

Inferring the metabolic rate of zebrafish from ventilation frequency

Tianjun Han^{1,2}, Giulia Lombardelli^{1,2}, Sean D. Peterson³, and Maurizio Porfiri^{1,2,4,*}

¹Department of Mechanical and Aerospace Engineering, Tandon School of Engineering, New
York University, Brooklyn, NY 11201, USA

²Center for Urban Science and Progress, New York University, Brooklyn, NY 11201, USA

³Department of Mechanical and Mechatronics Engineering, University of Waterloo, Waterloo,
ON N2L 3G1, Canada

⁴Department of Biomedical Engineering, Tandon School of Engineering, New York University,
Brooklyn, NY 11201, USA

* Corresponding author. E-mail: mporfiri@nyun.edu

Funding: This work was supported by the National Science Foundation award number CMMI-
1901697.

Abstract

Fish schooling has attracted the interest of the scientific community for centuries. Energy savings have been long posited to be a key determinant for the emergence of schooling patterns. Yet, current methodologies do not allow the precise quantification of the metabolic rate of specific individuals within the school, typically leaving the researchers with only a single, global measurement of metabolic rate for the collective. In this paper, we demonstrated the feasibility of inferring metabolic rate of swimming fish using the mouth opening frequency, a simple proxy that can be scored utilizing video recordings in the laboratory or in the field, even for small fish. The mouth opening frequency is independent of hydrodynamic interactions within the school, thereby mitigating potential confounding factors that arise when using locomotory measures associated with tail-beat motion. We assessed the reliability of mouth opening frequency as a proxy for metabolic rate by conducting experiments on zebrafish (*Danio rerio*) using swimming respirometry. We varied the flow speed from 0.8 to 3.2 body lengths per second and extracted tail-beat motion and mouth opening from video recordings. Our results revealed a strong correlation between oxygen uptake and mouth-opening frequency for non-zero flow speeds, but not in quiescent water. Contrary to our expectations, we did not find evidence in favor of the use of tail-beat frequency as a proxy of metabolic rate. Overall, our results open the door to the study of individual metabolic rates in fish schools without confounding factors related to hydrodynamic interactions.

Keywords: *Danio rerio*, mouth opening, metabolism, respirometry, swim tunnel, and tail beat.

Introduction

Enhanced predator avoidance and foraging behavior are some of the well-understood advantages that are bestowed upon individuals living in a group (Sumpter, 2006). Akin to insects and birds, fish attain these advantages as they propel themselves through a fluid environment (Pavlov & Kasumyan, 2000; Pitcher, 1986). Swimming in close proximity to one another, fish are subject to the flow environment created by their neighbors (Liao, 2007; Ligman et al., 2024; Timm et al., 2024). Whether they swim side-by-side or in-line, neighboring fish will interact hydrodynamically, mutually affecting their motion and influencing the forces they experience. Researchers have long proposed that these interactions reduce metabolic costs of the school (Belyayev & Zuyev, 1969; Breder, 1967; Weihs, 1973).

Swimming respirometry is the gold standard to study aerobic metabolism of fish and other aquatic organisms in laboratory settings (Liao, 2007; Mainardis et al., 2021; Zhang & Lauder, 2023a). Through swimming respirometry, one can estimate the metabolic rate of one or more subjects by measuring the rate of oxygen uptake in a swim tunnel. Swimming respirometry played a pivotal role in numerous investigations spanning from exploring the effect of climate change on aerobic scope to studying bioenergetic defects during development (Clark et al., 2013; Stackley et al., 2011), consequences of acute and chronic exposure to toxicants on swimming ability (Johansen & Esbaugh, 2017), relationships between positional preference in a school and individual metabolic phenotype (Killen et al., 2012), and obesity-related changes in locomotor performance (Seebacher et al., 2017), among others.

Several studies utilized swimming respirometry to elucidate the energetic consequences of swimming in a school. For example, (Parker Jr, 1973) and (Abrahams & Colgan, 1985) reported reduced energetic costs when schooling than when swimming in isolation; however, only a single

speed was used. When multiple speeds were considered, differing results have been observed (Burgerhout et al., 2013; Currier et al., 2021; Hvas & Oppedal, 2019). Whereas (Currier et al., 2021) confirmed energetic benefits of schooling at different swimming speeds, (Burgerhout et al., 2013) determined that these benefits were restricted to a specific range of swimming speed, and (Hvas & Oppedal, 2019) did not register variations in metabolic rates between fish swimming in isolation or in schools. Recently, (Zhang & Lauder, 2023b) put forward a comprehensive study across a broad range of swimming speeds, up to eight body lengths per second, pointing at an improved ability of schools to sustain aerobic swimming at high speeds, even in turbulent flows (Zhang et al., 2024).

As metabolic rate measurements cannot be collected from individuals within a school using swimming respirometry (that is, only a singular group average is measured), one cannot address important questions, such as: Would a fish occupying a leading position in the school experience a higher metabolic rate? Would following fish gain more energy savings? How would energy distribute in a diamond versus a phalanx formation?

A potential strategy to extract individual-level information about metabolic rate entails the use of behavioral proxies that can be scored from video observations. Tail-beat frequency is likely the most widely adopted proxy, with extant research demonstrating a robust correlation between tail-beat frequency and metabolic rate (measured using swimming respirometry). For example, (Steinhausen et al., 2005) showed a strong correlation between tail-beat frequency and oxygen consumption in saithe and whiting, suggesting that “[t]ail beat frequency may therefore serve as a predictor of swimming speed and oxygen consumption of saithe and whiting in the field.” A similar relationship has been documented for a wide variety of species, including channel catfish

(*Ictalurus punctatus*) (Vaughn et al., 2024), carp (*Cyprinus carpio*) and roach (*Rutilus rutilus*) (Ohlberger et al., 2007), and Japanese sea bass (*Lateolabrax japonicus*) (Mori et al., 2015).

All these findings were obtained for fish swimming in isolation, whether they are applicable to animals swimming in a school remains elusive. It is tenable that tail-beat motion would lock-in with coherent fluid structures in the flow, such as vortices shed by neighboring fish in the school (Weihs, 1973), skewing inferences of metabolic rate based on tail-beat frequency. It has been shown, for example, that fish swimming in the wake of a bluff body exhibit “Kármán gaiting” (Akanyeti & Liao, 2013b; Liao et al., 2003), wherein they actively adjust their lateral positions and synchronize their body movements with the shed vortices to gain thrust and reduce the energy required for locomotion (Akanyeti & Liao, 2013a; Li et al., 2017; Tong et al., 2021). (Liao, 2004) observed that the body motion of a dead trout exposed to the vortex street generated by a solid cylinder is qualitatively similar to freely swimming live trout, and, perhaps more profoundly, the dead animal generates thrust akin to the live one. The same tail-beat frequency could thus correspond to two different metabolic rates (zero for the dead animal and a non-zero value for the live one). These observations strain the assumption that tail-beat frequency is a valid proxy of metabolic rate in schools, calling for the exploration of alternative behavioral proxies.

In this study, we explored mouth opening as a proxy for metabolic rate that would not be affected by the hydrodynamic interactions that underpin the formation of a school. Previous research observed an association between metabolic rate and ventilation frequency for pikeperch (*Perca lucioperca*) (Frisk et al., 2012), Atlantic salmon (*Salmo salar*) (Millidine et al., 2008), and stoplight parrotfish (*Sparisoma viride*) (Van Rooij & Videler, 1996). These studies lacked standardization of swimming conditions, thereby challenging our ability to assess the accuracy of ventilation frequency as a proxy of metabolic rate. Specifically, (Frisk et al., 2012) measured gill

ventilation frequency of pikeperch at rest, during progressive hypoxia, and following exhaustion for temperatures ranging from 13 to 25 °C. (Millidine et al., 2008) examined gill ventilation frequency of Atlantic salmon during different behaviors including settling, feeding, and disturbance. (Van Rooij & Videler, 1996) probed gill ventilation frequency of stoplight parrotfish when stimulated by darkening the respirometer with black foil or knocking the wall of the respirometer.

We chose to experiment on zebrafish (*Danio rerio*), a popular model organism for the study of obesity and diabetes (Zang et al., 2018), social behavior (Fontana et al., 2022), and complex brain disorders (Kalueff et al., 2014). There is a vast literature on zebrafish social behavior, from laboratory studies (Miller & Gerlai, 2012) to field observations (Shelton et al., 2020), which documented the emergence of robust schooling patterns. For example, recent field observations by (Shelton et al., 2020) reported the formation of torpedo-like long schools of 2,000 individuals in natural fast flowing flows, in contrast to small schools of 3-30 individuals in quiescent flows. Several studies previously investigated zebrafish swimming against a flow in swim tunnels (Bek et al., 2021; Lucon-Xiccato et al., 2021; Mwaffo et al., 2017) and characterized their metabolism via swimming respirometry (Huang et al., 2020; Massé et al., 2013; Thomas & Janz, 2011). We performed experiments for swimming speeds from 0.8 to 3.2 body lengths per second, for which (Thomas & Janz, 2011) identified an approximately constant metabolic rate. Such a nearly flat metabolism-speed curve is likely due to some balance in the energy costs associated with the activation of pink and white muscle fibers in unsteady swimming and postural maintenance (Altringham & Ellerby, 1999; Coughlin, 2002). From video recordings, we characterized fish kinematics in terms of both the tail-beat motion and the mouth opening. We hypothesized that both

the tail-beat and ventilation frequencies would capture individual variations in metabolic rate among the subjects.

Materials and Methods

Ethics

All animal procedures were approved by the University Animal Welfare Committee of New York University under the protocol number 13-1424.

Animal care and maintenance

In this study, 32 wild-type adult zebrafish (*Danio rerio*; 16 males and 16 females) were tested. Fish were purchased from Carolina Biological Supply Co. (Burlington, NC, USA). The average body length (total length) and mass of the fish were 3.51 ± 0.05 cm and 0.46 ± 0.03 g (mean $\pm$ standard error), respectively. Following recommended protocols (Aleström et al., 2020; Avdesh et al., 2012), fish were housed in an 89.6 L aquarium [180 cm (length) $\times$ 60 cm (height) $\times$ 87 cm (width)], at a stocking density of approximately 1 fish/L. Male and female fish were housed in separate tanks, thereby easing the process of selecting sexes across conditions and avoiding breeding. Temperature, pH, and air saturation of the vivarium were maintained at 26 °C, 7.2 pH, and above 95%, respectively. Additionally, 50% of the water in the vivarium was replaced weekly with tap water. Stress coat was added into the tap water to remove chlorine and chloramines (API stress coat Aquarium Conditioner, API® Fishcare). Fish were kept under a 12 h light/12 h dark photo-period and fed with commercial flake food (TetraMin Tropical Flakes) ad libitum once a day, at approximately 7 pm. Prior to the experiments, fish were acclimatized in the aquarium for one month. Fish were fasted for 24h prior to starting each trial to avoid feeding metabolism interactions on the metabolic rate measurements. After testing, each fish was hand-netted to a

different vivarium with the same conditions. Tested fish were kept in groups to prevent social isolation.

Experimental setup and procedure

A swim tunnel respirometer was used (Loligo[®] Systems, Tjele, Denmark) was used to measure the metabolic rate of fish, $\dot{M}O_2$ (mgO₂ kg⁻¹ h⁻¹) (Figure 1a). The respirometer consisted of a 20-L water bath [575mm (length) x 200mm (width) x 250 (height)], incorporating a 170-mL swim tunnel [26.4mm (internal diameter) x 100mm (length)], a propeller coupled with an electric motor, an optical oxygen sensor (accuracy of +/- 0.5% air saturation), a temperature sensor (accuracy of +/- 0.15°C), and a flush pump – all part of the system. The flow speed in the swim tunnel was controlled by the propeller and calibrated using particle image velocimetry (Raffel et al., 2018). The water bath maintained the temperature of the swim tunnel and supplied fully aerated water to the tunnel when the flush pump was turned on. The temperature of the bath was maintained at 26 °C by a thermostat, and the oxygen saturation was maintained above 98% by an air pump. The oxygen sensor measured the temperature-corrected oxygen concentration (mg/L) inside the chamber at a frequency of 1 Hz. To measure fish kinematics, a mirror was installed at an angle of 45° behind the swim tunnel, allowing a high-speed camera (Model Flea3 high-speed camera., FLIR Systems Inc, San Diego, CA, USA) to record the top view. Video recordings were made at 60 fps, at a spatial resolution of 1530 by 670 pixels (47 um per pixel). To ensure minimum disturbance to fish during the experiments, the entire experimental setup was covered by black plastic sheets. Additionally, the room lights were turned off, and the test section was illuminated by a fluorescent tube above it. The control of the entire system and data acquisition were performed using AutoResp[™] 2 software from Loligo[®] Systems.

Four fish (two males and two females) were tested at flow speeds of 0.8, 1.2, 1.8, 2.2, 2.8, and 3.2 body lengths per second (BL/s), totaling 24 subjects. To avoid false inference of significance due to multiple conditions, we grouped these speeds into three groups: low speed (L, 0.8 and 1.2 BL/s), medium speed (M, 1.8 and 2.2 BL/s), and high speed (H, 2.8 and 3.2 BL/s). To verify the J-shape in the metabolism-speed relationship documented in the literature,^{10,22,44} we tested eight additional fish (four males and four females) in placid water (Z, speed of 0 BL/s). Each fish was only tested once. Two experiments were conducted per day, one approximately at 10 am and one at 2 pm, which balanced the execution times of the experiments. Furthermore, the order of testing speeds was randomized to avoid accidental biases. Throughout one trial at the flow speed of 2.8 BL/s and another at 1.8 BL/s, fish touched the end of the swim tunnel and remained still: these two trials were excluded from the dataset.

Before each experiment, the respirometer was cleaned and filled with pre-filtered fresh water to remove any residue from previous tests. At the beginning of each experiment, a fish was hand-netted from the vivarium. The mass and body length of the fish were gently measured, after which the fish was introduced into the swim tunnel. Based on previous studies by (Huang et al., 2020; Massé et al., 2013), fish were given two hours of acclimation at a flow speed of 0.7 BL/s for any test at a speed different from zero. When tested at zero speed, they were, instead, acclimatized in still water. The flush pump was turned on at the beginning of the acclimation time to flush fully aerated water into the swim tunnel. After the acclimation, the flow in the swim tunnel was set to the prescribed testing speed, and the flush pump was kept on. Flushing lasted for two minutes. After this period, the flush pump was turned off, and the swim tunnel was closed to allow the oxygen concentration inside the swim tunnel to stabilize for five minutes. During those five minutes, the oxygen concentration was not measured. The 5-min stabilization period was based on

pilot experiments, wherein we found that this time was sufficient for fish to continually orient their body parallel to the flow after the flushing period. After the stabilization period, the oxygen concentration was measured over a five-minute window. When measuring the oxygen concentration, the kinematics and ventilation of the fish were simultaneously recorded using the high-speed camera. The closed and open periods of the respirometer were seven and five minutes, respectively. After the measurement, the fish was removed from the respirometer and placed back in the aquarium. The background respirometry was then measured following the same procedures as the measurement with fish (measurement for five minutes).

For each minute of observation ($\Delta t = 60$ s), the metabolic rate was calculated as

$$\dot{M}O_2 = \frac{1}{m} \frac{(\Delta O_2 - \Delta O_{2,background}) \cdot (V_r - V_f)}{\Delta t}$$

where ΔO_2 is the change of oxygen concentration over the one-minute time window, $\Delta O_{2,background}$ is the change of oxygen concentration for the background respirometry, V_r is the swim tunnel volume, V_f is the fish volume (1g body mass = 1 ml water), and m is the fish mass. Thus, five data points per experiment were generated. The R^2 of the linear regression of oxygen concentration versus time during a one-minute observation ranges from 0.96 to 0.99 across the entire dataset with and without fish.

Video tracking

Tail-beat motion and mouth opening were studied by tracking fiducial points from the high-speed video. Specifically, four fiducial points – the tip of the fish’s mouth, the fish’s head, and the leading and trailing edges of the caudal fin – were tracked using DeepLabCutTM v2.3.8 (Figure 1b) (Mathis et al., 2018, 2021; Nath et al., 2019). Mouth opening was calculated from the increase in the

distance between the tip of the mouth and the head relative to the distance with the mouth closed. Such a relative head-to-mouth distance was normalized by the fish body length; instances of mouth opening were identified as the local peaks of such a distance. We applied a threshold of 0.3% the body length to filter out noise in the identification of the local peaks of the relative head-to-mouth distance so that movements smaller than this threshold would not confound the mouth opening estimation. For each minute of observation, the normalized mouth-opening amplitude (MOA) was calculated by averaging the prominence (how much a peak stands out from the neighboring baseline) of the identified peaks over that minute-long observation, and the mouth opening frequency (MOF) by dividing the number of those peaks by the observation length – see Figure S1a in Supplementary Material.

To score tail-beat motion, a neutral line was drawn between the tip of the fish's mouth and its head. The tail-beat amplitude was calculated as the perpendicular distance from the trailing edge of the caudal fin to the neutral line and was normalized by the body length and averaged over one minute to compute the normalized tail-beat amplitude (TBA). The tail-beat frequency (TBF) was calculated by Fourier transforming the one-minute time-series data of the tail-beat motion waveform⁴⁸ – see Figure S1b in Supplementary Material.

Statistical analyses

Analyses of $\dot{M}O_2$, MOA, MOF, TBA, and TBF were conducted using two-way mixed ANOVA considering the flow speed (Z, L, M, and H) as a between-subject factor and the time bin (one-minute intervals) as a within-subject factor. Tukey-Kramer *post-hoc* tests were performed in the case of significant effects. The significance level of all statistical tests in the study was set at 0.05. All statistical analyses were conducted in MATLAB R2023b (The MathWorks, Inc. Natick,

Massachusetts, United States) using functions `fitrm`, `ranova`, and `fitlme` – the complete dataset for the analysis is included as a Supplementary Table.

Linear multilevel regressions were conducted to examine the relationship between $\dot{M}O_2$ and either MOF or TBF. Following (Rogers & Weatherley, 1983), who found that both the intercept and slope of the linear relationship between metabolism and ventilation frequency vary with fish identity, we considered fish identity as a random effect in both the intercept and slope in our model. Therefore, the linear multilevel regression for MOF was formulated as

$$\dot{M}O_2 = [\beta_0 + (\beta_0 | \text{Fish})] + [\beta_1 + (\beta_1 | \text{Fish})] \text{MOF},$$

where β_0 and β_1 are the intercept and slope of the fixed effect (general proxy-relationship under investigation), respectively, and $(\beta_0 | \text{Fish})$ and $(\beta_1 | \text{Fish})$ are the intercept and slope of the random effect (fish identities), respectively – a similar model was used for TBF. Similar to (Rogers & Weatherley, 1983; Weatherley et al., 1982), we performed independent regressions for the zero and non-zero speed data to acknowledge expected differences in fish ventilation at zero and non-zero speeds. For each regression, we report conditional and marginal R^2 (R^2_c and R^2_m , respectively).

To assess the prediction error of the linear regression, we applied a jack-knife approach to the linear relationships that were found to be statistically significant (Halsey et al., 2009, 2011; Lear et al., 2016). Within this approach, we excluded data for one fish at a time and computed a new linear regression using the data of the remaining fish. The new linear relationship for the fixed effect was used to predict the data of the excluded fish. Finally, we calculated the coefficient of variability (COV) for the excluded fish as the ratio of the standard error of the predicted value over the mean measured value (Green, 2011).

Results

Metabolism

We identified a significant effect of the flow speed on the metabolic rate (Figure 2, flow speed: $F(3, 26) = 30.90, p < 0.001$). We did not register a statistically significant effect of time bin or an interaction between flow speed and time bin (time bin: $F(4, 104) = 2.19, p = 0.075$; flow speed $\times$ time bin: $F(12, 104) = 1.25, p = 0.258$), so that one may argue that the effect of the flow on the metabolic rate was consistent over time. The metabolic rate was maximized at zero speed ($p < 0.001$ in *post-hoc* comparisons).

Inspecting the motion of fish at zero or non-zero flow speeds, we identified key locomotory differences. In quiescent water, fish actively oscillated its pectoral fins, and passive oscillation of its caudal fin was induced, exhibiting a maneuvering motion (Figure 3a). During this maneuvering motion, fish could orient either to the left or right in the swim tunnel. At non-zero flow speeds, fish actively oscillated their caudal fins and swam against the flow with their pectoral fins very close to their bodies (Figure 3b).

Tail-beat motion

In agreement with one's expectations (Videler, 1993), the tail-beat frequency varied with the flow speed (Figure 4a, flow speed: $F(3, 26) = 34.01, p < 0.001$) and such a dependence was consistent over time, whereby we did not observe a significant effect of the time bin or an interaction between the flow speed and time bin on the tail-beat frequency (time bin: $F(4, 104) = 1.05, p = 0.386$; flow speed $\times$ time bin: $F(12, 104) = 0.65, p = 0.798$). The tail-beat frequency increased when varying the flow speed from low to high ($p < 0.001$ in *post-hoc* comparisons).

No statistically significant effect of the flow speed, time bin, or their interaction was observed on the normalized tail-beat amplitude as a function of the flow speed (Figure 4b, flow speed: $F(3, 26) = 0.65, p = 0.382$; time bin: $F(4, 104) = 0.95, p = 0.437$; flow speed $\times$ time bin: $F(12, 104) = 0.90, p = 0.554$).

Mouth opening

We did not observe any significant effect of the flow speed, time bin, or their interaction on the mouth-opening frequency (Figure 5a, flow speed: $F(3, 26) = 0.90, p = 0.456$; time bin: $F(4, 104) = 2.00, p = 0.099$; flow speed $\times$ time bin: $F(12, 104) = 0.74, p = 0.708$). Likewise, we did not register any significant effect of the flow speed, time bin, or their interaction on the normalized mouth-opening amplitude (Figure 5b, flow speed: $F(3, 26) = 0.15, p = 0.928$; time bin: $F(4, 104) = 0.29, p = 0.885$; flow speed $\times$ time bin: $F(12, 104) = 0.85, p = 0.600$). A synoptic presentation of all the average values $\pm$ standard error and sample sizes at each speed is presented in Table S1.

Linear multilevel regressions

Linear multilevel regression revealed a dependence of $\dot{M}O_2$ on MOF at non-zero flow speeds (Figure 6a, $p < 0.001, R^2_c = 0.69$), whereas no significant linear relationship between $\dot{M}O_2$ and MOF was identified at zero flow speed (Figure 6b, $p = 0.969, R^2_c = 0.01$). The linear relationship of the fixed effect between $\dot{M}O_2$ ($\text{mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$) and MOF (Hz) at non-zero speed is

$$\dot{M}O_2 = 240.97 + 226.80 \text{ MOF}.$$

Linear multilevel regressions did not yield any significant linear relationships between $\dot{M}O_2$ and TBF at non-zero (Figure 7a, $p = 0.483, R^2_c = 0.61$) or zero flow speed (Figure 7b, $p = 0.120, R^2_c = 0.08$). The COV of the linear relationship between $\dot{M}O_2$ and MOF at non-zero speeds range from

1% to 13%, as computed based on the jack-knife approach. Details of the regressions are presented in Tables 1 and 2.

Discussion

Swimming respirometry is the gold standard to study metabolic rate in fish, yet, its practical use for investigating the benefits and costs of swimming in a school is limited. Through swimming respirometry, one can only gather information about the global metabolic rate of the collective, without access to information about specific individuals in the school. Recent strides in computer vision enable high-resolution analysis of fish behavior, affording the potential for proxy measurement of metabolic rate from video tracking (Dell et al., 2014; Panadeiro et al., 2021).

In this study, we demonstrated the possibility of inferring metabolic rate of zebrafish swimming in a flow from their ventilation frequency, scored with a single camera observing the mouth opening of the subject – an original contribution of our work. Specifically, we simultaneously measured metabolic rate and ventilation frequency in a swimming respirometer at flow speeds, ranging from 0.8 to 3.2 body lengths per second. This observation is in agreement with previous work, which identified linear correlations between metabolic rate and ventilation frequency pikeperch (Frisk et al., 2012), Atlantic salmon (Millidine et al., 2008), and stoplight parrotfish (Van Rooij & Videler, 1996). Different from previous work, we scored ventilation frequency from mouth opening, rather than gill ventilation frequency. From a physiological point of view, mouth opening and gill ventilation are expected to encode analogous information about the oxygen uptake, as water passes through the mouth and then pumped over the gills (Hoar & Randall, 1984). Mouth opening is, however, easier to measure for small fish, like zebrafish, for which a single bird's eye view camera suffices to automatically score amplitude and frequency.

The availability of a behavioral proxy to infer metabolic rate of zebrafish from videos, combined with the growing body of literature on low-cost swim tunnels (Bek et al., 2021; Lucon-Xiccato et al., 2021; Mwaffo et al., 2017), opens the door to a new class of high-throughput experiments to detail links between aberrant metabolism and behavior.

In our experiments, we did not find indications of changes in the mouth-opening amplitude, which was approximately constant upon normalizing by the fish body length. Without the need for mouth-opening amplitude to predict metabolic rates, one may pursue simpler videography focusing on the frequency content of the pixels near the head of the subject with stationary or moving cameras. Such an approach may be valuable in less-controlled experimental conditions, in which the experimenter may not have access to high quality images or viewing angle with respect to the fish is unknown. It is presently unclear whether or not the predictive power of the mouth-opening amplitude of metabolic rate remains secondary as the flow speed increases. In our study, we focused on a relatively narrow range of flow speeds up to 3.2 body lengths per second, in which variations of the metabolic rate with the flow speed are minimal. Increasing the flow speed is expected to produce robust changes in the metabolic rate (Thomas & Janz, 2011), which may reverberate in variations of zebrafish breathing patterns.

While we were hoping for some associations between mouth-opening frequency and metabolic rate due to the extant use of quiescent water in behavioral assays on zebrafish (Fontana et al., 2022; Kalueff et al., 2014; Zang et al., 2018), the predictive value of mouth-opening frequency as a proxy of metabolic rate does not extend to experiments performed in quiescent water. Such a finding could have been expected based on prior observations on other fish species; in particular, for rainbow trout (*Oncorhynchus mykiss*), (Rogers & Weatherley, 1983) discovered a good linear association between metabolic rate and ventilation frequency only for forced

swimming. At zero flow speed, the authors identified a poor relationship between these variables, which they proposed to be related to the ability of fish to regulate oxygen uptake at the gill surfaces through the blood flow dynamics through the gills and/or changes in the cardiac.

At zero flow speed, zebrafish exhibit higher metabolic rate, following the typical J-curve observed in the literature for other fish species (Di Santo et al., 2017; Zhang & Lauder, 2023a, 2023b). For giant danio and skate, the higher metabolic rate in near-still flows was attributed to high postural costs (Di Santo et al., 2017; Di Santo & Kenaley, 2016; Zhang & Lauder, 2023a, 2023b), due to the need to exert higher stabilizing forces through fin and body movements to compensate for the loss of fluid-assisted stability (Di Santo et al., 2017; Dowis et al., 2003; He & Wardle, 1986; Lauder & Di Santo, 2015; Wilga & Lauder, 2000). This enhanced stability control was also observed in the present study, where at zero flow speed zebrafish actively oscillated their pectoral fins to maneuver in the swim tunnel. The pectoral fin motion was secondary when swimming in a flow, where fish actively oscillated their tail to propel against the incoming current.

Contrary to our expectations, we did not gather support for the use of tail-beat frequency as a proxy of metabolic rate even for isolated subjects. While tail-beat frequency increased with the flow speed to support the need for greater thrust generation, the extent of the change was not mirrored by a concomitant variation in the metabolic rate. A potential explanation could be the limited size of the dataset, which did not allow for bringing to light an association between tail-beat motion and metabolism. However, we do not favor such an explanation, based on power analyses conducted prior to the experiments, as well as the identification of a strong correlation between metabolic rate and the other candidate proxy, the mouth-opening frequency. Instead, we propose that the observed response is related to the limited capability of zebrafish to adapt to swim at moderately low speeds. Similar to trout, cetacean, blacktip shark, saith, mackerel, and dace,

zebrafish maintain a tail-beat amplitude of 10% its body length at any flow speed and increase their tail-beat frequency linearly with the flow speed (Saadat et al., 2017). The extent of the change of the tail-beat frequency was, however, much steeper for zebrafish, whereby the Strouhal number ($St = (2 \times \text{tail-beat amplitude}) \times (\text{tail-beat frequency}) / (\text{flow speed})$) for zebrafish was approximately 0.8, which is outside of the range between 0.2 and 0.4 for optimal thrust generation (Gazzola et al., 2014; Saadat et al., 2017; Triantafyllou et al., 2000). The Strouhal number of zebrafish swimming suggests that unlike other aquatic animals, zebrafish – indigenous to ponds and other bodies of standing water (Spence et al., 2008) – did not develop high propulsive efficiency through natural selection (Saadat et al., 2017).

Future work should explore a wider range of flow speed to further test the validity of mouth-opening and tail-beat frequencies as proxies of metabolic rate. We can neither exclude the possibility of a nonlinear relationship between mouth-opening frequency and metabolic rate nor of an improved predictive power of tail-beat frequency at higher flow speeds. Such a validation may entail the use of recent techniques developed by (Zhang et al., 2024; Zhang & Lauder, 2023b) to address anaerobic metabolic rate, whereby traditional swimming respirometry is limited to aerobic metabolic rate. We should also mention that the validity of mouth-opening frequency as a proxy of metabolic rate has only been tested at a given temperature; temperature changes are known to affect metabolic rate (Clarke & Johnston, 1999) and dissolved oxygen content (Ficke et al., 2007), thereby likely modulating the observed relationships. We also envision research into the gill ventilation frequency to better detail respiration of zebrafish and its relationship to metabolic rate. Our current setup is not suited to perform such an analysis due to the trade-off between the image resolution and acquisition frequency we adopted to accurately score mouth-opening frequency. Gills would be barely visible at our current camera resolution. An improved

resolution of the gills might facilitate the understanding of gas exchange taking place across the gills epithelium, which is dictated by the volume of water drawn to the gills and the degree of resistance that the lamellae oppose to the water flow, among other factors (Hughes, 1960; Hughes & Shelton, 1958).

Most importantly, future work should leverage the proposed proxy of metabolic rate to elucidate energy budgeting in zebrafish schools and shed light on the relationship between positional preference and energy savings. Experiments should commence with the study of fish pairs, as an archetypical, minimalistic model of a school (Ashraf et al., 2016; De Bie et al., 2020; Lombana & Porfiri, 2022). Using mouth-opening frequency, one may attempt at quantifying the specific energy savings and costs for the leader and follower in in-line swimming and exploring variations in energy budgeting when transitioning to tandem, side-by-side configurations. In addition, by comparing the tail-beat motions of the two subjects, one may attempt at assessing the role of synchronization of tail beating, or lack thereof, into energy budgeting for the pair.

Acknowledgements

The authors would like to thank Rayan Succar, Christopher Buglino, Fabiana Sofia Ricci, Dr. Roni Barak Ventura, and Dr. Simone Macri for helpful discussions on the design of the experiments and statistics and Dr. Mert Karakaya for preliminary work in the laboratory on the topic.

CRedit authorship contribution statement

Conceptualization, SDP and MP; Data Curation, TH; Formal Analysis, TH, GL, and MP; Funding Acquisition, SDP and MP; Investigation, TH, GL, and MP; Methodology, TH and MP; Project Administration, TH and MP; Resources, MP; Software, TH; Supervision, MP; Validation, SDP

and MP; Visualization, TH, SDP, and MP; Writing – Original Draft Preparation, TH, GL, SDP, and MP; Writing – Review & Editing, SDP and MP.

Disclosure Statement

The authors report no conflict of interest.

References

- Abrahams, M. V., & Colgan, P. W. (1985). Risk of predation, hydrodynamic efficiency and their influence on school structure. *Environmental Biology of Fishes*, 13(3), Article 3. <https://doi.org/10.1007/BF00000931>
- Akanyeti, O., & Liao, J. C. (2013a). A kinematic model of Kármán gaiting in rainbow trout. *Journal of Experimental Biology*, jeb.093245. <https://doi.org/10.1242/jeb.093245>
- Akanyeti, O., & Liao, J. C. (2013b). The effect of flow speed and body size on Kármán gait kinematics in rainbow trout. *Journal of Experimental Biology*, jeb.087502. <https://doi.org/10.1242/jeb.087502>
- Aleström, P., D’Angelo, L., Midtlyng, P. J., Schorderet, D. F., Schulte-Merker, S., Sohm, F., & Warner, S. (2020). Zebrafish: Housing and husbandry recommendations. *Laboratory Animals*, 54(3), Article 3. <https://doi.org/10.1177/0023677219869037>
- Altringham, J. D., & Ellerby, D. J. (1999). Fish swimming: Patterns in muscle function. *Journal of Experimental Biology*, 202(23), 3397–3403.
- Ashraf, I., Godoy-Diana, R., Halloy, J., Collignon, B., & Thiria, B. (2016). Synchronization and collective swimming patterns in fish (*Hemigrammus bleheri*). *Journal of The Royal Society Interface*, 13(123), Article 123. <https://doi.org/10.1098/rsif.2016.0734>

445 Avdesh, A., Chen, M., Martin-Iverson, M. T., Mondal, A., Ong, D., Rainey-Smith, S., Taddei, K.,
 446 Lardelli, M., Groth, D. M., Verdile, G., & Martins, R. N. (2012). Regular care and
 447 maintenance of a zebrafish (*Danio rerio*) laboratory: An introduction. *Journal of*
 448 *Visualized Experiments*, 69, Article 69. <https://doi.org/10.3791/4196>
 449 Bek, J. W., De Clercq, A., Coucke, P. J., & Willaert, A. (2021). The ZE-Tunnel: An affordable,
 450 easy-to-assemble, and user-friendly benchtop zebrafish swim tunnel. *Zebrafish*, 18(1),
 451 Article 1. <https://doi.org/10.1089/zeb.2020.1948>
 452 Belyayev, V., & Zuyev, G. (1969). Hydrodynamic hypothesis of school formation in fishes.
 453 *Problems of Ichthyology*, 9(27), Article 27.
 454 Breder, C. M. (1967). On the survival value of fish schools. *Zoologica : Scientific Contributions*
 455 *of the New York Zoological Society.*, 52(4), Article 4. <https://doi.org/10.5962/p.203258>
 456 Burgerhout, E., Tudorache, C., Brittiijn, S. A., Palstra, A. P., Dirks, R. P., & Van Den Thillart, G.
 457 E. E. J. M. (2013). Schooling reduces energy consumption in swimming male European
 458 eels, *Anguilla anguilla* L. *Journal of Experimental Marine Biology and Ecology*, 448, 66–
 459 71. <https://doi.org/10.1016/j.jembe.2013.05.015>
 460 Clark, T. D., Sandblom, E., & Jutfelt, F. (2013). Aerobic scope measurements of fishes in an era
 461 of climate change: Respirometry, relevance and recommendations. *Journal of*
 462 *Experimental Biology*, 216(15), Article 15. <https://doi.org/10.1242/jeb.084251>
 463 Clarke, A., & Johnston, N. M. (1999). Scaling of metabolic rate with body mass and temperature
 464 in teleost fish. *Journal of Animal Ecology*, 68(5), 893–905.
 465 Coughlin, D. J. (2002). Aerobic muscle function during steady swimming in fish. *Fish and*
 466 *Fisheries*, 3(2), 63–78.

467 Currier, M., Rouse, J., & Coughlin, D. J. (2021). Group swimming behaviour and energetics in
 468 bluegill *Lepomis macrochirus* and rainbow trout *Oncorhynchus mykiss*. *Journal of Fish*
 469 *Biology*, 98(4), Article 4. <https://doi.org/10.1111/jfb.14641>

470 De Bie, J., Manes, C., & Kemp, P. S. (2020). Collective behaviour of fish in the presence and
 471 absence of flow. *Animal Behaviour*, 167, 151–159.
 472 <https://doi.org/10.1016/j.anbehav.2020.07.003>

473 Dell, A. I., Bender, J. A., Branson, K., Couzin, I. D., De Polavieja, G. G., Noldus, L. P. J. J., Pérez-
 474 Escudero, A., Perona, P., Straw, A. D., Wikelski, M., & Brose, U. (2014). Automated
 475 image-based tracking and its application in ecology. *Trends in Ecology & Evolution*, 29(7),
 476 Article 7. <https://doi.org/10.1016/j.tree.2014.05.004>

477 Di Santo, V., & Kenaley, C. P. (2016). Skating by: Low energetic costs of swimming in a batoid
 478 fish. *Journal of Experimental Biology*, jeb.136358. <https://doi.org/10.1242/jeb.136358>

479 Di Santo, V., Kenaley, C. P., & Lauder, G. V. (2017). High postural costs and anaerobic
 480 metabolism during swimming support the hypothesis of a U-shaped metabolism–speed
 481 curve in fishes. *Proceedings of the National Academy of Sciences*, 114(49), Article 49.
 482 <https://doi.org/10.1073/pnas.1715141114>

483 Dowis, H. J., Sepulveda, C. A., Graham, J. B., & Dickson, K. A. (2003). Swimming performance
 484 studies on the eastern Pacific bonito *Sarda chiliensis*, a close relative of the tunas (family
 485 Scombridae) II. Kinematics. *Journal of Experimental Biology*, 206(16), Article 16.
 486 <https://doi.org/10.1242/jeb.00496>

487 Ficke, A. D., Myrick, C. A., & Hansen, L. J. (2007). Potential impacts of global climate change
 488 on freshwater fisheries. *Reviews in Fish Biology and Fisheries*, 17, 581–613.

Fontana, B. D., Müller, T. E., Cleal, M., De Abreu, M. S., Norton, W. H. J., Demin, K. A.,
 Amstislavskaya, T. G., Petersen, E. V., Kalueff, A. V., Parker, M. O., & Rosemberg, D. B.
 (2022). Using zebrafish (*Danio rerio*) models to understand the critical role of social
 interactions in mental health and wellbeing. *Progress in Neurobiology*, 208, 101993.
<https://doi.org/10.1016/j.pneurobio.2021.101993>

Frisk, M., Skov, P. V., & Steffensen, J. F. (2012). Thermal optimum for pikeperch (*Sander
 lucioperca*) and the use of ventilation frequency as a predictor of metabolic rate.
Aquaculture, 324–325, 151–157. <https://doi.org/10.1016/j.aquaculture.2011.10.024>

Gazzola, M., Argentina, M., & Mahadevan, L. (2014). Scaling macroscopic aquatic locomotion.
Nature Physics, 10(10), Article 10. <https://doi.org/10.1038/nphys3078>

Green, J. A. (2011). The heart rate method for estimating metabolic rate: Review and
 recommendations. *Comparative Biochemistry and Physiology Part A: Molecular &
 Integrative Physiology*, 158(3), Article 3. <https://doi.org/10.1016/j.cbpa.2010.09.011>

Halsey, L. G., Jones, T. T., Jones, D. R., Liebsch, N., & Booth, D. T. (2011). Measuring energy
 expenditure in sub-adult and hatchling sea turtles via accelerometry. *PLoS ONE*, 6(8),
 Article 8. <https://doi.org/10.1371/journal.pone.0022311>

Halsey, L. G., Shepard, E. L. C., Quintana, F., Gomez Laich, A., Green, J. A., & Wilson, R. P.
 (2009). The relationship between oxygen consumption and body acceleration in a range of
 species. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative
 Physiology*, 152(2), Article 2. <https://doi.org/10.1016/j.cbpa.2008.09.021>

He, P., & Wardle, C. S. (1986). Tilting behaviour of the Atlantic mackerel, *Scomber scombrus* , at
 low swimming speeds. *Journal of Fish Biology*, 29(sA), Article sA.
<https://doi.org/10.1111/j.1095-8649.1986.tb05013.x>

512 Hoar, W., & Randall, D. (1984). *Gills: Anatomy, Gas Transfer, and Acid-Base Regulation; Part*
513 *A*.

514 Huang, S.-H., Tsao, C.-W., & Fang, Y.-H. (2020). A miniature intermittent-flow respirometry
515 system with a 3D-printed, palm-sized zebrafish treadmill for measuring rest and activity
516 metabolic rates. *Sensors*, 20(18), Article 18. <https://doi.org/10.3390/s20185088>

517 Hughes, G. (1960). A comparative study of gill ventilation in marine teleosts. *Journal of*
518 *Experimental Biology*, 37(1), 28–45.

519 Hughes, G., & Shelton, G. (1958). The mechanism of gill ventilation in three freshwater teleosts.
520 *Journal of Experimental Biology*, 35(4), 807–823.

521 Hvas, M., & Oppedal, F. (2019). Influence of experimental set-up and methodology for
522 measurements of metabolic rates and critical swimming speed in Atlantic salmon *Salmo*
523 *salar*. *Journal of Fish Biology*, 95(3), Article 3. <https://doi.org/10.1111/jfb.14087>

524 Johansen, J. L., & Esbaugh, A. J. (2017). Sustained impairment of respiratory function and swim
525 performance following acute oil exposure in a coastal marine fish. *Aquatic Toxicology*,
526 187, 82–89. <https://doi.org/10.1016/j.aquatox.2017.04.002>

527 Kalueff, A. V., Stewart, A. M., & Gerlai, R. (2014). Zebrafish as an emerging model for studying
528 complex brain disorders. *Trends in Pharmacological Sciences*, 35(2), Article 2.
529 <https://doi.org/10.1016/j.tips.2013.12.002>

530 Killen, S. S., Marras, S., Steffensen, J. F., & McKenzie, D. J. (2012). Aerobic capacity influences
531 the spatial position of individuals within fish schools. *Proceedings of the Royal Society B:*
532 *Biological Sciences*, 279(1727), Article 1727. <https://doi.org/10.1098/rspb.2011.1006>

533 Lauder, G. V., & Di Santo, V. (2015). Swimming Mechanics and Energetics of Elasmobranch
 534 Fishes. In *Fish Physiology* (Vol. 34, pp. 219–253). Elsevier. [https://doi.org/10.1016/B978-](https://doi.org/10.1016/B978-0-12-801289-5.00006-7)
 535 0-12-801289-5.00006-7

536 Lear, K. O., Whitney, N. M., Brewster, L. R., Morris, J. J., Hueter, R. E., & Gleiss, A. C. (2016).
 537 Correlations of metabolic rate and body acceleration in three species of coastal sharks
 538 under contrasting temperature regimes. *Journal of Experimental Biology*, jeb.146993.
 539 <https://doi.org/10.1242/jeb.146993>

540 Li, C., Yang, W., Xu, X., Wang, J., Wang, M., & Xu, L. (2017). Numerical investigation of fish
 541 exploiting vortices based on the Kármán gaiting model. *Ocean Engineering*, 140, 7–18.
 542 <https://doi.org/10.1016/j.oceaneng.2017.05.011>

543 Liao, J. C. (2004). Neuromuscular control of trout swimming in a vortex street: Implications for
 544 energy economy during the Kármán gait. *Journal of Experimental Biology*, 207(20),
 545 Article 20. <https://doi.org/10.1242/jeb.01125>

546 Liao, J. C. (2007). A review of fish swimming mechanics and behaviour in altered flows.
 547 *Philosophical Transactions of the Royal Society B: Biological Sciences*, 362(1487), Article
 548 1487. <https://doi.org/10.1098/rstb.2007.2082>

549 Liao, J. C., Beal, D. N., Lauder, G. V., & Triantafyllou, M. S. (2003). The Kármán gait: Novel
 550 body kinematics of rainbow trout swimming in a vortex street. *Journal of Experimental*
 551 *Biology*, 206(6), Article 6. <https://doi.org/10.1242/jeb.00209>

552 Ligman, M., Lund, J., & Fürth, M. (2024). A comprehensive review of hydrodynamic studies on
 553 fish schooling. *Bioinspiration & Biomimetics*, 19(1), Article 1.
 554 <https://doi.org/10.1088/1748-3190/ad1335>

555 Lombana, D. A. B., & Porfiri, M. (2022). Collective response of fish to combined manipulations
 556 of illumination and flow. *Behavioural Processes*, 203, 104767.
 557 <https://doi.org/10.1016/j.beproc.2022.104767>
 558 Lucon-Xiccato, T., Bella, L., Mainardi, E., Baraldi, M., Bottarelli, M., Sandonà, D., & Bertolucci,
 559 C. (2021). An automated low-cost swim tunnel for measuring swimming performance in
 560 fish. *Zebrafish*, 18(3), Article 3. <https://doi.org/10.1089/zeb.2020.1975>
 561 Mainardis, M., Buttazzoni, M., Cottés, M., Moretti, A., & Goi, D. (2021). Respirometry tests in
 562 wastewater treatment: Why and how? A critical review. *Science of The Total Environment*,
 563 793, 148607. <https://doi.org/10.1016/j.scitotenv.2021.148607>
 564 Massé, A. J., Thomas, J. K., & Janz, D. M. (2013). Reduced swim performance and aerobic
 565 capacity in adult zebrafish exposed to waterborne selenite. *Comparative Biochemistry and*
 566 *Physiology Part C: Toxicology & Pharmacology*, 157(3), Article 3.
 567 <https://doi.org/10.1016/j.cbpc.2012.12.004>
 568 Mathis, A., Biasi, T., Schneider, S., Yuksekgonul, M., Rogers, B., Bethge, M., & Mathis, M. W.
 569 (2021). Pretraining boosts out-of-domain robustness for pose estimation. *2021 IEEE*
 570 *Winter Conference on Applications of Computer Vision (WACV)*, 1858–1867.
 571 <https://doi.org/10.1109/WACV48630.2021.00190>
 572 Mathis, A., Mamidanna, P., Cury, K. M., Abe, T., Murthy, V. N., Mathis, M. W., & Bethge, M.
 573 (2018). DeepLabCut: Markerless pose estimation of user-defined body parts with deep
 574 learning. *Nature Neuroscience*, 21(9), Article 9. [https://doi.org/10.1038/s41593-018-0209-](https://doi.org/10.1038/s41593-018-0209-y)
 575 [y](https://doi.org/10.1038/s41593-018-0209-y)
 576 Miller, N., & Gerlai, R. (2012). From schooling to shoaling: Patterns of collective motion in
 577 zebrafish (*Danio rerio*). *PloS One*, 7(11), e48865.

578 Millidine, K. J., Metcalfe, N. B., & Armstrong, J. D. (2008). The use of ventilation frequency as
 579 an accurate indicator of metabolic rate in juvenile Atlantic salmon (*Salmo salar*). *Canadian*
 580 *Journal of Fisheries and Aquatic Sciences*, 65(10), Article 10. [https://doi.org/10.1139/F08-](https://doi.org/10.1139/F08-118)
 581 118

582 Mori, T., Miyata, N., Aoyama, J., Niizuma, Y., & Sato, K. (2015). Estimation of metabolic rate
 583 from activity measured by recorders deployed on Japanese sea bass *Lateolabrax japonicus*.
 584 *Fisheries Science*, 81(5), Article 5. <https://doi.org/10.1007/s12562-015-0910-7>

585 Mwaffo, V., Zhang, P., Romero Cruz, S., & Porfiri, M. (2017). Zebrafish swimming in the flow:
 586 A particle image velocimetry study. *PeerJ*, 5, e4041. <https://doi.org/10.7717/peerj.4041>

587 Nath, T., Mathis, A., Chen, A. C., Patel, A., Bethge, M., & Mathis, M. W. (2019). Using
 588 DeepLabCut for 3D markerless pose estimation across species and behaviors. *Nature*
 589 *Protocols*, 14(7), Article 7. <https://doi.org/10.1038/s41596-019-0176-0>

590 Ohlberger, J., Staaks, G., & Hölker, F. (2007). Estimating the active metabolic rate (AMR) in fish
 591 based on tail beat frequency (TBF) and body mass. *Journal of Experimental Zoology Part*
 592 *A: Ecological Genetics and Physiology*, 307A(5), Article 5. <https://doi.org/10.1002/jez.384>

593 Panadeiro, V., Rodriguez, A., Henry, J., Wlodkowic, D., & Andersson, M. (2021). A review of 28
 594 free animal-tracking software applications: Current features and limitations. *Lab Animal*,
 595 50(9), Article 9. <https://doi.org/10.1038/s41684-021-00811-1>

596 Parker Jr, F. R. (1973). Reduced metabolic rates in fishes as a result of induced schooling.
 597 *Transactions of the American Fisheries Society*, 102(1), Article 1.

598 Pavlov, D., & Kasumyan, A. (2000). Patterns and mechanisms of schooling behavior in fish: A
 599 review. *Journal of Ichthyology*, 40(2), Article 2.

600 Pitcher, T. J. (Ed.). (1986). *The Behaviour of Teleost Fishes*. Springer US.
 601 <https://doi.org/10.1007/978-1-4684-8261-4>
 602 Raffel, M., Willert, C. E., Scarano, F., Kähler, C. J., Wereley, S. T., & Kompenhans, J. (2018).
 603 *Particle Image Velocimetry: A Practical Guide*. Springer International Publishing.
 604 <https://doi.org/10.1007/978-3-319-68852-7>
 605 Rogers, S. C., & Weatherley, A. H. (1983). The use of opercular muscle electromyograms as an
 606 indicator of the metabolic costs of fish activity in rainbow trout, *Salmo gairdneri*
 607 Richardson, as determined by radiotelemetry. *Journal of Fish Biology*, 23(5), Article 5.
 608 <https://doi.org/10.1111/j.1095-8649.1983.tb02933.x>
 609 Saadat, M., Fish, F. E., Domel, A. G., Di Santo, V., Lauder, G. V., & Haj-Hariri, H. (2017). On
 610 the rules for aquatic locomotion. *Physical Review Fluids*, 2(8), Article 8.
 611 <https://doi.org/10.1103/PhysRevFluids.2.083102>
 612 Seebacher, F., Tallis, J., McShea, K., & James, R. S. (2017). Obesity-induced decreases in muscle
 613 performance are not reversed by weight loss. *International Journal of Obesity*, 41(8),
 614 Article 8. <https://doi.org/10.1038/ijo.2017.81>
 615 Shelton, D. S., Shelton, S. G., Daniel, D. K., Raja, M., Bhat, A., Tanguay, R. L., Higgs, D. M., &
 616 Martins, E. P. (2020). Collective behavior in wild zebrafish. *Zebrafish*, 17(4), 243–252.
 617 Spence, R., Gerlach, G., Lawrence, C., & Smith, C. (2008). The behaviour and ecology of the
 618 zebrafish, *Danio rerio*. *Biological Reviews*, 83(1), Article 1.
 619 <https://doi.org/10.1111/j.1469-185X.2007.00030.x>
 620 Stackley, K. D., Beeson, C. C., Rahn, J. J., & Chan, S. S. L. (2011). Bioenergetic profiling of
 621 zebrafish embryonic development. *PLoS ONE*, 6(9), Article 9.
 622 <https://doi.org/10.1371/journal.pone.0025652>

623 Steinhausen, M. F., Steffensen, J. F., & Andersen, N. G. (2005). Tail beat frequency as a predictor
 624 of swimming speed and oxygen consumption of saithe (*Pollachius virens*) and whiting
 625 (*Merlangius merlangus*) during forced swimming. *Marine Biology*, 148(1), Article 1.
 626 <https://doi.org/10.1007/s00227-005-0055-9>

627 Sumpter, D. J. T. (2006). The principles of collective animal behaviour. *Philosophical*
 628 *Transactions of the Royal Society B: Biological Sciences*, 361(1465), Article 1465.
 629 <https://doi.org/10.1098/rstb.2005.1733>

630 Thomas, J. K., & Janz, D. M. (2011). Dietary selenomethionine exposure in adult zebrafish alters
 631 swimming performance, energetics and the physiological stress response. *Aquatic*
 632 *Toxicology*, 102(1–2), Article 1–2. <https://doi.org/10.1016/j.aquatox.2010.12.020>

633 Timm, M. L., Pandhare, R. S., & Masoud, H. (2024). Multi-body hydrodynamic interactions in
 634 fish-like swimming. *Applied Mechanics Reviews*, 76(3), Article 3.
 635 <https://doi.org/10.1115/1.4062219>

636 Tong, Y., Xia, J., & Chen, L. (2021). Study on energy extraction of Kármán gait hydrofoils from
 637 passing vortices. *Physics of Fluids*, 33(12), Article 12. <https://doi.org/10.1063/5.0070862>

638 Triantafyllou, M. S., Triantafyllou, G. S., & Yue, D. K. P. (2000). Hydrodynamics of fishlike
 639 swimming. *Annual Review of Fluid Mechanics*, 32(1), Article 1.
 640 <https://doi.org/10.1146/annurev.fluid.32.1.33>

641 Van Rooij, J., & Videler, J. (1996). Estimating oxygen uptake rate from ventilation frequency in
 642 the reef fish *Sparisoma viride*. *Marine Ecology Progress Series*, 132, 31–41.
 643 <https://doi.org/10.3354/meps132031>

644 Vaughn, A. J., Street, G. M., Boudreau, M. R., & Allen, P. J. (2024). The relationship among
 645 acceleration, metabolism, and tail beat frequency of channel catfish (*Ictalurus punctatus*)

during critical swimming speed tests. *Aquaculture*, 580, 740280.
<https://doi.org/10.1016/j.aquaculture.2023.740280>

Videler, J. J. (1993). *Fish Swimming*. Springer Netherlands.
https://books.google.com/books?id=qTm_EWMIUH8C

Weatherley, A. H., Rogers, S. C., Pincock, D. G., & Patch, J. R. (1982). Oxygen consumption of active rainbow trout, *Salmo gairdneri* Richardson, derived from electromyograms obtained by radiotelemetry. *Journal of Fish Biology*, 20(4), Article 4.
<https://doi.org/10.1111/j.1095-8649.1982.tb03941.x>

Weihs, D. (1973). Hydrodynamics of fish schooling. *Nature*, 241, 290–291.

Wilga, C. D., & Lauder, G. V. (2000). Three-dimensional kinematics and wake structure of the pectoral fins during locomotion in Leopard sharks *Triakis semifasciata*. *Journal of Experimental Biology*, 203(15), Article 15. <https://doi.org/10.1242/jeb.203.15.2261>

Zang, L., Maddison, L. A., & Chen, W. (2018). Zebrafish as a model for obesity and diabetes. *Frontiers in Cell and Developmental Biology*, 6, 91.
<https://doi.org/10.3389/fcell.2018.00091>

Zhang, Y., Ko, H., Calicchia, M., Ni, R., & Lauder, G. V. (2024). *Collective movement of schooling fish reduces locomotor cost in turbulence*.
<https://doi.org/10.1101/2024.01.18.576168>

Zhang, Y., & Lauder, G. V. (2023a). Energetics of collective movement in vertebrates. *Journal of Experimental Biology*, 226(20), Article 20. <https://doi.org/10.1242/jeb.245617>

Zhang, Y., & Lauder, G. V. (2023b). *Energy conservation by group dynamics in schooling fish* [Preprint]. *elife*. <https://doi.org/10.7554/eLife.90352.1>

Figures

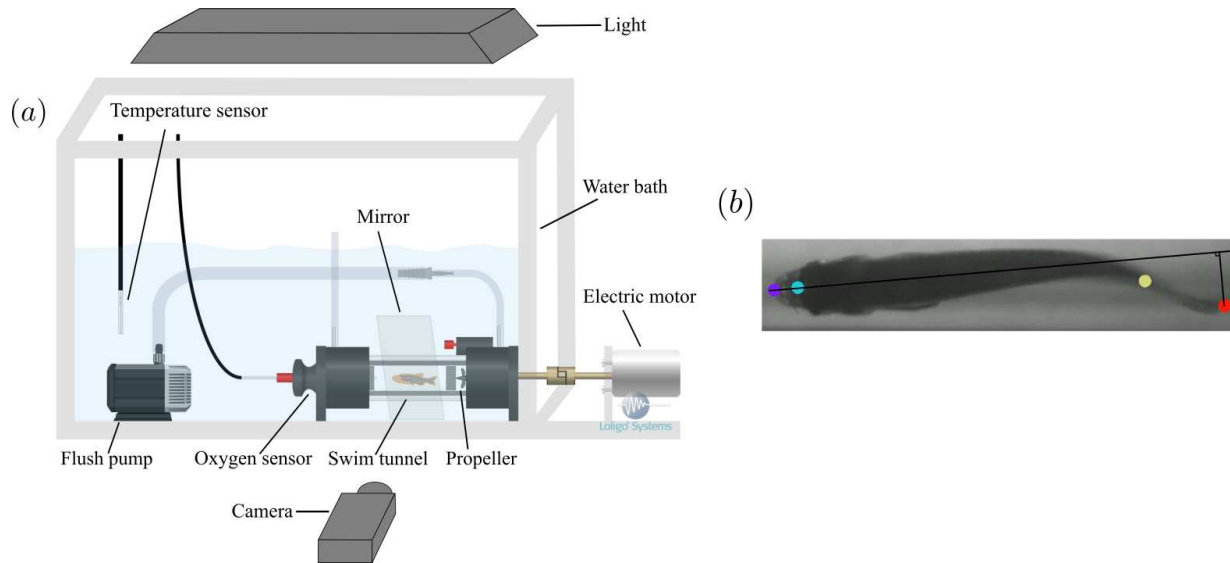
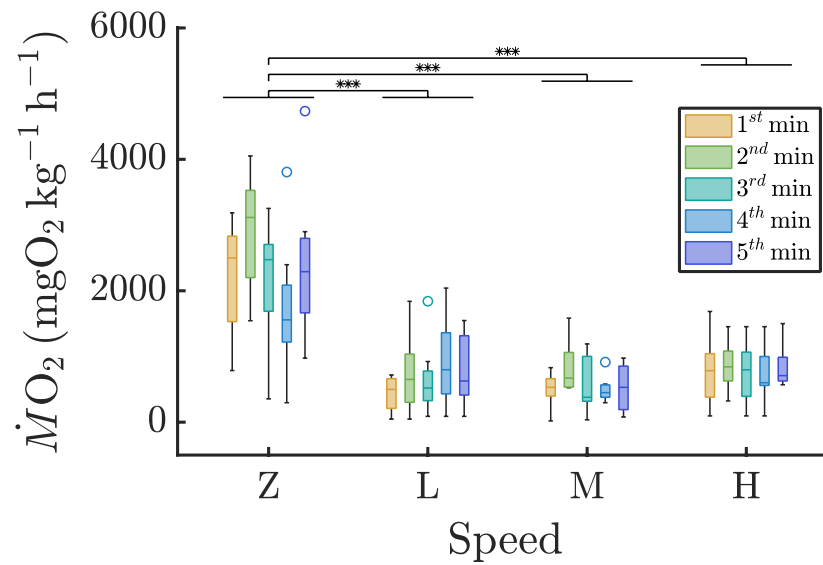


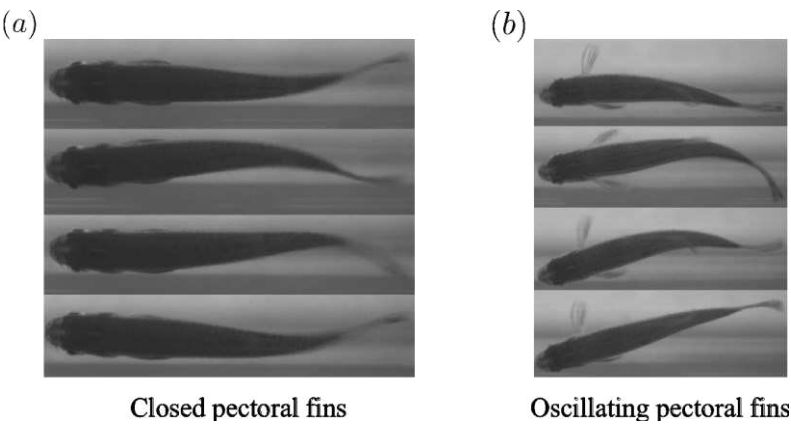
Figure 1. Simultaneous characterization of fish metabolic rate and kinematics. (a) Schematic of the experimental setup for swimming respirometry. (b) Tracking of the tail-beat motion (red dot with respect to black line) and mouth opening (purple dot) from the video. Cyan and red dots represent the fish head and leading edge of the caudal fin, respectively. *Image credit to Loligo[®] Systems.*



678

679 **Figure 2. Metabolism-speed relationship.** Box plots of the metabolic rate for different speed
 680 groups (Z, L, M, and H represent zero, low, medium, and high flow speeds, respectively). The
 681 color legend indicates different time bins within the 5-min measurement. The median is shown as
 682 the line in the middle of a box. The first and third quartiles are shown as the limits of a box. The
 683 top and bottom whiskers of a box extend to the smallest and largest values within 1.5 times the
 684 interquartile from the first and third quartiles, respectively. Circles identify outliers. Statistical
 685 significance is denoted by *** for p -value < 0.001 .

686



687

688

689

690

691

Figure 3. Difference in locomotory patterns at zero versus non-zero speed. Snapshots of zebrafish motion over one oscillating cycle, showing (a) maneuvering motion at zero speed, and (b) propulsive motion at non-zero speed.

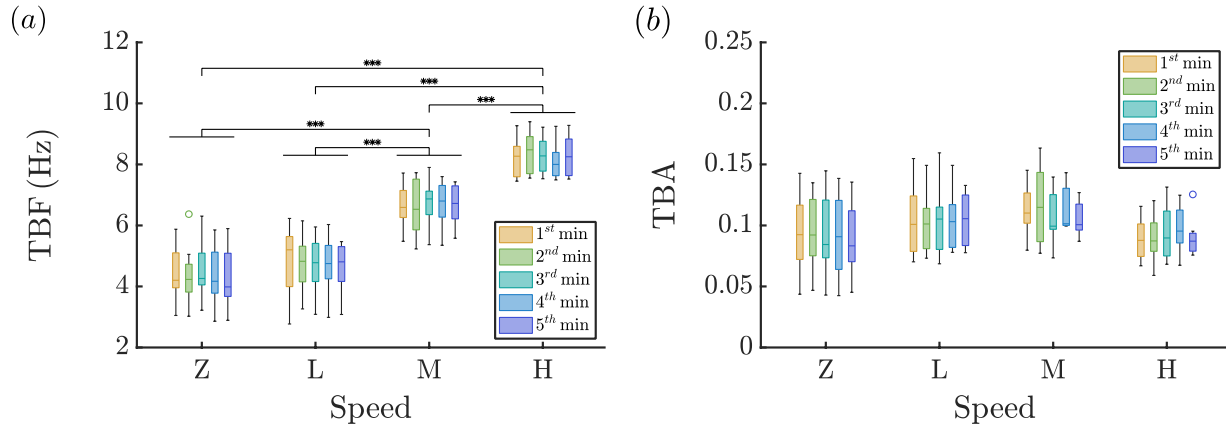


Figure 4. Tail-beat motion as a function of speed. Box plots of (a) tail-beat frequency (TBF) and (b) normalized tail-beat amplitude (TBA) for different speed groups. The median is shown as the line in the middle of a box. The first and third quartiles are shown as the limits of a box. The top and bottom whiskers of a box extend to the smallest and largest values within 1.5 times the interquartile from the first and third quartiles, respectively. Circles identify outliers. Statistical significance is denoted by *** for p -value < 0.001. For the non-zero flow speeds, the tail-beat frequency increases with the flow speed (that is, $TBF \sim 3.09 + 1.71 \text{ Speed}$), while the normalized tail-beat amplitude is nearly constant and equal to 0.10 – see Figure S2 in Supplementary Material. The mean Strouhal number ($St = (2 \times \text{tail-beat amplitude}) \times (\text{tail-beat frequency}) / (\text{flow speed})$) is approximately 0.76 – see Figure S3 in Supplementary Material.

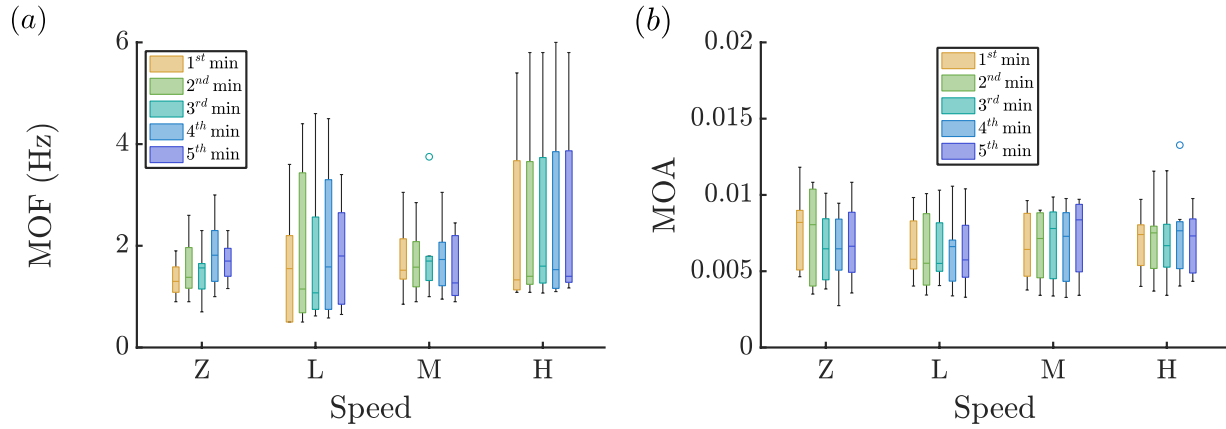


Figure 5. Mouth opening as a function of speed. Box plots of (a) mouth-opening frequency (MOF) and (b) normalized mouth-opening amplitude (MOA) for different speed groups. The median is shown as the line in the middle of a box. The first and third quartiles are shown as the limits of a box. The top and bottom whiskers of a box extend to the smallest and largest values within 1.5 times the interquartile from the first and third quartiles, respectively. Circles identify outliers.

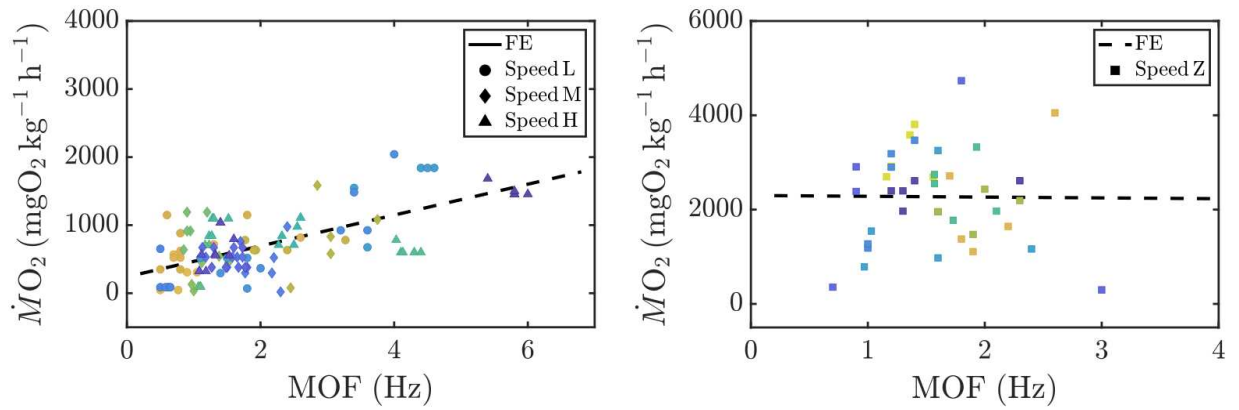
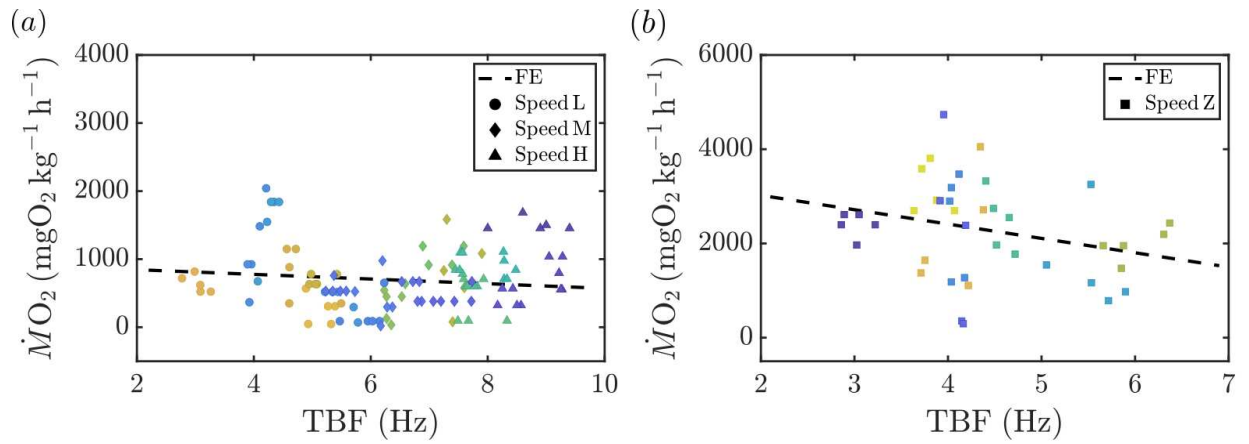


Figure 6. Mouth-opening frequency as a proxy of metabolic rate. Linear multilevel regression of $\dot{M}O_2$ versus mouth-opening frequency (MOF) at (a) non-zero ($R^2_c = 0.48$ and $R^2_m = 0.69$) and (b) zero speed ($R^2_c = 0.01$ and $R^2_m = 0.00$). For each fish, a different color is used and five observations are reported, one per minute. Black dashed lines represent the linear relationships with the fixed effect (FE).



718

719 **Figure 7. Mouth-opening frequency as a proxy of metabolic rate.** Linear multilevel regression

720 of $\dot{M}O_2$ versus tail-beat frequency (TBF) at (a) non-zero ($R^2_c = 0.61$ and $R^2_m = 0.02$) and (b) zero

721 speed ($R^2_c = 0.08$ and $R^2_m = 0.07$). For each fish, a different color is used and five observations are

722 reported, one per minute. Black dashed lines represent the linear relationships with the fixed effect

723 (FE).

724

Tables

725 **Table 1. Mouth-opening as a proxy of metabolic rate.** Results of the linear multilevel
 726 regressions for $\dot{M}O_2$ versus mouth-opening frequency (MOF), showing predictive power of MOF
 727 for non-zero speeds (β_1 row for non-zero speed – see bolded row). CI and SD represent confidence
 728 level and standard deviation of the coefficients, respectively. R^2_c and R^2_m represent the conditional
 729 and marginal R^2 , respectively.

		Coefficient	<i>p</i> -value	95% CI	SD (random effect)
Non-zero speed $R^2_c = 0.69$ $R^2_m = 0.48$	β_0	240.97	0.015	[48.15, 433.78]	165.65
	β_1	226.80	< 0.001	[123.86, 329.74]	53.27
Zero speed $R^2_c = 0.01$ $R^2_m = 0.00$	β_0	2299.3	< 0.001	[1134.5, 3464.1]	254.93
	β_1	-17.07	0.959	[-691.29, 657.15]	112.89

730

Table 2. Tail-beat frequency as a proxy of metabolic rate. Results of the linear multilevel regressions for $\dot{M}O_2$ versus tail-beat frequency (TBF), indicating lack of predictive power of TBF at any speed. CI and SD represent confidence level and standard deviation of the coefficients, respectively. R^2_c and R^2_m represent the conditional and marginal R^2 , respectively.

		Coefficient	<i>p</i> -value	95% CI	SD (random effect)
Non-zero speed $R^2_c = 0.61$ $R^2_m = 0.02$	β_0	913.66	< 0.001	[386.52, 1440.8]	144.69
	β_1	-34.27	0.483	[-130.74, 62.20]	83.22
Zero speed $R^2_c = 0.08$ $R^2_m = 0.07$	β_0	3630.1	< 0.001	[1894.9, 5365.3]	288.90
	β_1	-304.63	0.120	[-692.32, 83.06]	60.97