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EVALUATING METHODS TO DETERMINE THE MAXIMUM OXYGEN CONSUMPTION BY THE GULF KILLIFISH, *FUNDULUS GRANDIS*

An Honors Thesis

Presented to

The Department of Biological Sciences

of the University of New Orleans

In Partial Fulfillment

of the Requirements for the Degree of

Bachelor of Science, with University High Honors

And Honors in Biology

by

Sylvia Mullen

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ABSTRACT

Metabolic rate is an essential feature of animal physiology and ecology. The rate of aerobic metabolism, as determined by oxygen consumption rate (MO_2), is influenced by a variety of factors, including body size, temperature, and activity levels. Maximum aerobic metabolic rate (MMR) reflects the physiological capacity of an animal for oxygen extraction and utilization. As such, MMR is argued to be an important feature of an animal's life history. For fish, MMR is frequently estimated as the peak MO_2 immediately following an exhaustive chase, although several studies indicate that this value may underestimate MMR. Rather, MMR may be attained during sustained activity or following ingestion of a large meal. In this study, I used intermittent-flow respirometry to quantify MO_2 by the Gulf killifish, *Fundulus grandis*, after chasing, after ingestion of a meal, or during swimming. MMR estimates obtained by the three techniques were repeatable over two trials ($r \geq 0.74$). However, MMR estimates after chasing were significantly lower than those obtained during swimming ($P = 0.001$); MMR estimates after feeding were marginally ($P = 0.06$) higher than those obtained after chasing and significantly lower than those during swimming ($P = 0.02$). Additionally, the MMR estimates among methods were uncorrelated with one another ($r \leq 0.55$). The results demonstrate that MO_2 after an exhaustive chase or during digestion underestimate MMR in this species, and, importantly, such estimates may be poor predictors of inter-individual variation in maximum aerobic metabolism.

Keywords: metabolism, oxygen consumption, metabolic rate, fish, *Fundulus*, exercise, feeding

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INTRODUCTION

Metabolism is the sum of all the chemical reactions in a living organism (Norin and Clark, 2016). In animals, metabolism reflects the energy that is used to maintain the organism as well as support activities such as locomotion, growth, and reproduction. To fuel metabolism, animals obtain and break down complex food molecules and eliminate waste products into their environment. Metabolism reflects energy flow through organisms and, on a larger scale, through ecological systems (Rodgers and Clark, 2016) and thus it is an essential component of animal physiology and ecology.

The rate of animal metabolism can be measured in various ways. Energy budgets estimate metabolism as the difference between energy entering the animal (food) and energy leaving the animal (wastes). Calorimetry measures metabolic rate by determining the heat produced by an animal. The final method, respirometry, estimates metabolic rate by the rate of oxygen consumption, or alternatively the rate of carbon dioxide production. Respirometry is also referred to as indirect calorimetry because rates of gas exchange can be directly related to heat production, if the animal is using aerobic processes to support metabolism (Treberg et al., 2016).

The metabolic rate of an animal is influenced by various factors including body size, activity, nutrition, sex, reproductive state, and environmental conditions. For an animal of a given physiological condition in a defined environment, its maximum (aerobic) metabolic rate (MMR) is its capacity for oxygen uptake in support of energetically-costly activities such as growth, locomotion, and digestion (Norin and Clark 2016). MMR is also influenced by an animal's anatomy (e.g, gill, lung, or heart size), physiology (e.g, cardiac output), and

biochemical capacities (e.g., tissue enzyme activities and mitochondrial density) (Clark et al., 2013; Hvas and Oppedal et al., 2019).

Traditionally, MMR is estimated as the peak rate of oxygen consumption (MO_2) while fish are swimming at increasing speeds (Fry and Hart 1948; Brett 1964). This can be done by use of a swim tunnel respirometer. One type of swim tunnel respirometer, designed by Blazka et al. (1960), utilizes a "tube-within-a-tube design" in which a current generated by a propeller moves water through the inner tube and circulates the water back to the front of the respirometer between the space in the inner and outer tube (Blazka 1960). Another type of swim tunnel respirometer developed by Brett et al. (1964), is comprised of a circular or oval flume with an in-line pump to propel water past a fish in a separate section of the flume. In both types of swim tunnels, oxygen consumption by the fish is determined at increasing water speeds. The trial is concluded when the fish becomes exhausted as determined by failure to maintain position in the generated current (Norin and Clark 2016). However, there are drawbacks to this type of approach when estimating MMR. Swim tunnel respirometers are expensive and only allow for one individual to be measured at one time, limiting sample size and prolonging experiments. In addition, some species of fish may be poor swimmers and reluctant or incapable of swimming against a current.

Due to these drawbacks, MMR is commonly estimated as the peak MO_2 immediately after an exhaustive chase (Soofiani and Preide, 1995; Clark, 2013; Reidy 1995). In this approach, the fish is placed in a circular tub and manually chased by the experimenter (via net or hand) until exhaustion (Norin and Clark, 2016). Sometimes, fish are briefly exposed to air for a short period of time which aims to further increase metabolism (Clark 2013; Roche, 2013). Air exposure leads to increases in MO_2 in some species (Roche et al., 2013) but not in others

(Reemeyer and Rees, 2020). Either with or without air exposure, the fish is rapidly transferred to a static respirometer and MO_2 measurements begin (Norin and Clark, 2016). It is critical that respirometry starts quickly to capture the highest MO_2 value and avoid missing MMR (Rummer et al., 2016). The chase to exhaustion protocol is favored due to the ability to chase and use intermittent-flow respirometry on several fish concurrently, making this approach less expensive and time-consuming (Norin and Clark, 2016).

Less frequently, MMR is estimated by the peak MO_2 after ingestion of a large meal. Specific dynamic action (SDA) is the increase in metabolic rate after a meal, which reflects the cost of breaking down food and assembling simple molecules into complex macromolecules (McCue et al., 2006; Secor et al., 2008). For some ectothermic animals, the maximum MO_2 after a meal approaches their MMR (Secor et al., 2008). For some benthic, sit-and-wait predatory species of fish, MMR could occur during the period of SDA (Jordan and Steffensen, 2007; McKenzie et al., 2013). Like the chase method, the advantages of the feeding method are that many fish can be fed and measured simultaneously. However, one disadvantage is ensuring fish eat a large and reproducible ration of food.

The method best suited to determine MMR may differ between species (Rummer et al., 2016). For example, species that cannot sustain swimming for prolonged periods of time may not be good candidates for a swim tunnel protocol and may be better suited for a chase protocol (Rummer et al., 2016). On the other hand, athletic species that readily swim for long periods of time, MMR may be best estimated using the swim tunnel method (Rummer et al., 2016). Other species, for example, benthic sluggish fishes might reach MMR after a meal rather than during swimming or after an exhaustive chase.

The goal of my thesis is to evaluate techniques for determining the MMR in the Gulf killifish, *Fundulus grandis*. *Fundulus grandis* is a small estuarine fish that is abundant in environmentally dynamic habitats along the Gulf of Mexico. In addition, they are easy to collect and maintain in the laboratory. Because of their ecological importance, physiological tolerances, and suitability for laboratory research, the genus *Fundulus* is a model for environmental and evolutionary biology (Burnett et al., 2007). Here, MMR was estimated and compared after an exhaustive chase, after feeding, and during swimming. I addressed three objectives: to determine the reproducibility of each method, to determine which method gives the highest MO_2, and to determine if individual MO_2 is correlated among methods.

MATERIALS AND METHODS

Fish Collection and Housing

Fundulus grandis (n = 8) were collected from Bayou Cumbest, in the Grand Bay National Estuarine Research Reserve, Mississippi, U.S.A., as part of an earlier study (Reemeyer and Rees, 2020). Several months prior to and during this study, fish were housed individually in ~30 l acrylic tanks connected to a shared 200 l sump. The system contained dechlorinated tap water made to a salinity of ~10.7 ppt (Table 1) using Instant Ocean Synthetic Sea Salt (www.instantocean.com). The dissolved oxygen was maintained between 83 and 100% air saturation by aeration (Table 1). The temperature was approximately 25ºC (Table 1), and the photoperiod was 12:12 (light: dark). Fish were fed an amount of Tetramarine large saltwater flakes (www.tetra-fish.com) equal to ~ 1.5% of their body mass three times per week and occasionally supplemented with frozen chopped shrimp. This ration was sufficient for fish to

maintain mass over the course of the experiment, which was conducted from June to October, 2021 (Table 2). Food was withheld for 48 hours prior to and during trials, except for those evaluating post-feeding MO_2 (see below). All procedures with live animals were approved by the UNO Institutional Animal Care and Use Committee (18-006).

Experimental Design

The experiment consisted of measuring the MO_2 of the same 8 fish using three methods to elicit MMR, chasing (Reemeyer and Rees, 2020), feeding (McKenzie et al. 2013), and swimming (Kolok and Sharky, 1997). In addition, two trials of each method were performed to assess the repeatability of the three methods (Table 2). The two feeding trials were conducted 67 days apart, and the two swim trials were conducted 70 days apart. The time between the two chasing trials was longer (102 days) due to a delay from Hurricane Ida. The time between two consecutive trials of any type was at least 2 weeks, during which time fish were held in the maintenance system described above. Except as noted below, all trials began between 08:00 and 12:00 to ensure that all MMR determinations were during the light phase.

The chasing trials consisted of gently netting two fish from their holding tanks, transporting them to an adjacent room, and placing them separately into black circular tubs (40 cm diameter) containing 6.5 l of water having the same composition as used in respirometry (salinity ~10 ppt, temperature ~25°C). Fish were allowed to habituate to the black tubs for 27 min, after which they were individually chased by hand for 3 min. Immediately after chasing, fish were quickly transferred through air (< 2 s) and individually placed into static respirometry chambers. MO_2 measurements by intermittent-flow respirometry (see below) began within 30 s of the end of the chase protocol. The highest MO_2 measured within 1 h after chasing was

retained as the fish's peak $MO_{2\text{-chase}}$. In the first trial MO_2 measurements continued until the next morning (ca. 20 h), when fish were removed from the chambers, weighed, and returned to the maintenance system with each fish. In the second trial, fish were weighed and returned to the maintenance system after 1 h.

The first feeding trial consisted of gently netting two fish from their holding tanks and placing them separately into black circular tubs, as described above. Fish were allowed to habituate for 10 min. Over the next 20 min, each fish was offered a ration of chopped shrimp up to 5% of its body mass. The fish were transferred into static chambers for intermittent-flow respirometry as described above. The amount of food remaining in the tanks was weighed and subtracted from the amount offered. Although a similar approach has been used for other killifish species (McKenzie et al., 2013), fish in the current study were agitated and ate variable amounts of shrimp. Therefore, in the second feeding trial, fish were left in their individual tanks in the maintenance system, where they were offered up to 5% of their mass in chopped shrimp over 20 min. As before, uneaten shrimp was removed and subtracted from the amount offered. In this trial, fish were netted, transported in a small amount of water to the respirometry system in an adjacent laboratory, and transferred through air into the respirometry chambers. This introduced a delay of approximately 2 min between feeding and the start of respirometry. For both feeding trials, the highest MO_2 over the first 6 h after feeding was retained as the fish's peak $MO_{2\text{-feeding}}$. Intermittent-flow respirometry continued overnight (~14 h), when fish were removed from the chambers, weighed, and returned to the maintenance system.

The swimming trials were modeled after a critical swim test previously used for *F. grandis* (Kolok and Sharky, 1997) and utilized a Blazka-type swim respirometer. In the first swim trial, fish were individually netted, transported to an adjacent room, and transferred

through air (< 2 s) into the swim tunnel having water of the same composition as the maintenance system. Fish were allowed to adjust to a water velocity of 10 cm s^{-1} for 20 min, after which MO_2 measurements began. Swim trials ended when fish were pinned on the rear grate for 5 s. For the first trial, fish were transferred to static chambers and MO_2 measurements continued using intermittent-flow respirometry for another ~20 h. At the end of the trial, fish were weighed and returned to the maintenance system. For the second swim trial, fish were placed in the swim tunnel in the afternoon prior to the critical swim test and allowed to adjust to the swim tunnel for ~16 h at a water velocity of ~5 cm s^{-1} (~1/2 body length (BL) s^{-1}). The critical swim test began between 08:00 and 10:00 the next morning, and it was conducted exactly as it was during the first trial, except that fish were removed, weighed, and returned to the maintenance systems. In both trials, the highest MO_2 recorded during the critical swim test was taken as the fish's peak $MO2_{-swim}$.

Respirometry and System Description

Intermittent-flow respirometry was utilized to measure MO_2 as described by Svedsen et al. (2016). The system for chase and feeding treatments was comparable to Reemeyer and Rees (2020). This system consisted of two acrylic respirometry chambers (62 mm diameter) and end caps, each fitted to two sets of non-toxic, flexible PVC tubing. The first set of tubing was connected in a loop to a flow-through, fiber-optic oxygen sensor (Loligo Systems; www.loligosystems.com) and a water pump, which continuously circulated the water from the chamber past the oxygen sensor at a flow rate of approximately 2 l min^{-1}. The volume of the chamber, tubing, oxygen sensor, and water pump was 545 ml, which was between 30 and 70 times the mass of the fish (Table 2). The second set of tubing was connected to a second water

pump that periodically flushed the chamber with the surrounding water at approximately 3 l min^{-1}. If the combined flow when both pumps were on was uniformly distributed through the cross-sectional area of the chamber (30 cm^2), then the water velocity in the respirometry chambers never exceeded 3 cm sec^{-1} (< ½ BL s^{-1}). The respirometry chambers, tubing, and pumps were immersed in a large tank containing approximately 150 l of well-aerated water having the same composition as the maintenance tanks, with the exception that the temperature was maintained at 25 ± 0.1ºC by computer-controlled aquarium heaters. Water in the system was continuously circulated through an ultraviolet filter and an external water bath set to 24.5ºC. The two respirometry chambers were not visually shielded from one another, but both were shielded from the investigator by black plastic. Computer software (AutoResp, Loligo Instruments) controlled the water pumps as follows: both pumps on for 120 s (flush phase), flush pump off and recirculation pump on for 30 s (wait phase), flush pump off and recirculation pump on for 300 s (measure phase). This "loop" design was repeated for the duration of the respirometry trial (from 1 to >20 h). Typically, the oxygen content of water rose above 94% air saturation during the flush phase and did not drop below 81% during the measure phase. The oxygen content of water was collected once per second by a Witrox-4, and MO_2 was determined as the rate of decline in oxygen content during the measure phase as described below.

The swimming respirometry system consisted of a Blazka- type swim tunnel, with an acrylic outer cylinder (95 mm diameter) and an inner glass cylinder (88 mm diameter). The swim tunnel volume was 1600 ml, representing a volume to fish mass ratio from 90 to 210 for the largest and smallest fish, respectively. A fiber optic dipping probe oxygen sensor was inserted through one end cap and extended into the inner glass cylinder. A motor-powered propeller projected into the other end of the inner glass tube. Laminar flow was ensured by two plastic

honeycombs on either side of the inner glass tube. Before each swim trial, water velocity was calibrated using digital particle velocimetry (Loligo Systems; www.loligosystems.com) of videos of neutrally buoyant fluorescent particles captured at increasing motor voltage. The swim tunnel was immersed in a reservoir of 45 liters having the same composition as the maintenance system. Water in the reservoir was maintained at 25 ± 0.1° by computer-controlled aquarium heaters, and it was continuously circulated through an ultraviolet filter and an external water bath set to 24.5ºC. Measurement loops consisted of a 299 s flush phase, 1 s wait phase, and a 300 s measure phase. At the end of each measure phase, water velocity was increased by 5 cm s^{-1}. The trial ended when fish were not able to maintain position in the chamber and were pinned against the rear grate for 5 s, which was usually at water velocities of 30-45 cm s^{-1} as previously documented for this species (Kolok and Sharky,1997). Thus, swim trials including the 20 min adjustment period, lasted between 60 and 90 min. The flush water pump, aquarium heaters, and propeller power supply were connected to a DAQ-M relay system (Loligo Systems; www.loligosystems.com) and controlled by AutoResp software (Loligo Instruments). The oxygen content of water was collected once per second by a Witrox-4. Typically, the oxygen content of water rose above 96% air saturation during the flush phase and did not drop below 85% during the measure phase. MO_2 was determined as the rate of decline in oxygen content during the measure phase as described below.

Background Respiration

Background respiration was determined by measuring MO_2 in each chamber in the absence of a fish before and after each trial. Background MO_2 was taken as the average rate of change in oxygen content determined in 2 or 3 loops of intermittent-flow respirometry. The loop

design comprised of a 90 s flush, 30 s wait and 1080 s measure phase. Background MO_2 was assumed to increase linearly over the duration of each trial and a time-corrected value was subtracted from the MO_2 measured in the presence of a fish to obtain the fish's MO_2 (Svendsen et al., 2016; Rosewarne et al., 2016). Background MO_2 ranged from <5% to ~20% of the fish's MO_2, depending upon the duration of the trial.

Determining Peak MO_2

The rolling regression method described by Zhang et al. (2020) was used to determine each fish's peak MO_2 in each trial. For each 5-min measurement interval, ordinary least-squared linear regression was used to determine the slope of oxygen concentration (in percent air-saturation) versus time (in seconds) over a variety of periods ranging from 30 to 240 s (Zhang et al., 2020). For chase and feeding trials, the slopes determined over 60 s were statistically higher than those estimated over the entire 5-min measurement interval while still achieving high coefficients of determination (average $r^2 = 0.89$). Because of the larger volume of the swim tunnel, 120 s intervals were required to achieve a similar r^2 (average $r^2 = 0.91$). The peak $MO_{2\text{-chase}}$ was the highest MO_2 over any 60 s period within 1 h after chasing. The peak $MO_{2\text{-feeding}}$ was the highest MO_2 over any 60 s period within 6 h after feeding, and the peak $MO_{2\text{-swim}}$ was the highest MO_2 over any 120 s period during a swim test. All values were expressed as μmol O_2 min^{-1} g body $mass^{-1}$ after accounting for barometric pressure, salinity, temperature, fish mass, and background respiration as described above.

Statistical Analysis

Pearson's correlation coefficients were used to assess the repeatability between trials. For each fish, the higher peak MO_2 measurement of the two trials was used to assess the difference between methods by repeated measures ANOVA and the consistency of peak MO_2 measured in the different methods with Pearson's correlation. All calculations, analyses, and graphing were carried out using Microsoft Excel and Graph-Pad Prism. In all cases, the level of statistical significance was taken as $P < 0.05$. All values of MO_2 are presented as averages and 1 standard deviation, unless otherwise specified.

RESULTS

The first objective of this study was to evaluate the repeatability of three techniques used to elicit MMR in fishes: chasing to exhaustion, feeding a large meal, and swimming at maximum sustainable levels. Pearson's correlation coefficients comparing the highest MO_2 measured in two trials of each method were similar across methods ($r \geq 0.74$; Fig. 1). This relationship was significant for peak $MO_{2\text{-feed}}$ and peak $MO_{2\text{-swim}}$ ($P = 0.03$ and 0.01, respectively). This relationship for peak $MO_{2\text{-chase}}$ failed to reach statistical significance ($P = 0.06$), primarily because it was tested with a smaller sample size ($n = 7$). During the second chase trial, one fish appeared to be injured and had abnormally low MO_2 measurements. Thus, it was removed from this analysis. In addition, the time between the chase trials was longer between the feeding or swimming trials. Nevertheless, it appears that all three methods are similarly repeatable over time.

The second objective of this study was to determine if each method yielded similar estimates of MMR. There was an overall effect of method when the higher of the two values

from each trial of chase, feeding, and swimming were compared ($P < 0.001$; Fig. 2). Peak $MO_{2\text{-chase}}$ (0.208 ± 0.039 μmol $min^{-1}g^{-1}$) and peak $MO_{2\text{-feed}}$ (0.276 ± 0.052 μmol $min^{-1}g^{-1}$) were significantly lower than peak $MO_{2\text{-swim}}$ (0.340 ± 0.056 μmol $min^{-1}g^{-1}$). Although there was a trend for peak MO_2 to be higher after feeding compared to chasing, this difference was not statistically significant ($P = 0.06$). If peak $MO_{2\text{-swim}}$ is considered to be MMR, then peak $MO_{2\text{-chase}}$ and peak $MO_{2\text{-feed}}$ underestimate MMR by 39% and 19%, respectively.

The third objective of this study was to evaluate whether the variation among individuals in peak MO_2 was similar across the three methods. Even if the mean values differed among methods, it is possible that a given individual would have high (or low) values of MO_2 in all three methods. The values of peak MO_2 measured in these fish were not correlated across the three methods ($r < 0.55$, $P > 0.15$; Fig. 3).

DISCUSSION

The primary goal of this experiment was to determine which method yields the highest estimate of MMR in *Fundulus grandis*. I measured the peak MO_2 after an exhaustive chase, after ingestion of a large meal, and during maximum sustainable swimming. In the current experiment, all three methods yielded similar correlations between MO_2 determined in two trials (Fig. 1). Although the correlation between the chase method trials did not reach statistical significance, one fish was removed from the analysis, which led to a smaller sample size. Additionally, the chasing trials were conducted farther apart in time than the other trials in the other treatments. Still, the correlations coefficients for all three methods were consistent with other studies on this species (Reemeyer and Rees, 2020), showing significant repeatability of MMR.

Although consistency across multiple trials is important, more crucial is the question of which method gives the fish's maximum attainable MO_2. My results show that the methods differ significantly in the peak MO_2 measured (Fig. 2). Specifically, the chase method yielded the lowest MO_2, and the swim protocol yielded the highest MO_2. The feeding protocol gave intermediate values of MO_2. Although peak MO_2 after feeding was not statistically different from the peak MO_2 after chase method, it was statistically lower than the swim tunnel method. Several considerations can account for these differences. First, each method depends, to a certain extent, on the motivation of the fish. If a fish was not motivated to escape from the investigator's chasing, the chase method would underestimate MMR; if the fish was not hungry or too anxious to eat a large meal, the feeding method could underestimate MMR; and if the fish was unable or unwilling to swim for an extended period of time (60-90 min), the swim tunnel method could underestimate MMR.

In addition to these differences in motivation, the delay between chasing or feeding fish and the start of respirometry could affect peak MO_2 by these methods. It is generally assumed that the delay between chasing and respirometry must be kept to a minimum in order to capture peak MO_2. Recently, Zhang et al. (2020), used a static respirometer chamber modified with the addition of a bottle brush to chase the fish inside the respirometer. When peak $MO_{2\text{-chase}}$ was measured during the chase it was 18% higher compared to MO_2 values by the same fish immediately after the chase. Thus, even a 30 s delay in starting respirometry may be too long after chasing to capture the peak MO_2 (Zhang et al., 2020). In contrast, other investigators reported that the maximum MO_2 does not occur immediately after a chase, but may occur hours later (Clark et al., 2013; Reemeyer and Rees, 2020). For example, Clark et al. (2013) found that MO_2 measurements peaked between 6 and 8 h post-chase in coho salmon, *Oncorhynchus*

kisutch. For peak MO_2 after feeding, the delay can also be considerable. Jordan and Steffenson (2007) found that MO_2 measurements reached a maximum at between 6 and 10 h subsequent to feeding in juvenile cod, *Gadus morhua*.

Finally, the physiological processes responsible for MO_2 by the fish differ in the three methods and these processes could differ in upper limits. The MO_2 measured during sustainable swimming is largely due to skeletal muscle activity, with contributions from other tissues that are active during swimming (e.g., heart). In contrast, the processes underlying MO_2 after an exhaustive chase are varied and include residual skeletal muscle activity, replenishment of blood and tissue oxygen stores, and the clearance of products of anaerobic metabolism (Norin and Clark, 2016). While the first two processes are likely to be quick, the last of these occurs slowly and may take hours to peak (Milligan 1996). Finally, the changes in MO_2 after a large meal reflect the energetic cost of breaking down food into smaller molecules and synthesizing complex macromolecules, which could have a different upper limit than energy use during or after exercise.

My results align with previous studies comparing methods to elicit MMR in other species of fishes. In black sea bass, peak MO_2 values from the swim tunnel method yielded an MMR that was 125% higher than the chase method (498 ±22 mg O_2 kg^{-1} hr^{-1} compared to 397 ±11 mg O_2 $kg^{-1}hr^{-1}$) (Slessinger et al., 2019). In Atlantic salmon, the difference was even larger: peak MO_2 during swimming was 152% higher than after chasing (511 ±15 mg O_2 kg^{-1} hr^{-1} compared to 337 ±9 mg O_2 kg^{-1} hr^{-1}) (Hvas and Oppedal, 2019). With regard to MO_2 after feeding, Brett and Zala (1975) reported a value (370 mg O_2 kg^{-1} hr^{-1}) very similar to the post-chase MO_2 of the closely related Atlantic salmon (Hvas and Oppedal, 2019). In addition, Von Herbing et al. (2004) found that the MO_2 by juvenile Atlantic cod following an exhaustive chase did not differ from the MO_2

following ingestion of a meal. These results are similar to my results: the chase and feeding methods yield similar values of peak MO_2, which were both lower than that measured during swimming.

It has been suggested that the best method to estimate MMR may vary among species, depending upon swimming ability and lifestyle. Avid, athletic swimmers, such as tuna, are predicted to reach MMR during sustainable swimming rather after chasing or feeding (Norin and Clark, 2016; Rummer et al. 2016). In contrast, species that are poor swimmers, benthic, or ambush predators may attain higher MO_2 after a chase or ingestion of a meal (McKenzie et al. 2013; Norin and Clark 2016). However, even within a given species, there can be conflicting data. For example, Soofiani and Priede (1985) found that in juvenile Atlantic cod, the chase method showed higher MO_2 compared to the swim tunnel method. In adults of this species, however, Tang et al. (1994) found that that the swim test yielded higher MO_2 than did an exhaustive chase in Atlantic cod. Conversely, Reidy (1995) reported that post chase MO_2 was 36% higher than MO_2 reached during a swim test. These discrepancies may arise from differences in experimental design and/or lifestage (juvenile vs adult) of the fish.

A novel finding of this study is that the peak MO_2 measured by these three methods were not correlated among this group of *F. grandis* (Fig. 3). As mentioned above, this result could be due to differences in motivation or physiology among individuals, or perhaps technical differences in the three methods. Nevertheless, because of this lack of correlation among techniques, they cannot be substituted for one another when determining either the mean MMR of a sample of fish or its variation among individuals within the sample. I propose that each method is appropriate in specific contexts. For example, in studies of the consequences of exhaustive, burst-type activities (e.g., predator escape), the chase protocol may be most

appropriate. For studies interested in the cost of digestion, then the MO_2 after feeding should be measured. Similarly, if the energetic cost of sustainable swimming is the goal, then the swim tunnel is most appropriate. Importantly, if the central goal is to understand the fish's maximum capacity for oxygen consumption under any condition, then a comparison among methods is critical to determine which yields the highest values. As suggested for other species, in *F. grandis* the swim tunnel method yielded the highest MO_2 and is likely to be the best estimate of this species "true" MMR.

CONCLUSION

This study used intermittent-flow respirometry to quantify oxygen consumption (MO_2) as a proxy of MMR by *Fundulus grandis* after chasing, feeding, and during sustained swimming. Over two trials of each method, MMR estimates obtained by three methods were repeatable ($r \geq 0.74$). Also, MMR estimates following the chase protocol significantly underestimated values obtained during swimming ($P = 0.001$); MMR estimated after feeding were slightly ($P=0.06$) higher than those obtained after chasing and significantly lower than those during swimming ($P=0.02$). Finally, there was no correlation between MMR estimates among the three methods ($r \leq 0.55$), suggesting that individual variation in metabolic rate varies among methods. Although the swim tunnel method yielded the highest MMR for *F. grandis*, the method that yields the highest MMR could vary among species depending upon experimental design, motivation, swimming abilities, lifestyle, and lifestage.

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TABLE 1: Water quality during maintenance of fish. Data were collected from May to October, 2021. Salinity, temperature, and O_2 concentration (mg/L and % air sat.) are shown as mean, SD, minimum and maximum values, and number of measurements.

	Salinity (ppt)	T (ºC)	O_2 concentration (mg/L)	O_2 concentration (% air sat.)
Average	10.7	25.1	7.43	95.0
SD	0.36	0.53	0.26	3.11
Minimum	9.8	24.0	6.47	83.4
Maximum	11.7	27.9	7.95	100
n	77	73	68	67

TABLE 2: Fish masses over the course of the experiment. Different trial types were conducted over several days beginning on the date indicated. The average, range, and SD of fish body mass are shown (n = 8).

Trial Type	Trial Dates	Mass Average (g)	Minimum (g)	Maximum (g)	SD (g)
Chase 1	6/8/21	12.39	8.78	15.14	2.46
Feed 1	6/29/21	12.13	7.78	15.21	2.64
Swim 1	7/30/21	11.97	7.68	15.28	2.62
Feed 2	9/4/21	12.27	8.11	15.96	2.67
Chase 2	9/18/21	12.63	8.12	17.05	2.88
Swim 2	10/8/21	12.35	8.22	16.94	2.76

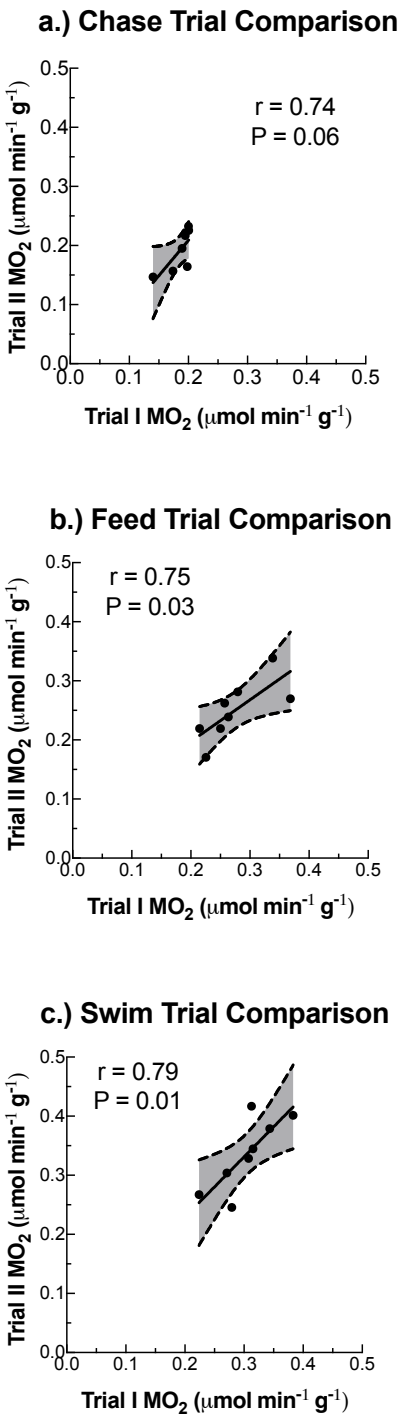

FIGURE 1: Comparison of Trial I and Trial II peak MO_2 values for *F. grandis* after an exhaustive chase, feeding to satiation, and during swimming. Pearson's correlation coefficients are similar across all three methods ($r \geq 0.74$). MO_2 for two trials were significantly correlated for feeding and swimming methods ($P > 0.05$).

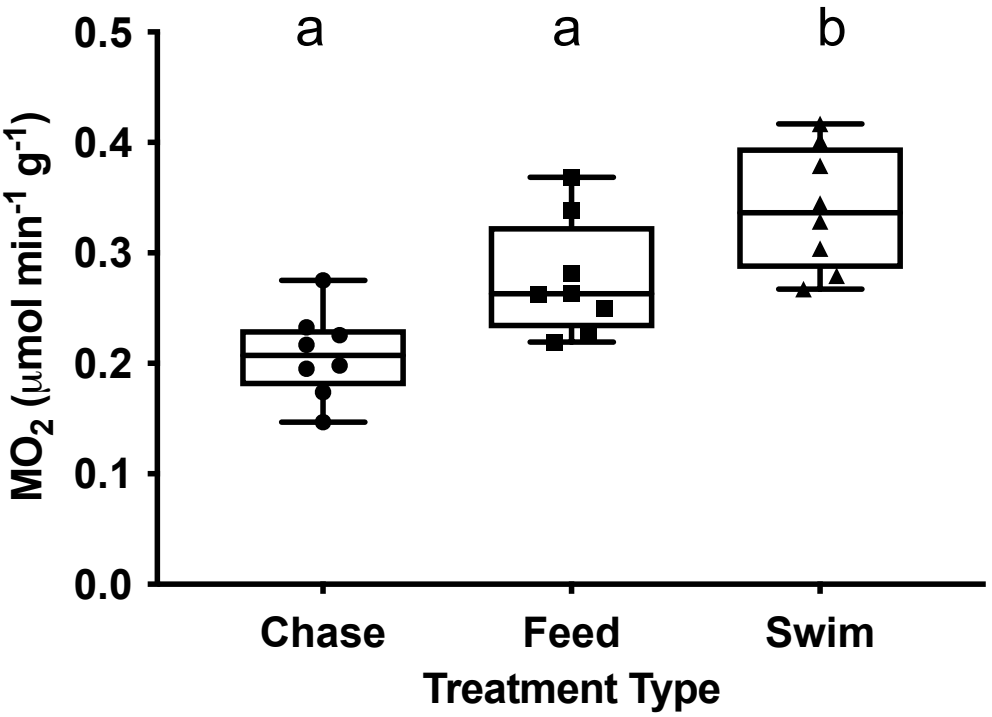

FIGURE 2: Comparison of peak MO_2 measured by three methods to estimate MMR. Peak MO_2 values following an exhaustive chase (solid circles), feed to satiation (solid squares), and during swimming (solid triangles). The higher of two trials of each method for each fish are shown. Boxes represent interquartile range. Lines represent the minimum and maximum values. Treatments bearing different letters are significantly different ($P < 0.05$).

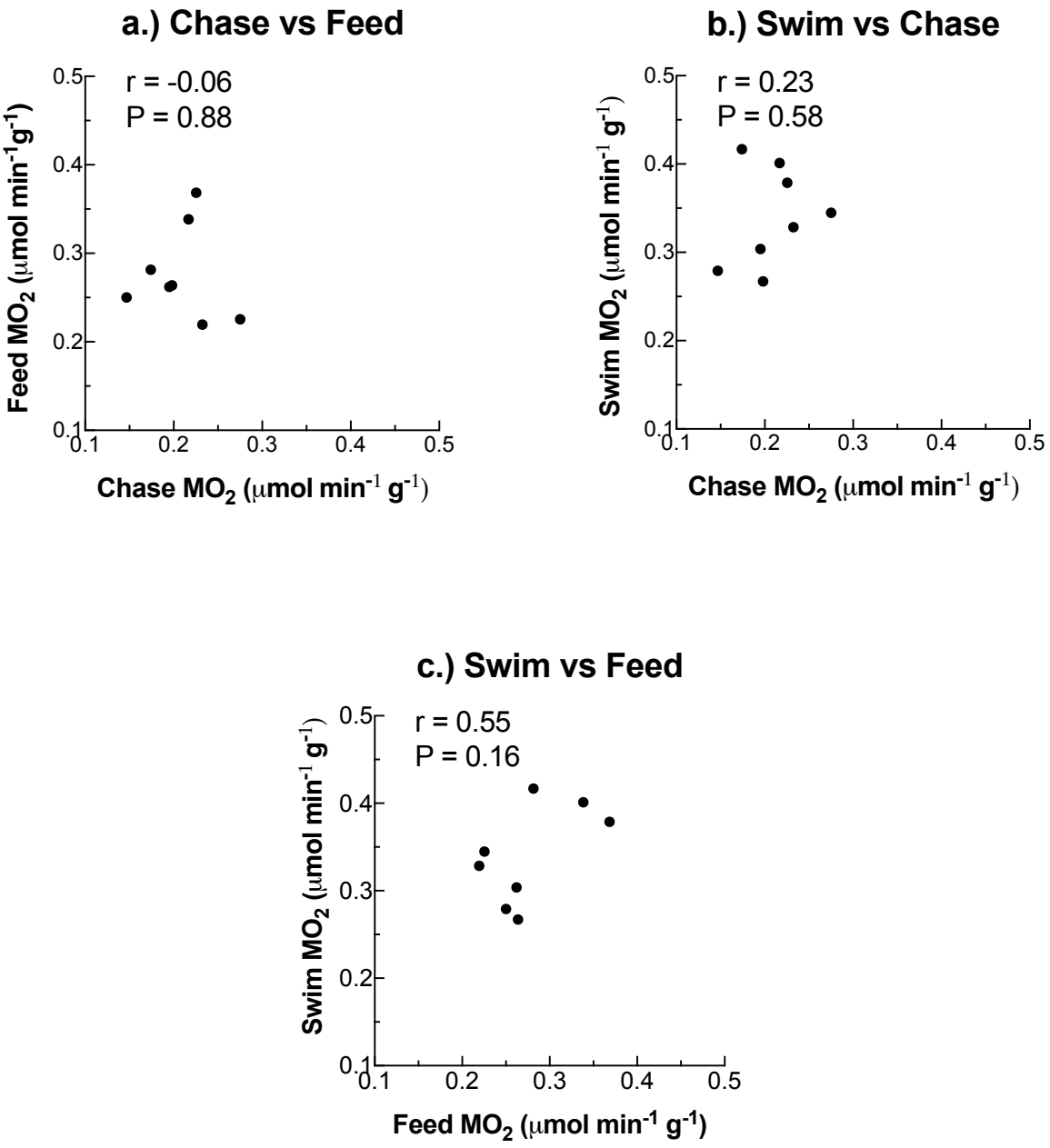

FIGURE 3: Correlations among methods used to estimate MMR a.) peak $MO_{2\text{-feed}}$ versus peak $MO_{2\text{-chase}}$, b.) peak $MO_{2\text{-swim}}$ versus $MO_{2\text{-chase}}$, and c.) peak $MO_{2\text{-siwm}}$ versus peak $MO_{2\text{-feed}}$. For each method, the MO_2 values presented are the higher of two values determined in Trial I and Trial II. The values of peak MO_2 were not correlation across the three methods ($P > 0.15$).