the contribution of lipid remained depressed at approximately 15%, whereas the balance was made up by a progressively increasing contribution from protein, which peaked at 52% at 7 h and remained close to this level through 10 h.

Although the relative changes in carbohydrate and protein/amino acid usage (Fig. 3A) were reflected in absolute changes (Fig. 3B), the depression of lipid usage on a relative basis (Fig. 3A) was not as marked on an absolute carbon usage rate (Fig. 3B). In the fed fish,

the average percentages of fuel usage for the10-h period were 14.2% for lipids, 46.5% for carbohydrates and 39.2% for proteins; for absolute carbon usage, the values were, respectively, 23.7, 109.8 and 90.1 µg C g^{-1} h^{-1}. These values represent the average contribution of each fuel to the whole SDA up to 10 h post-feeding.

However, all measured parameters (Fig. 1) remained significantly different from fasting levels at 10 h, and the SDA event was clearly ongoing at that time. Thus, metabolism and fuel use were altered for an extended period after feeding in zebrafish, at least under the conditions of our experiment. In the study of Lucas and Priede (1992), where the experimental conditions were very different and the post-feeding $\dot{M}_{O_2}$ peak was much faster, as noted earlier, it still took 12 to 24 h (depending on ration size) for the $\dot{M}_{O_2}$ to return to pre-feeding levels.

It is likely that the first 1–2 h after feeding, when the carbohydrate contribution was greatest, represents a transitional stage when nutrients from food have not yet been fully absorbed but aerobic metabolic costs are already elevated (Fig. 1). These costs can be quickly met from endogenous glycogen stores, reflected in the 55–70% carbohydrate usage at this time (Fig. 3A). Carbohydrate is clearly the main metabolic substrate used to support the early increase in metabolic costs.

The later SDA costs, in terms of aerobic fuel usage, were supported by an increasing contribution from protein/amino acids, which peaked at 7 h (53%), though the contribution of carbohydrate remained in the 35–40% range (Fig. 3A). Therefore, our original hypothesis that the metabolism of protein/amino acids would predominate during the later part of the SDA period was supported. However, integrated over the whole 10-h post-prandial period, the contribution of protein (39.2%) remained slightly less than that of carbohydrate (46.5%). Brown and Cameron (1991a,b) presented elegant experimental evidence that the major portion of SDA is due to the cost of protein synthesis and, therefore, the cost of growth. Although the anabolic building of protein requires a significant amount of O_2 to produce the ATP required to make peptide bonds (Brown and Cameron, 1991a,b), high levels of nitrogen excretion occur during protein catabolism (Ip and Chew, 2010). The present data suggest that in the later part of the SDA, a considerable quantity of protein was de-aminated and oxidized to support this cost of growth. This high usage of protein to fuel the building of protein seems counter-productive, but it should be remembered that proteins must be broken down before they can be absorbed, and the protein content of the diet was particularly high (51%). Fernandes et al. (2016) have established that the ideal percentage of dietary protein required for maximum protein retention in juvenile zebrafish is 44.8%. Therefore, it is possible that a lower-protein diet would have led to lower use of protein to power anabolic pathways.

By knowing the ration consumed (2.12±0.20% of body mass) and combining this with the $\dot{M}_N$ data (Fig. 1C), we were able to calculate 'nitrogen wastage' over the 10 h post-prandial period (see Wood et al., 2017, for calculation details and assumptions). In summary, these data revealed that 'nitrogen wastage' over 10 h amounted to approximately 20% (23.39±1.30 µmol N g^{-1}) of the total nitrogen consumed in the ration (123.76±14.72 µmol N g^{-1}). For the total nitrogen excreted, approximately 65% (15.28 µmol g^{-1} µmol N g^{-1}) was exogenous nitrogen (from food). These data indicate that when excess dietary amino acids are available to zebrafish, they are preferentially metabolized for energy production over other substrates, a pattern similar to that in many other teleost species, where there are large increases in $\dot{M}_N$ after a meal (reviewed by Wood, 2001).

In conclusion, we have used the respirometry apparatus of Harter et al. (2017), in combination with the instantaneous fuel usage calculation developed by Lauff and Wood (1996a,b), to quantitatively understand how zebrafish use their substrates during fasting and feeding. Our findings support the hypothesis that fasting is supported mainly by endogenous carbohydrates and lipids in zebrafish, though protein also contributes. Our findings also support our hypothesis that increased carbohydrate oxidation fuels the early part of the SDA, while during the later part, the oxidation of protein/amino acids becomes predominant. Additional studies of fuel usage patterns on a variety of species fed different diets under different environmental conditions are required to more fully understand the bioenergetics of fishes in ecological, evolutionary and aquacultural contexts.

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Competing interests
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Author contributions
Conceptualization: M.S.F., T.H, C.M.W., P.G.D.M. and A.L.V.; Methodology: M.S.F, T.H., C.M.W.,P.G.D.M. and A.L.V.; Formal analysis: M.S.F, T.H., C.M.W., and G.D.P.; Investigation: M.S.F., T.H., C.M.W., G.D.P., and P.G.D.M. Resources: A.L.V., C.M.W. and P.G.D.M.; Writing - original draft: M.S.F., C.M.W. and A.L.V.; Writing - review & editing: M.S.F., T.H., C.M.W., P.G.D.M., G.D.P. and A.L.V.; Visualization: M.S.F., T.H., C.M.W., P.G.D.M., G.D.P. and A.L.V.; Supervision: C.M.W., P.G.M.D. and A.L.V.; Project administration: A.L.V., C.M.W. and P.G.D.M.; Funding acquisition: C.M.W., P.G.D.M. and A.L.V.

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