The effect of hybridization on Killifish (*Fundulus* spp.) metabolic rate

by

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ABSTRACT

Interspecific hybridization occurs when two species successfully mate to produce offspring. This phenomenon is relatively common in nature and can produce hybrids with advantaged, disadvantaged, or intermediate physiology compared to parental species. To better understand the outcomes of hybridization, I studied two species of fish, the Banded Killifish (*Fundulus diaphanus*) and the Common Killifish (*F. heteroclitus*), and their inter-specific hybrids. In the wild, female *F. diaphanus* and male *F. heteroclitus* can naturally hybridize to produce all-female asexual clones. To compare the physiology of these Killifish hybrids to their parental species, I measured the resting metabolic rates (RMR) and maximal metabolic rates (MMR) of the three species to determine their aerobic scope (difference between RMR and MMR). I predicted that the hybrids would have an aerobic scope above that of *F. diaphanus* but below that of *F. heteroclitus*, as they display intermediate values in other physiological traits. The resting and maximal aerobic metabolic rates of Killifish caught from Porters Lake, Nova Scotia were measured using intermittent-flow respirometry. RMR was measured while fish were resting, after a short acclimation period, whereas MMR was measured after fish were swam to exhaustion using the chase method. It can be difficult to distinguish between *F. heteroclitus* and hybrids visually, so I also measured distinctive morphological traits and genotyped the mitochondrial DNA to confirm species identities. Unfortunately, I was unable to collect sufficient data for inter-specific MMR comparisons, but RMR data suggests that these Killifish hybrids have similar RMR to both parental species. To assess aerobic scope, additional MMR data is required.

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1. INTRODUCTION

1.1 Hybridization in animals can lead to asexuality

Interspecific hybridization is the mating and subsequent reproduction of two species to produce offspring with blended genomes (Runemark et al. 2019). It is a common phenomenon in nature on a per-species basis, with 10-30% of multicellular animal and plant species having the ability to hybridize. However, within these species only 0.01-1% of individuals will hybridize (Abbott et al. 2013). Hybridization among different species can lead to various outcomes that can have either positive or negative effects on hybrid evolutionary fitness and physiological capacity relative to parental species (Runemark et al. 2019). Botanists have traditionally stressed the potential of hybridization to generate diversity and adaptive variation, whereas zoologists have viewed hybridization as a factor reducing biodiversity because gene flow between branching species can counteract their divergence (Seehausen 2004; Runemark et al. 2019). However, recent evidence has shown that hybridization is indeed an important evolutionary process in animals and can foster increased diversity in some species (Grant and Grant 1994; Rieseberg et al. 1999; Runemark et al. 2019).

There are many potential outcomes of hybridization. Some hybridization events might produce infertile offspring, such as a cross between a horse (*Equus caballus*) and a donkey (*Equus asinus*) (Camillo et al. 2003) or between lions (*Panthera leo*) and tigers (*Panthera tigris*) (McKinnell and Wessel 2012). This reduces the reproductive success of the parent species, leading either to selection against hybrids or to an increased risk of extinction due to wasted reproductive effort (Runemark et al. 2019). Alternatively, fertile hybrids may also backcross with parental species, which can lead to introgression of alleles from one species into another and create a spectrum of genomic variation across a hybrid zone (Runemark et al. 2019). Finally,

offspring generated from hybridization may evolve into a separate species, which is more likely to occur when hybrids have polyploid genomes or engage in asexual reproduction (Abbott et al. 2013).

Hybridization leading to all-female lineages that reproduce asexually occurs in only a few vertebrate lineages, as most hybrids continue to reproduce sexually (Dawley 1992; Hernandez-Chavez and Turgeon 2007; Janko et al. 2017). Sexual reproduction involves both parents providing genetic material to produce progeny that are genetically unique. Meanwhile, asexual reproduction involves only one parent transferring genes so that the offspring is genetically identical to that single parent due to lack of recombination and fertilization (Otto 2008). The creation of all-female asexual offspring is a phenomenon which only occurs in approximately 100 known species of vertebrates and is normally the result of hybridization. These hybrid mothers produce daughters that are genetically identical to both the mothers and to each other (Dawley 1992; Avise 2015) and have no paternal genetic contribution (Klabacka et al. 2022).

In hybrid-produced asexual vertebrates studied to date, the eggs double the number of chromosomes they possess before meiosis begins, resulting in the preservation of ploidy after meiosis is complete, therefore allowing the cells to develop without fertilization or recombination (reviewed by Dalziel et al. 2020). This phenomenon freezes the genome in a hybrid state (Vrijenhoek and Pfeiler 2008; Warren et al. 2018). When looking at an asexual vertebrate's genome, they are typically heterozygous at most genes, a reflection of their hybrid origin (Vrijenhoek and Pfeiler 2008; Warren et al. 2018).

1.2 Potential evolutionary costs of asexuality

Evolutionary fitness is a representation of an organism's reproductive success and includes the organism's ability to survive long enough to reproduce. The benefits of asexual reproduction in terms of evolutionary fitness, including less time and effort spent on reproduction, should predict an abundance of asexual species; however, this is not the case (Maynard Smith 1958, 1978). The rareness of asexual reproduction and overall dominance of sexual reproduction among vertebrate species indicates an inherent cost to asexuality that outweighs its benefits (Speijer et al. 2015). Sexual reproduction provides more genetic diversity due to variation in combinations of alleles as a result of recombination during gamete formation and the union of egg from the mother and sperm from the father. This genetic diversity might help organisms combat deleterious mutations and parasitic infection, potentially explaining the benefit of sexual reproduction and its resulting dominance over asexual reproduction among vertebrate species (Sharp and Otto 2016).

In terms of their evolutionary fitness, asexual hybrids might fare better or worse relative to their parental generation or perform somewhere in between (e.g., Klabacka et al. 2022). Since asexual hybrids have high heterozygosity, an increase in fitness compared to their parental generation is frequently predicted (Mitton and Grant 1984; Bullini 1994; Cullum 1997). For example, fruit fly (*Drosophila melanogaster*) and copepod (*Tigriopus californicus*) F_1 hybrids experience increased fitness compared to their parental lineages, as evidenced by higher rates of coupled mitochondrial respiration (McDaniel and Grimwood 1971; Martinez and McDaniel 1979; Ellison and Burton 2008).

However, several other studies have found reduced hybrid fitness. For example, asexual *Aspidoscelis* lizards were found to have reduced endurance capacity and mitochondrial

respiration relative to the parental generation (Klabacka et al. 2022). Cullum (1997) also found that asexual *Cnemidophorus* lizards had significantly worse endurance than their sexual parent species despite their increased heterozygosity.

1.3 Measuring the effects of hybridization and asexuality: Metabolic rate as a measure of physiological capacity

To survive, an animal must be able to perform ecologically relevant tasks such as eluding perdators and catching prey, which involve physical feats such as running, jumping, flying, feeding, and vocalization, (Irschick and Higham 2015). For this thesis I aimed to use aerobic scope as a measure of physiological performance - and by extension, fitness - to compare asexual hybrids to their parental lineage. Aerobic scope is the difference between the resting and maximal metabolic rates of an organism and indicates the energy available for carrying out ecologically relevant tasks beyond basic maintenance metabolism (Clark et al. 2013). It can describe the capacity for activities such as catching prey, escaping predation, and defending territory, which are important for survival and reproduction in many animal species, and may indicate how well an animal will perform in the wild (Clark et al. 2013). I aimed to measure both resting and maximal metabolic rates to calculate aerobic scope. Resting metabolic rate (RMR) is defined as the minimum oxygen consumption rate of an individual in a relatively quiescent state (Burton et al. 2011). Maximal metabolic rate (MMR) is defined as the maximum rate of oxygen consumption that an animal can achieve under any ecological circumstance (Norin and Clark 2016), usually reached when the individual is under significant stress or running, swimming or flying.

1.4 *Fundulus* as a model system to study the effects of hybridization and asexuality on animal performance

Fish are one group of animals that commonly hybridize (reviewed by Dalziel et al. 2020). Hybridization is particularly common in fish species relative to other vertebrate groups largely due to their use of external fertilization for reproduction (Hernandez-Chavez and Turgeon 2007), which increases the chances of sperm and egg from different species coming into contact (Allendorf and Waples 1996). Teleost fish from the genus *Fundulus* are one clade that commonly hybridizes (e.g., MacPherson et al. 2023) and have long been a model taxon in physiology, genetics, biochemistry, population biology and toxicology (Eisler 1986; Bernardi 1993; Wood and Marshall 1994; Burnett et al. 2007). This genus is a favourite of researchers because of its evolutionary and ecological diversity, with over 35 species found throughout North America in both freshwater and marine habitats (Wiley 1986; Bernardi 1997). The common killifish or mummichog, *Fundulus heteroclitus*, lives in the brackish waters of tidal marshes and estuaries along the coast of eastern North America, spanning from Newfoundland to Texas (Wood and Marshall 1994). Its accessibility, high abundance in North America, and tolerance to various environmental stressors (e.g., water salinity, toxins) have contributed to widespread popularity throughout the biological sciences due to the resulting amenability to experimental manipulation (Burnett et al. 2007).

Another species of killifish, the banded killifish (*Fundulus diaphanus*), is also found on the eastern coast of North America but has not been studied as intensively as *F. heteroclitus* (Hernandez-Chavez and Turgeon 2007). The two species have different distributions along the Atlantic coast of Canada and the United States (Hernandez-Chavez and Turgeon 2007) and differ in their salinity tolerance, with *F. heteroclitus* being more salt tolerant in general than *F.*

diaphanus (Fritz and Garside 1974). Specifically, *F. diaphanus* occurs mainly in inland freshwater lakes in contrast to the brackish water habitats of *F. heteroclitus*, so it is assumed that the two species are mostly allopatric. However, both species can tolerate a wide range of water salinities, so in some areas their distributions overlap and hybridization can occur (Fritz and Garside 1974). Such sympatric populations have been found at several locations in Nova Scotia, Canada including Porters Lake and the Saint Mary's River (Fritz and Garside 1974; Dawley 1992).

Male *F. heteroclitus* and female *F. diaphanus* naturally hybridize in the wild where their distributions overlap to produce all-female asexual hybrid killifish (Mérette et al. 2009). One such area of overlap is Porters Lake, Nova Scotia, where Fritz and Garside (1974) found 170 of these hybrids which were distinctly intermediate between the two parental species in numerous morphological characteristics. These diploid hybrids only occupy about 10% of the *Fundulus* population at Porters Lake, suggesting them to be a rare species (Dawley 1992; Mérette et al. 2009). Jonah (2019) found that killifish hybrids were also intermediate between their parental species in terms of water salinity tolerance; these hybrids are more tolerant to environmental stress than their female parent *F. diaphanus*, yet less tolerant than their male parent *F. heteroclitus*. Dawley (1992) also determined that killifish hybrid "DNA values" tested at multiple loci were intermediate between the two parental lineages, suggesting that hybrids may be generally intermediate in most physiological traits compared to their parental species.

1.5 Thesis goals

The major goal for this thesis was to determine the effect of *F. heteroclitus–F diaphanus* hybridization on killifish standard and maximal metabolic rate and resulting scope for aerobic

activity as a means of assessing the hybrids' physiological capacities compared to their parental species. To accomplish these goals, I measured resting metabolic rates and maximal metabolic rates of 20 wild-caught killifish at a common salinity and temperature (10 ppt ± 1 ppt, and 20-22 °C): six *F. diaphanus*, seven hybrids, and seven *F. heteroclitus*. Aerobic metabolic rates were measured using intermittent-flow respirometry by monitoring O_2 consumed by the fish over time.

I predicted that the hybrid killifish would have a metabolic rate intermediate to those of their two parents: below that of the male parental species (*F. heteroclitus*) and above that of the female parental species (*F. diaphanus*). I made this prediction because F1 hybrid killifish are intermediate between their two parental species in numerous morphological characteristics (Fritz and Garside 1974) as well as water salinity tolerance (Jonah 2019), and do not show any evidence of hybrid breakdown, suggesting that most traits are controlled by additive genetic variation.

2. MATERIALS AND METHODS

2.1 Collection of killifish (*F. heteroclitus, F. diaphanus,* and hybrids)

Killifish were collected from Porters Lake, Nova Scotia (44.7433°N, 63.2972°W) in July and September of 2022 (Figure 2.1). A total of 99 female fish were caught – a mixture of *Fundulus diaphanus*, *Fundulus heteroclitus*, and their inter-specific hybrids. Fish were caught using both seine netting and Gee minnow traps. Traps were left to soak for approximately two hours. We first identified fish species visually and attempted to verify this identification in the field by measuring three morphological traits and using an algorithm that incorporated these measures, which was found to have a 91.5% success rate (Merette 2009) (Figure 2.2). However, this system of morphological measurements did not work well for smaller juvenile fish such as

ours, so we also conducted genetic identification (see section 2.5). All collection methods followed those approved by the Saint Mary's University Animal Care Committee protocols (SMU ACC AUPF 22-12) and Department of Fisheries and Oceans permit (Licence #343930) held by Dr. Anne Dalziel. The fish were transported back to Saint Mary's University Aquarium facilities where they were kept in two 30-gallon glass tanks and acclimated for at least one week at a salinity of approximately 10 ppt, a temperature of about 21°C, and a pH of 7.4-8.0. Fish were fed twice daily: in the morning before 10:00am with measured portions of bloodworms and TetraMin fish flakes, and in the afternoon after 2:00pm with brine shrimp. Water quality was measured weekly to assess the concentrations of ammonia, nitrite, and nitrate, as well as pH – water changes were conducted afterwards at a minimum of 20% of tank volume, with a higher percentage being changed if water quality concentrations exceeded levels deemed stressful for fish.

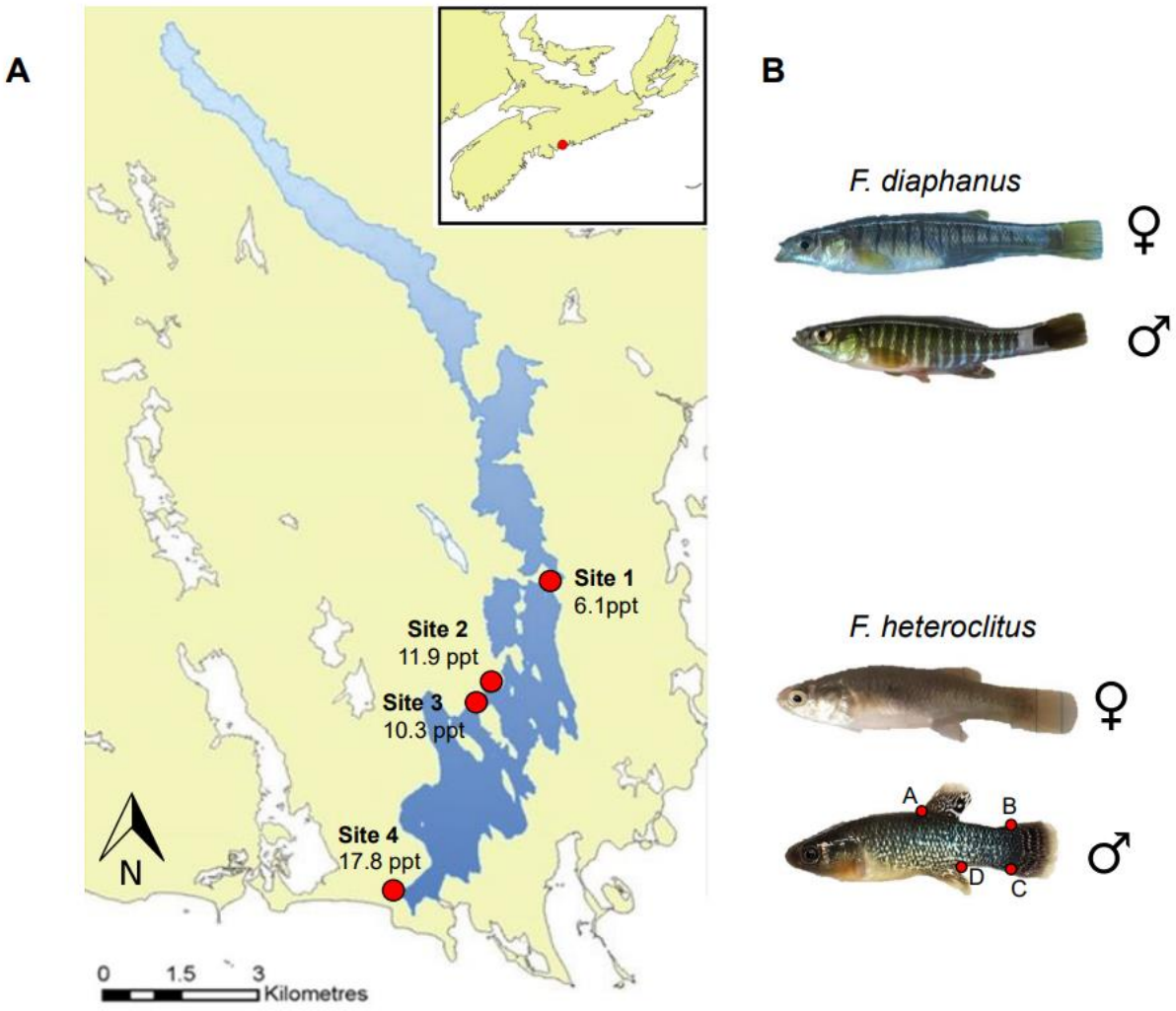

Figure 2.1. Map of Porters Lake, Nova Scotia, and images of adult male and female *Fundulus diaphanus* and *Fundulus heteroclitus* in breeding condition. Site 2 indicates the location where fish were collected in July and September of 2022. The gradient of blue indicates water salinity, with freshwater at the top in light blue and saltier water at the bottom in darker blue (image from MacPherson et al. 2023).

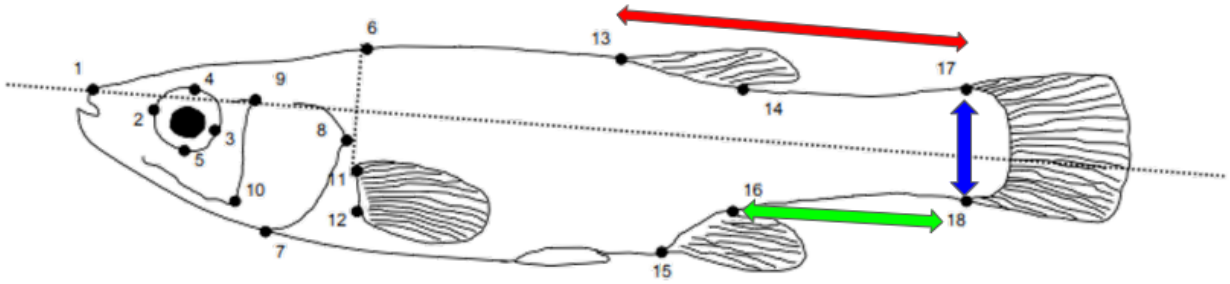

Figure 2.2. Diagram of a *Fundulus* fish from Mérette et al. (2009) with red, blue, and green arrows indicating the three different morphological measurements used to identify species with Merette et al.'s (2009) maximum likelihood method.

2.2 Tagging, fin-clipping, and morphological identification

The fish were allowed one week of acclimation in the lab after collection and before tagging, fin-clipping, and a second attempt at morphological identification. All fish were individually tagged using a different combination of Visible Implant Elastomer tags (Northwest Marine Technology). We then re-measured the morphological characteristics of the fish (Figure 2.2) using digital calipers and entered the data into the morphological identification algorithm once again to try to verify their species identity. However, the algorithm again proved to be inaccurate; therefore, small clippings of the tail fins were taken from each fish to be used for genotyping as an alternate method of species identification (Section 2.5). Fin clips were collected and stored in tubes containing 95% ethanol.

2.3 Resting metabolic rate measurements

Oxygen consumption was used as a measure of metabolic rate since O_2 is used in the electron transport chain to produce energy in the form of ATP during cellular respiration. The oxygen consumption of the acclimated fish was measured in the Fall of 2022 using two 170mL Loligo mini swim tunnel respirometers (SW10000; Loligo Systems, Tjele, Denmark) following

the methods of Healy and Schulte (2012). Oxygen consumption was measured by percentage of air saturation in the tunnel and then converted to μM O_2 for analysis by incorporating the daily barometric pressure, O_2 solubility coefficient, and the molar mass of oxygen (31.999 g/mol). Values were then converted to μmol O_2/g/hr by taking into account the volume of the chamber and the mass of the fish, producing the final mass-corrected metabolic rate value used in my data analysis.

The fish were fasted for 24 hours before experiments took place so that the metabolic costs of digestion would not interfere with resting metabolic rate measurements. Before beginning experiments each day the water salinity, water temperature, barometric pressure, and individual fish weight were recorded in the Loligo AutoResp© software (Loligo Systems, Tjele, Denmark) so that metabolic rates were adjusted to correct for these variables as mentioned above. At the beginning and end of each day of experiments we measured the bacterial oxygen consumption rate in the swim tunnels so that we could subtract these measurements to obtain the fish's actual oxygen consumption rate. To measure resting metabolic rate one fish was placed in each swim tunnel and the swim tunnels were wrapped with black garbage bags to reduce light exposure and relax the fish. The two fish were then left undisturbed for 30 minutes in the swim tunnel with oxygenated water flowing into the respirometry chamber from the outer tank. After 30 minutes the respirometer was shut automatically with AutoResp© and the water oxygen concentration was recorded in mg O_2 per L over a measurement period of 20 minutes. Dissolved oxygen was measured using a Witrox 4 oxygen meter (OX11875), sensor spots (OX11035), and fiber cables (OX11150) from Loligo (Loligo Systems, Tjele, Denmark).

2.4 Maximal metabolic rate measurements

Maximal metabolic rate was measured using the same Loligo system as for resting metabolic rate. To elicit maximal metabolism, fish were chased around in a bucket until they showed signs of exhaustion, a commonly used method (Healy and Schulte 2012; Norin and Clark 2016). Briefly, the fish were chased and pinched lightly at the tail for a period of approximately 10-20 minutes until the fish no longer made attempts to evade the pinching. At this point the fish was placed into a Loligo swim tunnel where its oxygen consumption was measured until it began to decrease back to a normal rate that was no longer maximal; usually no more than 10 minutes.

Unfortunately, the glass Loligo swim tunnel respirometer broke halfway through the maximal metabolic rate experiments, so I was not able to finish collecting this data. Due to this issue the maximal metabolic rate data are not included in my results, and aerobic scope could not be calculated.

2.5 DNA extraction and genotyping to confirm species identity

To confirm species identity we combined visual identification with morphological measures and genetic identification. Since the hybrids are all females we selected only female fish for this study, which could be distinguished from males that show bright breeding colouration (see Figure 2.1). We first identified the species of each fish visually as either *F. heteroclitus* or *F. diaphanus*. *F. heteroclitus* have a characteristic short snout and wide caudal peduncle, whereas *F. diaphanus* have a longer snout and longer, more streamlined body, while also exhibiting dark brown bands (Figure 2.1). Hybrids appear nearly identical to *F. heteroclitus* females. Second, we measured three morphological traits (Section 2.2; Figure 2.2). Finally, we

tested if fish had a mitochondrial genome from *F. heteroclitus* or *F. diaphanus* by using the assay designed by Merette et al. (2009) and optimized by Tirbhowan (2019). Fish that identified both visually and genetically with one species were assigned that species, and fish that identified visually as *F. heteroclitus* but had the mitochondrial D-loop of *F. diaphanus* were classified as hybrids, as >95% of hybrids at Porters Lake have an *F. diaphanus* mother (Hernandez-Chavez and Turgeon, 2007; Dalziel et al., 2020).

DNA was extracted from *Fundulus* spp. fin clippings using the Omega Bio-tek E.Z.N.A.® Tissue DNA Kit (D3396-02; Omega Bio-tek, Inc., Norcross, GA, USA) following the manufacturer's instructions for DNA extractions from tissue samples. DNA was then stored at -20°C for later use in polymerase chain reactions (PCR).

PCR was used to amplify the D-loop region of mitochondrial DNA for each sample in 25 µL reactions following Tirbowan's (2019) protocol. We used D-loop primers '71 Fundulus Dloop F1 (5'- TTAACCCCCACCCCTAGCTC -3')' and '73 Fundulus Dloop Rev1 (5'- GCACTGTGAAATGTCAACTGAA -3')', updated by Tirbowan (2019) from primers 'd-loopA' and 'd-loopE' originally designed by Lee et al. (1995). For each reaction, final PCR concentrations and quantities were as follows: 2.5 µL of 10X PCR buffer (Green GoTaq® Flexi buffer), 1 µL of 0.4 µM forward primer ('71'), 1 µL of 0.4 µM reverse primer ('73'), 0.5 µL of 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.25 µL of 0.025 U/µL GoTaq® DNA polymerase (Taq), and 19.75 µL of nuclease-free deionized distilled water (ddH_2O) to obtain a final volume of 25 µL. 1-2 µL of DNA was used for each reaction. Amplification conditions were as follows: initial denaturation of 3 minutes at 95°C, followed by 44 cycles of denaturation for 45 seconds at 95°C, primer annealing for 45 seconds at 54°C and primer extending for 1

minute at 72°C, and a final extension for 5 minutes at 72°C. After amplification we obtained a final product that was 660 base pairs long.

The PCR products were then subjected to restriction enzyme digestion to identify the maternal species (either *F. heteroclitus* or *F. diaphanus*). There are two HphI restriction sites in the amplified region of the *F. diaphanus* mitochondrial D-loop, but only one in that of *F. heteroclitus* (Hernandez-Chavez and Turgeon, 2007; Tirbhowan, 2019). Digestion of the mitochondrial D-loops of the two species produce bands at 211, 166, and 215 base pairs for *F. diaphanus* and bands at 211 and 381 base pairs for *F. heteroclitus*. PCR products were digested by adding a solution composed of 3 µL of 10X CutSmart® Buffer, 1 µL of 250 U/mL HphI restriction enzyme, and 1 µL of distilled water to each product and incubating at 37°C for 8 hours. The digested products were then run on a 3.5% agarose gel stained with ethidium bromide at 120 V for 1 hour and analyzed for the aforementioned band patterns (Figure 3.2).

2.6 Data analysis

Graphs of µM of O_2 in the swim tunnels over time (in minutes) were generated for all resting metabolic rate trials by converting air saturation of oxygen in the tunnel to µM O_2 using variables discussed in Section 2.3. I used the slope of each graph to calculate resting metabolic rate by converting µM O_2/min to µmol O_2/g/hr by incorporating other variables (see Section 2.3). The resting metabolic rates were then analyzed using separate one-way ANOVA tests in R 4.2.2 and RStudio 2022.07.2+576 with a p-value cutoff of 0.05 to test for significant differences in resting metabolic rate among parental species and hybrids, using species identification as my explanatory variable and metabolic rate as my response variable.

3. RESULTS

Figure 3.3 and Table 3.2 show the resting metabolic rates (RMR) of Killifish species, uncorrected for bacterial respiration, which did not significantly differ among groups (one-way ANOVA; $F_{2,15}$=0.290, p=0.752). Figure 3.4 and Table 3.3 show the bacterial respiration rates used to correct RMR of Killifish species, which also did not significantly differ among groups ($F_{2,15}$=0.565, p=0.579) and were on average about 10% of the total uncorrected RMR. Figure 3.5 and Table 3.4 show the final corrected RMR of Killifish species, which did not significantly differ among groups ($F_{2,15}$=0.223, p=0.803). As mentioned in Section 2.4, the maximal metabolic rate data was unable to be used due to equipment failure, so aerobic scope could not be compared among species.

Table 3.1. Species identification and resting metabolic rate data for each fish tested. *F. diaphanus* could be clearly identified visually, so were not genotyped except for fish #97, which was used as a positive control for the *F. diaphanus* mitochondrial genome. Size-corrected metabolic rate data are presented as μmol O_2/g/hr.

Fish #	Visual ID	Mitochondrial D-loop	Species	Uncorrected RMR	Bacterial Respiration	Corrected RMR
18	heteroclitus	heteroclitus	heteroclitus	10.88	1.50	9.38
29	heteroclitus	heteroclitus	heteroclitus	25.99	0.10	25.89
56	heteroclitus	diaphanus	hybrid	20.35	2.80	17.56
61	heteroclitus	diaphanus	hybrid	16.73	1.84	14.89
62	heteroclitus	diaphanus	hybrid	17.07	2.42	14.64
64	heteroclitus	diaphanus	hybrid	12.56	0.82	11.74
65	diaphanus		diaphanus	13.10	1.09	12.01
67	heteroclitus	heteroclitus	heteroclitus	7.80	1.07	6.74
70	heteroclitus	heteroclitus	heteroclitus	6.51	0.04	6.47
77	heteroclitus	heteroclitus	heteroclitus	5.04	0.14	4.91
81	diaphanus		diaphanus	14.57	0.40	14.18
82	heteroclitus	diaphanus	hybrid	12.02	2.61	9.41
83	heteroclitus	heteroclitus	heteroclitus	14.69	3.65	11.04
85	diaphanus		diaphanus	11.23	0.72	10.51
87	heteroclitus	diaphanus	hybrid	11.04	1.11	9.93
89	diaphanus		diaphanus	11.65	2.45	9.19
95	heteroclitus	heteroclitus	heteroclitus	29.44	1.53	27.91
96	heteroclitus	diaphanus	hybrid	14.55	0.15	14.40
97	diaphanus	diaphanus	diaphanus	11.09	1.60	9.49
99	diaphanus		diaphanus	12.73	0.68	12.04

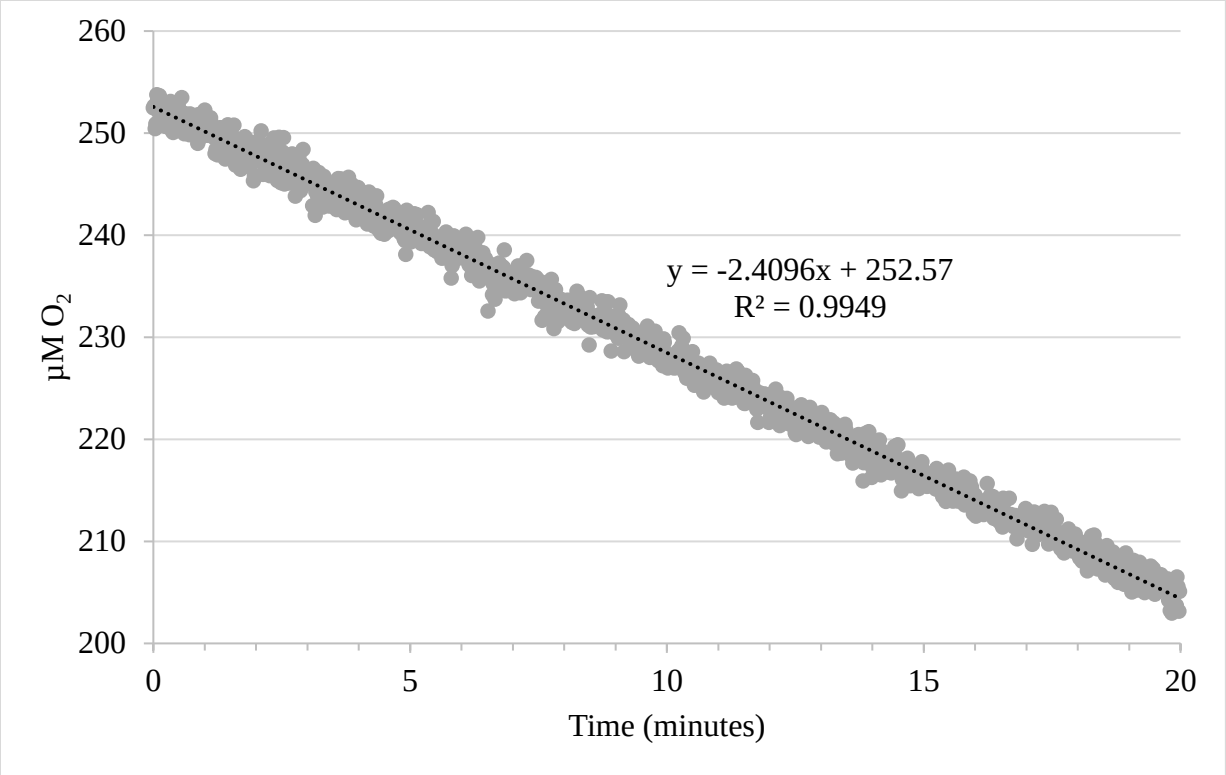

Figure 3.1. Example of oxygen depletion in the Loligo swim tunnel over time. The slope is used to calculate resting metabolic rate by converting µM O_2/min to µmol O_2/g/hr using variables discussed in Section 2.3.

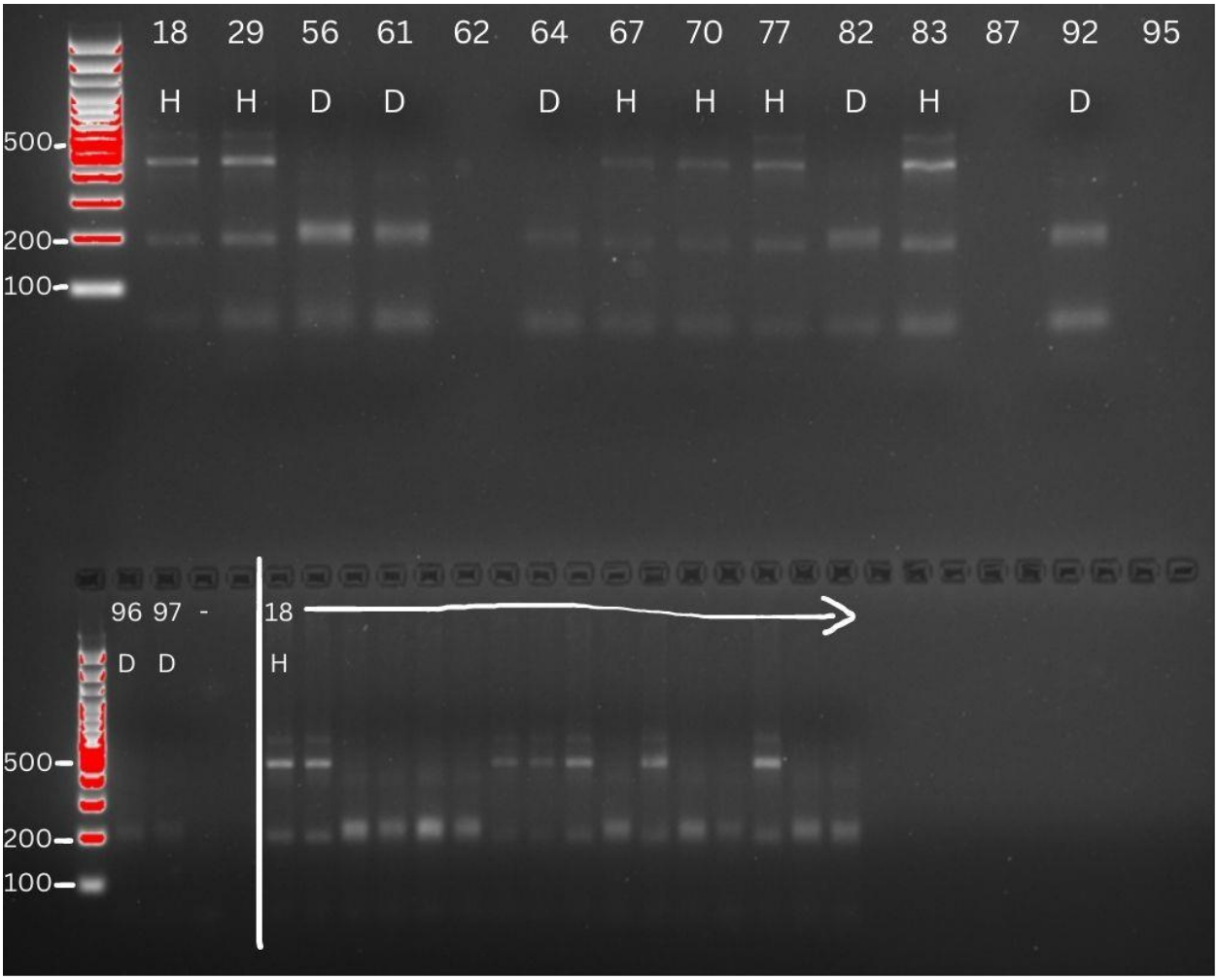

Figure 3.2. Example agarose gel electrophoresis image of HphI digested PCR amplified section of the mitochondrial D-loop in Killifish. The first lane in each row is loaded with a 100 base pair DNA ladder. Gel lanes labelled with fish identification number and letter denoting maternal species: "H"=*F. heteroclitus*, "D"=*F. diaphanus*. Samples not shown on this gel were re-run on other gels (data not shown).

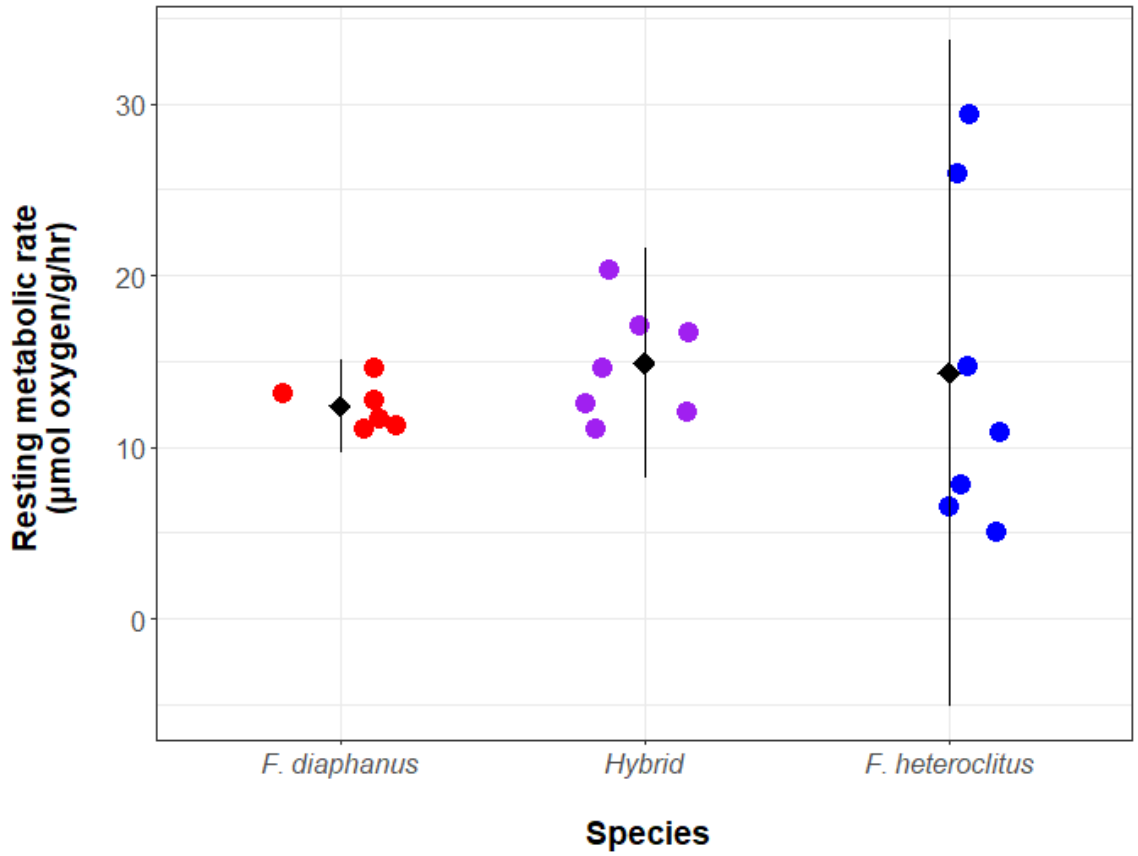

Figure 3.3. Uncorrected resting metabolic rates in µmol of oxygen/g/hour of Killifish species from Porters Lake in 2022 (n=6 *F. diaphanus*, n=7 hybrids, and n=7 *F. heteroclitus*).

Table 3.2. One-way ANOVA table for uncorrected RMR of Killifish species from Porters Lake in 2022.

	Df	Sum Sq	Mean Sq	F value	P value
Species	2	21.90	10.95	0.2901	0.7518
Residuals	17	641.74	37.75		

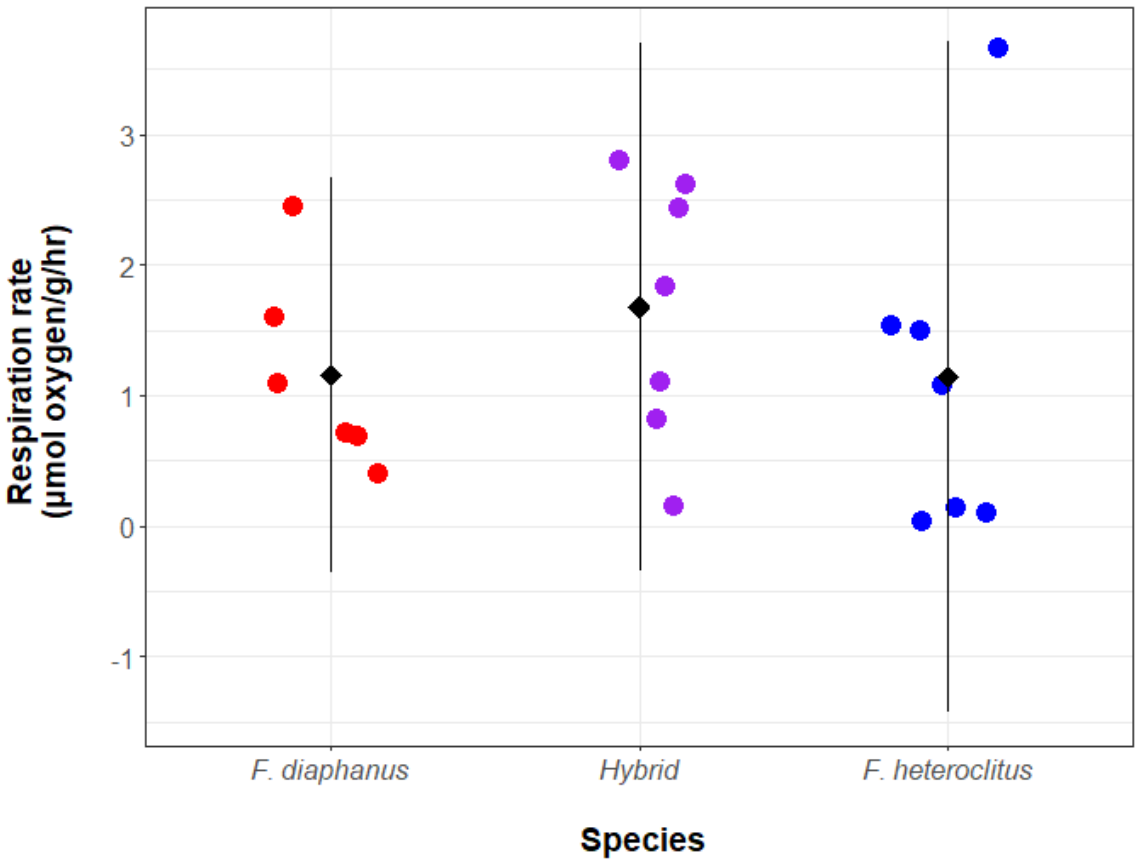

Figure 3.4. Bacterial respiration rates in µmol of oxygen/g/hour to correct for RMR of Killifish species from Porters Lake in 2022 (n=6 *F. diaphanus*, n=7 hybrids, and n=7 *F. heteroclitus*).

Table 3.3. One-way ANOVA table for bacterial respiration rates used to correct RMR of Killifish species from Porters Lake in 2022.

	Df	Sum Sq	Mean Sq	F value	P value
Species	2	1.2548	0.62741	0.5647	0.5789
Residuals	17	18.8892	1.11113		

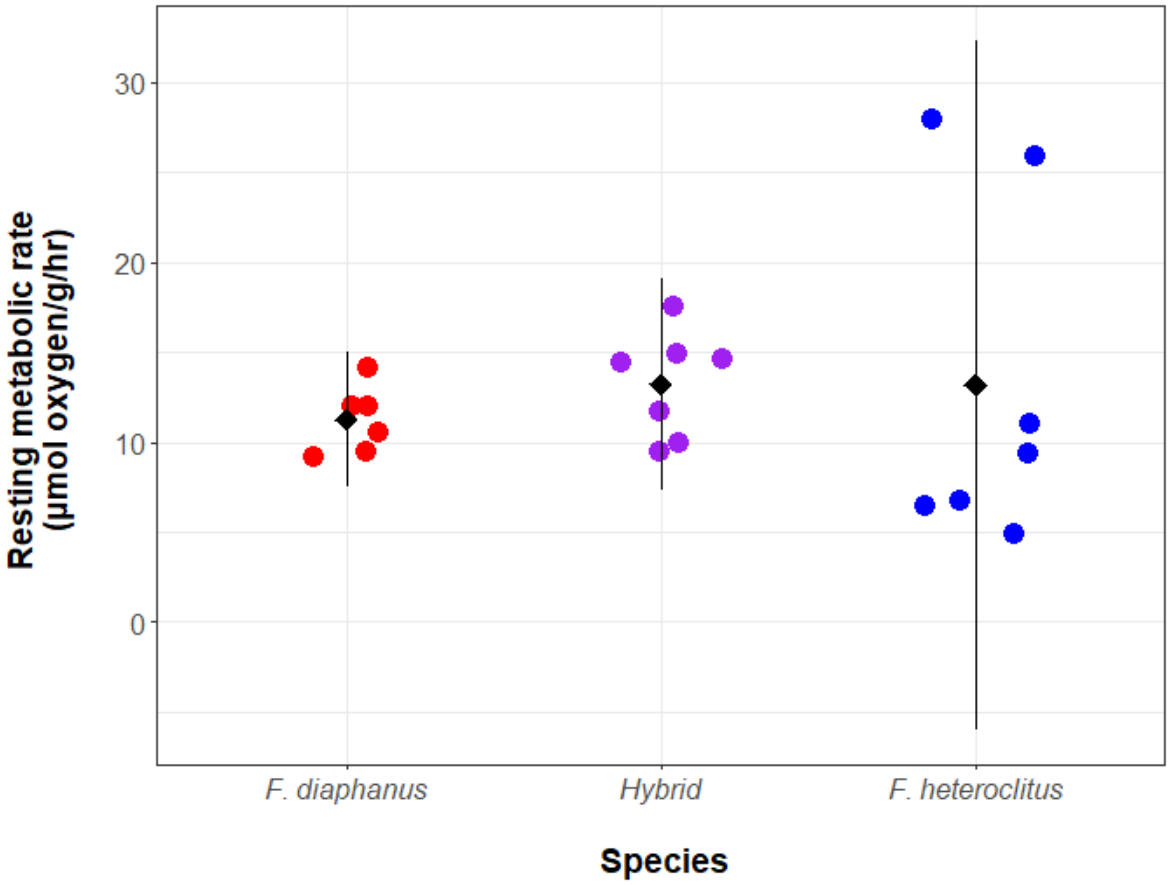

Figure 3.5. Corrected resting metabolic rates in µmol of oxygen/g/hour of Killifish species from Porters Lake in 2022. Data have been corrected to account for bacterial respiration rates (Figure 3.4, Table 3.3; n=6 *F. diaphanus*, n=7 hybrids, and n=7 *F. heteroclitus*).

Table 3.4. One-way ANOVA table for corrected RMR of Killifish species from Porters Lake in 2022.

	Df	Sum Sq	Mean Sq	F value	P value
Species	2	16.32	8.158	0.2229	0.8025
Residuals	17	622.32	36.607		

4. DISCUSSION

4.1 Resting metabolic rates

My results indicate that there is no significant difference between the resting metabolic rates (RMR) of Common Killifish, Banded Killifish, and their hybrid species (Figure 3.5; Table 3.4). This indicates that all three species require approximately the same amount of energy to survive at a resting state in the conditions that we studied them. The hybrids show no advantage nor disadvantage compared to their parental species. One possible explanation for this phenomenon is that all three species share the same environment and may have evolved to possess similar RMR levels over time. Although studies of RMR in asexual fish on its own are scarce, my results agree with those of Cullum (1997) which found that asexual lizard hybrids had no difference in size-corrected standard metabolic rate compared to their parental species. My results, and those of Cullum (1997) disagree with studies that predicted hybrids to have a physiological advantage compared to their parent species due to their higher heterozygosity (Mitton and Grant 1984; Bullini 1994; Cullum 1997), as well as studies that found increased mitochondrial respiration and fitness in F_1 hybrids of fruit flies and copepods (McDaniel and Grimwood 1971; Martinez and McDaniel 1979; Ellison and Burton 2008). These data suggest that the effects of hybridization and asexuality may be species or clade specific.

As I could not measure maximal metabolic rate (MMR), I do not know if aerobic scope varies among these species. Hybrid asexual lizards (*Aspidoscelis* spp.) are predicted to have lower aerobic scopes than parental species, as they have similar resting metabolic rates and lower endurance capacity and maximal muscle mitochondrial respiration (often positively correlated with MMR) than in sexual parental species (Klabacka et al. 2022; Cullum 1997). However, the parental *Aspidoscelis* lizard species have similar endurance capacities and maximal

mitochondrial respiration rates, while we predict that *F. diaphanus* will have a lower MMR than *F. heteroclitus*. Thus, we predict a higher chance of heterosis (i.e., high aerobic scope) in asexual *Fundulus* F_1 hybrids than in *Aspidoscelis* lizards. If RMR is constant across species, aerobic scope could only differ between species or individuals if MMR varies. Indeed, my predictions for aerobic scope might still be correct if *F. diaphanus* have a lower MMR than *F. heteroclitus* and the hybrids are intermediate between the two; future studies must be conducted on MMR and aerobic scope to find out.

For an animal, having a low RMR could provide both advantages and disadvantages depending on the environmental conditions. The organism does not have to expend as much energy to simply survive, which could be beneficial in environments where food is scarce. However, low RMR might be detrimental in environments where food is plentiful and resting energy could be used to grow, thereby increasing body size and strength to avoid predation (reviewed by Cullum 1997). As for MMR and aerobic scope, it is usually more advantageous to have a higher rate as this allows for more energy to perform ecologically relevant activities (Clark et al. 2013).

The similar levels of RMR seen in the two Killifish species and their hybrids indicate a similar level of animal performance, as well as physiological capacity available to perform ecologically relevant activities important for basic survival in a resting state. As physiological capacity can be an indicator of evolutionary fitness (Irschick and Higham 2015), my data may suggest that hybridization in these Killifish yields no cost or benefit to fitness while the organism is in a resting state.

4.2 Limitations

Unfortunately, I encountered a few issues that prevented me from obtaining maximal metabolic rate data. The glassware of both of our Loligo swim tunnels broke midway through MMR experiments and prevented me from measuring MMR and calculating the aerobic scope of the killifish as originally planned. We also did not have enough time to genotype the fish as thoroughly as we had hoped; we had planned to supplement the mitochondrial D-loop genotyping with species-specific nuclear microsatellites to confirm species identity further, as performed in Hernandez-Chavez and Turgeon (2007) and Dalziel et al. (2020). This method could have provided a more accurate species identification by confirming hybrids were heterozygous at specific nuclear loci and their parental lineages were homozygous.

4.3 Future directions

The next step required to determine the effect of hybridization on Killifish metabolic rate would be to finish the work that I set out to achieve and successfully measure maximal metabolic rates combined with resting metabolic rates to calculate aerobic scope of the fish. As per my hypothesis, I would predict that the maximal metabolic rates, and therefore the aerobic scopes, of these three species would differ: *F. diaphanus* would have a lower aerobic scope than *F. heteroclitus*, and their hybrid species would be intermediate between the two. This is due to similar patterns seen for these species in numerous morphological characteristics (Fritz and Garside 1974) and upper salinity tolerance (Jonah 2019). I would also genotype the fish at species-specific nuclear microsatellites to confirm Killifish species identity as previously mentioned.

It would also be interesting to see how aerobic scope varies with ecologically relevant changes in temperature and salinity. My study maintained a constant salinity of 10 ppt and air temperature of 21°C, but exploring metabolic rates in different conditions could allow us to determine which conditions maximize aerobic scope. In this environment they would be at their maximum physiological performance and be able to perform the most activities important to survival and reproduction, such as catching prey, escaping predation, and defending territory. Salinity in Porters Lake ranges widely from 0.8-28.2 ppt, and water temperature ranges from 6.0-24.3°C (CBCL Limited 2013). Due to this large variation it is worth testing more salinities and temperatures to analyze ecologically relevant measures of energetic needs.

4.4 Conclusions

Common Killifish, Banded Killifish, and their hybrid species do not differ significantly in terms of their resting metabolic rates, indicating that they require similar amounts of energy to survive in a relaxed state at 10 ppt and 21°C. This suggests that there may be no major metabolic costs or benefits to hybridization, but measurements of maximal metabolic rates across a range of ecologically relevant conditions are needed to further test this hypothesis.

5. LITERATURE CITED

Abbott R, Albach D, Ansell S, Arntzen JW, Baird SJE, Bierne N, Boughman J, Brelsford A, Buerkle CA, Buggs R, et al. 2013. Hybridization and speciation. Journal of Evolutionary Biology. 26(2): 229-246.

Allendorf FW, Waples RS. 1996. Conservation and genetics of salmonid fishes. Nature, 2380280.

Avise JC. 2015. Evolutionary perspectives on clonal reproduction in vertebrate animals. Proceedings of the National Academy of Sciences. 112(29): 8867-8873.

Bernardi G, Sordino P, Powers DA. 1993. Concordant mitochondrial and nuclear DNA phylogenies for populations of the teleost fish *Fundulus heteroclitus*. Proceedings of the National Academy of Sciences. 90(20): 9271-9274.

Bernardi G. 1997. Molecular phylogeny of the Fundulidae (Teleostei, Cyprinodontiformes) based on the cytochrome b gene. In: Molecular systematics of fishes. San Diego (CA): Academic Press. p. 189-198.

Bullini L. 1994. Origin and evolution of animal hybrid species. Trends in Ecology and Evolution. 9(11): 422-426.

Burnett KG, Bain LJ, Baldwin WS, Callard GV, Cohen S, Di Giulio RT, Evans DH, Gómez-Chiarri M, Hahn ME, Hoover CA, et al. 2007. *Fundulus* as the premier teleost model in environmental biology: opportunities for new insights using genomics. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics. 2(4): 257-286.

Burton T, Killen SS, Armstrong JD, Metcalfe NB. 2011. What causes intraspecific variation in resting metabolic rate and what are its ecological consequences?. Proceedings of the Royal Society B: Biological Sciences. 278(1724): 3465-3473.

Camillo F, Vannozzi I, Rota A, Di Luzio B, Romagnoli S, Aria G, Allen WR. 2003. Successful non-surgical transfer of horse embryos to mule recipients. Reproduction in Domestic Animals. 38(5): 380-385.

CBCL Limited. 2013. Porters Lake Watershed Servicing Study.

Clark TD, Sandblom E, Jutfelt F. 2013. Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. The Journal of Experimental Biology. 216(15): 2771-2782.

Cullum AJ. 1997. Comparisons of physiological performance in sexual and asexual whiptail lizards (genus *Cnemidophorus*): implications for the role of heterozygosity. American Naturalist. 150(1): 24-47.

Dawley RM. 1992. Clonal hybrids of the common laboratory fish *Fundulus heteroclitus*. Proceedings of the National Academy of Sciences. 89(6): 2485-2488.

Eisler R. 1986. Use of *Fundulus heteroclitus* in pollution studies. American Zoologist. 26(1): 283-288.

Ellison CK, Burton RS. 2008. Interpopulation hybrid breakdown maps to the mitochondrial genome. Evolution. 62(3): 631-638.

Fritz ES, Garside ET. 1974. Identification and description of hybrids of *Fundulus heteroclitus* and *F. diaphanus* (Pisces: Cyprinodontidae) from Porters Lake, Nova Scotia, with evidence for absence of backcrossing. Canadian Journal of Zoology. 52(12): 1433-1442.

Grant PR, Grant BR. 1994. Phenotypic and genetic effects of hybridization in Darwin's finches. Evolution. 48(2): 297-316.

Healy TM, Schulte PM. 2012. Thermal acclimation is not necessary to maintain a wide thermal breadth of aerobic scope in the common killifish (*Fundulus heteroclitus*). Physiological

and Biochemical Zoology: Ecological and Evolutionary Approaches. 85(2): 107-119.

Hernandez-Chavez C, Turgeon J. 2006. Asexual and sexual hybrids between *Fundulus diaphanus* and *F. heteroclitus* in the Canadian Atlantic region. Molecular Ecology. 16(7): 1467-1480.

Irschick DJ, Higham TE. 2015. Animal performance: An overview. In: Animal athletes: An ecological and evolutionary approach. Oxford: Oxford Academic. 1-23.

Janko K, Pačes J, Wilkinson-Herbots H, Costa RJ, Roslein J, Drozd P, Iakovenko N, Rídl J, Hroudová M, Kočí J, et al. 2017. Hybrid asexuality as a primary postzygotic barrier between nascent species: on the interconnection between asexuality, hybridization and speciation. Molecular Ecology. 27(1): 248-263.

Jonah LS. 2019. Evolutionary variation in salinity tolerance among species of killifishes (*Fundulus* spp.) [Unpublished master's thesis]. Saint Mary's University.

Klabacka RL, Parry HA, Nian Yap K, Cook RA, Herron VA, Horne LM, Wolak ME, Maldonado JA, Fujita MK, Kavazis AN, et al. 2022. Reduced mitochondrial respiration in hybrid asexual lizards. The American Naturalist. 199(5): 719-728.

MacPherson N, Champion CP, Weir LK, Dalziel AC. 2023. Reproductive isolating mechanisms contributing to asymmetric hybridization in Killifishes (*Fundulus* spp.). Journal of Evolutionary Biology. 36(3): 605-621.

Martinez AO, McDaniel RG. 1979. Mitochondrial heterosis in aging *Drosophila* hybrids. Experimental Gerontology. 14(5): 231-238.

Maynard Smith J. 1958. The theory of evolution. Cambridge University Press, Cambridge.

Maynard Smith J. 1978. The evolution of sex. Cambridge University Press, Cambridge.

McDaniel RG, Grimwood BG. 1971. Hybrid vigor in *Drosophila*: Respiration and mitochondrial

energy conservation. Comparative Biochemistry and Physiology. 38(2): 309-314.

McKinnell Z, Wessel G. 2012. Ligers and tigons and .....what? ....oh my!. Molecular Reproduction & Development. 79(8): Fm i.

Mérette D, Bourret A, Turgeon J. 2009. Diversity and distribution of gynogenetic hybrids between *Fundulus diaphanus* and *Fundulus heteroclitus* in Porter's Lake (Nova Scotia) in relation to salinity. Journal of Fish Biology. 75(5): 1115-1122.

Mitton JB, Grant MC. 1984. Associations among protein heterozygosity, growth rate, and developmental homeostasis. Annual Review of Ecology and Systematics. 15: 479-499.

Norin T, Clark TD. 2016. Measurement and relevance of maximum metabolic rate in fishes. Journal of Fish Biology. 88(1): 122-151.

Otto SP. 2008. Sexual reproduction and the evolution of sex. Nature Education. 1(1): 182.

Rieseberg LH, Archer MA, Wayne RK. 1999. Transgressive segregation, adaptation and speciation. Heredity. 83: 363-372.

Runemark A, Vallejo-Marin M, Meier JI. 2019. Eukaryote hybrid genomes. PLOS Genetics. 15(11): e1008404.

Seehausen O. 2004. Hybridization and adaptive radiation. Trends in Ecology & Evolution. 19(4): 198-207.

Sharp NP, Otto SP. 2016. Evolution of sex: using experimental genomics to select among competing theories. Bioessays. 38(8): 751-757.

Speijer D, Lukeš J, Eliáš M. 2015. Sex is a ubiquitous, ancient, and inherent attribute of eukaryotic life. Proceedings of the National Academy of Sciences. 112(29): 8827-8834.

Tirbhowan S. 2019. Temporal stability of Killifish hybrid clonal lineages. Halifax, NS: Saint Mary's University.

Vrijenhoek RC, Pfeiler E. 2008. Differential survival of sexual and asexual *Poeciliopsis* during environmental stress. Evolution. 51(5): 1593-1600.

Warren WC, García-Pérez R, Xu S, Lampert KP, Chalopin D, Stöck M, Loewe L, Lu Y, Kuderna L, Minx P, et al. 2018. Clonal polymorphism and high heterozygosity in the celibate genome of the Amazon molly. Nature Ecology & Evolution. 2: 669–679.

Wiley EO. 1986. A study of the evolutionary relationships of *Fundulus* topminnows (Teleostei: Fundulidae). American Zoologist. 26(1): 121-130.

Wood CM, Marshall WS. 1994. Ion balance, acid-base regulation, and chloride cell function in the common killifish, *Fundulus heteroclitus*–a euryhaline estuarine teleost. Estuaries. 17(1): 34-52.