SPERM MORPHOMETRY AND MOTILITY IN AN AFRICAN CICHLID, *PSEUDOCRENILABRUS MULTICOLOR VICTORIAE*, ACROSS DIVERGENT HABITATS

by

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ABSTRACT

There is a paucity of studies on the natural variation in sperm morphometry and motility in a single species across dissolved oxygen regimes. This study measured the natural variation in reproductive traits including sperm morphometry and motility of male *Pseudocrenilabrus multicolor victoriae* across a wide range of habitats in Uganda, Africa. I investigated whether fish displayed differences in testes mass, testes asymmetry, sperm morphometry, and sperm motility. *P. multicolor* were collected from nine sites characterized by three oxygen regimes: seasonally fluctuating, hypoxic and normoxic. Fish traits were measured and analyzed by site and by oxygen regime. I found that *P. multicolor* display variation in reproductive traits across habitats, and that males in hypoxic regimes are smaller bodied, have lower testes asymmetry, shorter sperm, and a higher sperm velocity relative to males in normoxic or fluctuating regimes. Males may be able to invest more energy into reproduction in hypoxic sites due to a lack of predators in these sites. Additionally, over the long term, males in hypoxic sites may have locally adapted to chronic conditions, allowing them to invest more energy into testes and sperm to offset the costs of living under hypoxia. In contrast, males from the fluctuating regime were large bodied, had high testes asymmetry, and the longest sperm with the lowest sperm velocity. Males in fluctuating regimes may be experiencing energetic trade-offs between growth and reproduction, due to the less predictable oxygen levels in their habitat. Future studies should assess *P. multicolor* for trait differences across oxygen regimes in a split-brood laboratory study to control for confounding effects of food availability, predation risk, and mating competition on reproductive traits.

Keywords

Sperm morphology, sperm motility, *Pseudocrenilabrus multicolor victoriae*, testis asymmetry

RÉSUMÉ

Il y a peu d'études traitant la variation naturelle morphologique des spermatozoïdes et leur motilité dans une seule espèce dans divers régimes d'oxygène dissous. Cette étude a mesuré la variation naturelle des traits reproductifs, y compris la morphométrie des spermatozoïdes et leur motilité chez les mâles du poisson *Pseudocrenilabrus multicolor victoriae* habitant une diversité d'habitats d'Ouganda, en Afrique. J'étudiée si les poissons présentent des différences au niveau d'indices gonadosomatiques, d'asymétrie testiculaire, ainsi que la morphométrie et la motilité des spermatozoïdes. Les mâles *P. multicolor* ont étaient recueillies dans neuf sites caractérises par trois régimes d'oxygène: saisonnier fluctuante, hypoxique et normoxique. Les différents traits chez les poissons ont étaient mesures et analyses par site ainsi que selon le régime d'oxygène dissous. J'ai trouvé une variation des traits reproductifs chez *P. multicolor* selon l'habitat où ils se trouvent. De plus, les mâles habitant dans des régimes hypoxiques ont un corps plus petit, possèdent un index gonadosomatique supérieur, avec de spermatozoïdes plus courts, et possédant une plus grande vitesse de nage que les ceux des mâles habitant des régimes normoxiques ou fluctuants. Il est possible que les mâles puissent être en mesure d'investir plus d'énergie dans la reproduction dans des sites hypoxiques en raison d'un manque de prédateurs dans ces sites. En outre, il est possible aussi à long terme, que les mâles des sites hypoxiques se sont adaptés localement aux conditions chroniques, leur permettant ainsi d'investir plus d'énergie dans le développement des testicules et la qualité des spermatozoïdes pour compenser les coûts d'habiter sous des conditions d'hypoxiques. En revanche, les mâles du régime fluctuant ont de grands corps, ayant le deuxième plus haut index gonadosomatique, une grande asymétrie testiculaire, et le plus long des spermatozoïdes mais avec la vitesse de nage la plus basse. Il est fort probable que les mâles des régimes fluctuants éprouvent des challenges énergétiques compromettant la croissance et la reproduction, en raison des niveaux d'oxygène moins prévisibles dans leur habitat. Les études futures devraient évaluer différents traits chez *P. multicolor* entre différents régimes d'oxygène dans des conditions contrôlées de laboratoire pour ainsi contrecarrer les possibles effets de confusion de

la disponibilité alimentaire, le risque de prédation, et la concurrence d'accouplement sur les traits reproductifs.

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GENERAL INTRODUCTION

There are over 32,000 fish species known today, classified into approximately 50 taxonomic Orders and thousands of Families, making them the most widespread and diverse group of vertebrates in the world (Froese and Pauly 2011). As a result of such diversity, there is a wide variety of morphological and physiological adaptations across groups (Rocha *et al.,* 2008). Sexual selection and adaptation to habitat combine to shape the reproductive morphology, physiology, and behaviour of fish species (Reynolds 1996; Seehausen and Alphen 1999; Basilone *et al.,* 2006). Sexual selection occurs when an individual who possesses certain morphological or behavioural traits is able to secure more mates or produce more offspring than others (Darwin 1859; 1871; Andersson 1994). Males who possess traits that allow them to produce more offspring are 'selected' as more reproductively 'fit' than other males in the population (Darwin 1859; Carson 1987: Beaver *et al.,* 2002). For example, across species, fish that experience higher competition due to density or mating system type invest more in spermatogenesis in order to increase their fitness, and thus have a higher gonadosomatic index (GSI) than males in less competitive systems (Stockley *et al.,* 1997; Balshine *et al.,* 2001; Awata *et al.,* 2008).

Aside from mating systems, other biotic and abiotic factors can directly affect reproductive fitness. Examples include water temperature (Jafri 1989; Lahnsteiner and Mansour 2012), substrate characteristics (Sternecker *et al.,* 2013), water flow (Coma and Lasker 1997; Petersen *et al.,* 2001), oxygen availability (Wu *et al.,* 2003), population density (Lee and Ban 1999), predation risk (Pavlová *et al.,* 2010; Cothran *et al.,* 2012), and the quality and availability of food (Collins and Anderson 1999;

Okamoto *et al.,* 2012). For example, the cycling of food abundance from low to high is the only trigger for oocyte growth and maturation in the golden perch (*Macquaria ambigua*), due to the unpredictable conditions of their natural habitat (Collins and Anderson 1999). Similarly, the availability and abundance of prey species is an important predictor of the amount of offspring produced by the black surfperch, *Embiotoca jacksoni*, (Okamoto *et al.,* 2012). In order to understand male reproductive traits such as sperm morphometry and motility, I will first give an overview of fish mating systems and modes of reproduction. Secondly, I will describe the study species, and provide a review of the literature investigating the relationships between male fish reproductive traits, their mating systems, and the environment.

1.1 Mating systems, sexual selection, and fitness

A 'mating system' encompasses mode of reproduction, courtship behaviour, average number of mates, and the extent of parental care in a species (Emlen and Oring 1977). The mating system which occurs when one male or female attempts to mate with one other partner is called monogamy (Rocha *et al.,* 2008). Polygamy occurs when one or both members of a mating pair attempt to mate with more than one partner (Rocha *et al.,* 2008). However, it is now known that there is a distinction between social and genetic monogamy (Rocha *et al.,* 2008). Many fish species that are socially monogamous are not genetically monogamous, because they mate outside of their pair bond (Rocha *et al.,* 2008). For example, the cichlid *Variabilichromis moorii,* forms a social pair bond, and both parents guard a nest

together during the breeding period (Sturmbauer *et al.,* 2008). Nonetheless, some offspring from each nest are sired by outside males (Sefc *et al.,* 2008). Another example is the Australian Seahorse (*Hippocampus subelongatus*), which was previously considered to 'mate for life' with one partner, and was found to have both monogamous and polygamous males in one population (Kvarnemo *et al.,* 2000).

Pre-copulatory sexual selection occurs when a male secures a mate through interactions with females or other males (Andersson 1994; Møller and Ninni 1998). Male fish characteristics that convey reproductive fitness to females can include body size (Reynolds and Gross 1992) and carotenoid colouration (Evans *et al*., 2003). In a laboratory courtship study, female guppies (*Poecilia reticulata*) selected males with relatively longer total length (Reynolds and Gross 1992). In a later study, female guppies selected male guppies with a relatively larger area of orange body colouration (Evans *et al.,* 2003). The honest signal hypothesis states that males that possess traits attractive to females also produce more or larger offspring (Reynolds and Gross 1992; Evans *et al.,* 2003). Male guppies that were longer produced relatively larger and faster growing offspring (Reynolds and Gross 1992), and more colourful males fertilized more eggs when competing at equal sperm densities with others under laboratory conditions (Evans *et al.,* 2003).

Furthermore, male competition for mates in some species has resulted in the development of alternate reproductive tactics (ARTs), defined by differences in behaviour or physiology between males in one species (Henson and Warner 1997; Fitzpatrick *et al.,* 2007). The most common ART is the 'sneaker', which is a male that fertilizes eggs guarded by a dominant male (Henson and Warner 1997; Leach

and Montgomerie 2000; Fitzpatrick *et al.,* 2007). In the Bluegill Sunfish (*Lepomis macrochirus*), for example, there are three different ARTs (Gross 1982). Smaller males act as sneakers and do not build nests, intermediate sized males mimic females to gain access to the nest and fertilize the eggs; finally, there are large dominant males that build nests to attract females (Gross 1982). Some cichlid (Cichlidae) species have 'pirate' males that, attempt to fertilize eggs by chasing the dominant male away from the nest (Ota *et al.,* 2012). In some salmonids (*Salmonidae*), sneakers mature early, stay small, and do not build nests; whereas nest builders mature slowly, grow larger, and do not sneak (Avise *et al.,* 2002). In some species such as the Bluehead Wrasse (*Thalassoma bifasciatum*), males switch from being a sneaker to a dominant male as they grow larger (Hoffman *et al.,* 1985).

Where attractiveness to females is a part of pre-copulatory sexual selection, the competition between sperm to fertilize eggs is a form of post-copulatory sexual selection (Parker 1970). The two types of sperm competition that may determine how much energy males invest in sperm production are sperm competition risk and sperm competition intensity (Byrne 2004). Males perceive sperm competition risk when there is a chance that their partner has or will mate with other males, and sperm competition intensity is associated with the number of males mating when males always compete (Parker *et al.,* 1996). Different species respond to sperm competition risk and intensity in different ways, but many studies have found that males will increase sperm production or expenditure (reviewed in Byrne 2004).

Aside from increasing sperm expenditure or production, sperm competition can be a strong determinant of sperm quality traits such as morphometry and motility

(Parker 1998; Gage *et al.*, 2004). Fish sperm morphometry varies across species and reproductive strategies, but the basic design includes a rounded head, a midpiece that contains mitochondria and adenosine triphosphate (ATP), and a flagellum, or tail, to propel sperm forward (Thünken *et al.*, 2007).

1.3 African cichlid evolution and mating systems

There are more than 3000 cichlid species across Central and South America, and Africa (Kocher 2004). Africa contains the most cichlid species; these cichlids have displayed massive adaptive radiations and rapid speciation, and have very diverse and unique mating systems (Kornfield and Smith 2000). For these reasons, cichlids have become a model system to study modes of evolution and sexual selection (Kornfield and Smith 2000). Particularly, Lake Victoria has been home to about 500 species of haplochromine cichlids, which diversified in the last 15,000 years: one of the most rapid radiations known today (Kaufman 1992).

Pseudocrenilabrus multicolor victoriae is a haplochromine cichlid, native to the Nile River basin of East Africa. It is wide-ranging species, living in a diversity of habitats, including small streams, larger rivers, lakes, and swamps (Chapman *et al.*, 2000). During courtship, males perform a "wiggle" or "quiver" to attract females, and have pronounced yellow ventral colouration, and a red spot on their anal fin (Gotanda *et al.*, 2012; Gray *et al.*, 2012). Males create territorial pits in sandy substrate and display aggressive behaviour toward other males (Fernö 1986; Gray *et al.*, 2012). During spawning, one male *P. multicolor* releases sperm before or during the period that the female is retrieving eggs from the substrate, and it is unknown if

some fertilization occurs in the female's mouth in some cases (Reardon and Chapman 2010). Females incubate developing embryos in their mouths for 10-25 days, and then release the offspring as juveniles (Reardon and Chapman 2010).

1.4 Reproductive fitness and the environment

There are multiple anthropogenic stressors that have the potential to negatively affect fish reproduction (Wu 2002; Gray *et al.*, 2012). For example, deforestation and intense agriculture along the Mpanga River in Uganda has exposed *P. multicolor* to increased levels of turbidity in its natural habitat, which has the potential to affect the species' reproductive behaviour (Gray *et al.*, 2012). Soil and other particles are more easily washed into nearby waterways after tree removal exposes the soil surface, decreasing the ability of light to penetrate the water and possibly darkening the colour of water through absorption and scattering, reducing visibility (Utne-Palm 2002). Under conditions of increased turbidity, male *P. multicolor* increase aggressive behaviour towards other males, potentially as a mechanism to attract females in a visually disrupted environment (Gray *et al.*, 2012).

Another anthropogenic stressor affecting fish survival and reproduction is hypoxia, or low dissolved oxygen (Diaz 2001). Hypoxia occurs naturally in aquatic habitats of low light and low water mixing (Chapman and Liem 1995; Richards *et al.*, 2009) such as swamps, flooded forests and plains, and in deep, stratified lakes (Carter 1955; Chapman and Liem 1995; Friesen *et al*., 2012). In these habitats, hypoxia varies temporally and spatially with photoperiod (Chapman *et al.*, 2008), as well as the rates of: water flow, photosynthesis, respiration, temperature change, and

decomposition (Rabalais *et al.,* 2010). However, the frequency and extent of hypoxia is increasing globally, and is associated with the use of agricultural fertilizers and the release of human waste runoff that leads to eutrophication (Diaz 2001; Rabalais *et al.,* 2010). For example, nitrogen loading into the Mississippi River from nearby corn crops has doubled in the last century, sometimes more than tripling the size of the seasonal hypoxic "dead" zone in the Gulf of Mexico from 5000 km^2 to up to 22,000 km^2 (Turner *et al*., 2008). The recent global increase in hypoxia is resulting in mass fish kills and extirpations in these dead zones (Diaz 2001; Gray *et al.,* 2002; Rabalais *et al.,* 2010). Evidence suggests that hypoxia may also be increasing and spreading due to global climate change (Richards *et al.,* 2009; Lyons *et al.,* 2010).

Sufficient levels of dissolved oxygen (DO) are necessary for aerobic cellular respiration and ATP production in fish (Richards *et al.,* 2009). For most freshwater species, the minimum concentration of oxygen in water that does not cause physiological stress (known as the oxygen threshold) is between 5 and 6 mg L^{-1}, whereas the threshold for most marine species is 2 mg L^{-1} (Landry *et al.,* 2007; Richards *et al.,* 2009). Dissolved oxygen concentrations above average threshold levels are generally considered to be normoxic, whereas concentrations below the average threshold are generally considered hypoxic. The oxygen levels in *P. multicolor's* habitats can be highly variable or relatively stable depending on the site, and range from as low as 0.4 mg L^{-1} in swamps (Friesen *et al.,* 2012), and as high as 9.1 mg L^{-1} in streams.

1.5 General effects of hypoxia on fishes

When dissolved oxygen levels are below threshold concentrations, fish will use relatively more energy to maintain homeostasis, causing them direct or indirect stress (Heath 1995). Fish respond to hypoxia with behavioural, physiological, and morphological changes (Chapman *et al.,* 2002; Pollock *et al.,* 2007; Crispo and Chapman 2008). These responses will differ depending on the species and the severity and duration of exposure (Timmerman and Chapman 2004; Martínez *et al*. 2006). Short term exposure has been shown to first induce behavioural changes such as avoidance (Pihl *et al.,* 1991), increased gill ventilation, or air surface respiration in some species (Chapman *et al.,* 2002; Timmerman and Chapman 2004), as well as reduced activity levels (Schurmann and Steffensen 1994; Dalla Via *et al.,* 1998). Secondly, short term physiological responses include bradycardia, or slowing of the heart rate (Milsom 2012), increased red blood cell production, hemoglobin levels, or hematocrit levels, in an attempt to maintain oxygen levels or raise oxygen carrying capacity in the blood (Chapman *et al.,* 2002; Martínez *et al.,* 2004; Timmerman and Chapman 2004).

On the other hand, long-term exposure (i.e., months or multiple generations) to hypoxia in *P. multicolor* has resulted in morphological changes like increased gill size and surface area, as well as longer gill filaments (Chapman *et al.,* 2000; 2002). *P. multicolor* in hypoxic sites also have larger heads to accommodate larger gills (Crispo and Chapman 2011), and smaller brains to reduce oxygen demands, relative to populations living in normoxia (Chapman *et al.,* 2008; Crispo and Chapman 2010;

2011). Long-term physiological responses can also include metabolic changes, such as higher levels of liver lactate dehydrogenase (LDH) in species like the Gulf Killifish, *Fundulus grandis*, (Martínez *et al.*, 2009). However, results from studies on a larger array of metabolic enzymes in species adapted to long term hypoxia such as *P. multicolor* (Crocker *et al.*, 2013*a,b*), the Gulf killifish, *Fundulus grandis,* (Martínez *et al.*, 2006) and the Common Carp, *Cyprinus carpio*, (Zhou *et al.*, 2000) have shown no significant trend in metabolic responses to hypoxia. This suggests that some species can adapt metabolically to decreased oxygen, when living under hypoxia for many generations (Zhou *et al.*, 2000; Martínez *et al.*, 2009; Crocker *et al.*, 2013 a, b).

1.6 Hypoxia and reproduction

High reproductive fitness depends on proper gonad development, high quality and quantity of gametes, the ability to achieve fertilization, and the ability of the fertilized eggs to hatch and survive (Wu *et al.*, 2003). Gonad development begins with the system of endocrine glands known as the hypothalamus– pituitary–gonadal (HPG) axis (Thomas and Rahman 2009). A cascade of hormone stimulation and generation occurs, beginning with gonadotropins and ending with ovulation and spermiation, or the release of eggs and sperm (Thomas and Rahman 2009). In a study on the Common Carp, short-term hypoxia exposure disrupted this pathway by altering sex hormone function and impairing the quality of eggs and sperm (Wu *et al.*, 2003). Sex hormone levels, gonad size, total fertilization, and juvenile survival rates were all reduced by 50% or more in carp exposed to hypoxia (1 mg O_2 L^{-1}) for

eight weeks (Wu *et al.,* 2003). After one month of laboratory induced hypoxia exposure, Gulf Killifish were producing 50% less hormones than fish in normoxic conditions (Landry *et al.*, 2007). They also produced fewer eggs and had a stunted spawning time (Landry *et al.*, 2007). If severity or duration of hypoxia increase in the Gulf Killifish natural habitat, it could have negative effects on the reproductive capacity of populations in this species (Landry *et al.,* 2007)

1.7 Male reproductive traits and the environment

Organisms experience life history trade-offs because they cannot produce sufficient energy to maximize fitness in all physical or behavioural traits at once (Fisher 1930; Trivers 1972; Skibiel *et al.,* 2013). For example, fish must balance energy investment between growth and reproduction, where higher investment in growth or homeostasis represents energy that became unavailable to invest in sperm quality (Taborsky 1998; Awata *et al.*, 2008; Franssen *et al.,* 2008). Therefore, reproductive characteristics can vary between individuals in a population who have invested energy differently (Burness *et al.,* 2008). For example, male Whitefish (*Coregonus clupeaformis*) that have a relatively higher Fulton's body condition (K) before spawning often develop larger gonads and larger ejaculates than males in lower condition (Burness *et al.*, 2008). However, at the time of spawning, these higher condition males can exhibit reduced Fulton's condition relative to males that did not invest as much energy into initial gonad development (Burness *et al.,* 2008). Individual investment in gonads and ultimately spermatogenesis (the production of sperm), can also depend on mating systems (Gage *et al.,* 1995; Reynolds 1996).

Therefore, the level of competition and the availability of females can play a role in how much a species invests in testes development (Rocha *et al.*, 2008). For example, sneaker Atlantic Salmon (*Salmo salar*) males, or parr, have been shown to have a higher gonadosomatic index (GSI), a higher percent of motile sperm, and increased sperm swimming longevity, relative to anadromous males (Gage *et al.*, 1995).

Since testes development is energetically costly, testes asymmetry can be an indicator of energetic trade-offs (Clarke 1995). The development of paired structures and whether or not they are symmetrical in size and shape is dependent on an organism's genes, and on the environment (Clarke 1995; Harrod and Griffiths 2005). Many species of fish, including whitefish (*Coregonus* spp.), salmonids, and anchovies (Engraulidae family) display some level of testes asymmetry on a population scale (reviewed in Harrod and Griffiths 2005). In a study of testes asymmetry in a Whitefish population, males had a larger left testis, and that testis also had a higher ATP content when controlling for gonad mass (Burness *et. al.*, 2008). Because ATP content is an indicator of energy production, this study showed that testes asymmetry may have evolved as a means to produce good quality sperm in the face of energy constraints in this species (Burness *et al.*, 2008).

Sperm morphometry, velocity, and longevity are expected to affect sperm quality, among other traits (Parker 1990; Gomendio and Roldan 1991). Overall, sperm quality is determined by the ability to fertilize an egg and produce viable offspring (Rurangwa *et al.*, 2004). Additionally, studies have shown that fertilization success can depend on motility, internal spermatocrit levels, pH, chemical composition of seminal plasma, enzyme activity, and ATP production or

concentration, to name a few (reviewed in Rurangwa *et al.,* 2004). However, in externally fertilizing species, the velocity of sperm seems to be the main determinant of fertilization success when sperm from different males are released at the same time, from the same location (Levitan 2000; Gage *et al.,* 2004). It has been hypothesized that longer sperm flagella should have increased wave propagation relative to shorter flagella, giving sperm with longer flagella more power to propel sperm forward (Katz *et al.,* 1989). Therefore, sperm with longer flagella should be able to swim at higher velocities than sperm with shorter flagella (Gomendio and Roldan 1991; 2008). In a study of 35 fish species from different taxonomic orders, Ishijima (2012) found that the main determinant of sperm velocity was beat frequency of the flagellum, and longer flagella produced more beats per unit time than shorter flagella (Ishijima 2012). In support of these hypotheses, sperm velocity has been correlated with flagellum length in some species (Tuset *et al.,* 2008; Simpson *et al.,* 2014; Bakker *et al.,* 2014). Simpson *et al.,* (2014) found that in a species of Rainbowfish (*Melanotaenia australis),* sperm that had longer flagella and a shorter head relative to flagellum length swam faster than other sperm from the same individual. Tuset *et al.,* (2008) found that Rainbow Trout (*Oncorhynchus mykiss*) displayed two different fertilization strategies within individuals, which affected both morphometry and velocity. Sperm with longer flagella had a faster velocity, and the trajectory of faster sperm was more linear than slower sperm (Tuset *et al.,* 2008).

The hypothesis that faster sperm having longer flagella was later amended to include the idea that faster swimming sperm may face a trade-off with swimming

longevity, having a shorter time to find and fertilize an egg than slower sperm (Gomendio and Roldan 1993; Ball and Parker 1996). Bakker *et al.,* (2014) found that Three-spined Stickleback sperm possessed intraspecific variation in flagellum length, with longer sperm in one individual initially being more successful fertilizers, but with a reduced swimming longevity. Sea urchin (*Lytechinus variegatus*) sperm that swam faster fertilized more eggs, but also had lower swimming longevity than slower sperm (Levitan 2000). A later study found that in sea urchin (*Heliocidaris erythrogramma*) faster sperm have longer flagella (Fitzpatrick *et al.,* 2010).

1.7 STUDY PROBLEM

Multiple studies confirm that the physiological and morphological responses to hypoxia in *P. multicolor* are a combination of genetic and phenotypically plastic responses, which vary across populations (Chapman *et al.,* 2000; Chapman *et al.,* 2008; Crispo and Chapman 2008; Martínez *et al.,* 2009; Crispo and Chapman 2010; 2011; Crocker *et al.,* 2013 *a, b*). To date, several studies have investigated the effects of hypoxia on female *P. multicolor*. In a laboratory study of *P. multicolor* hormone levels, testosterone levels were higher in fish from hypoxic sites (Friesen *et al.,* 2012). Also, the ratio between testosterone and estradiol was higher in *P. multicolor* from hypoxic sites (Friesen *et al.,* 2012), suggesting that hypoxia disrupts the enzyme mediated conversion of testosterone to estradiol (Landry *et al.,* 2007). Brooding females living under long-term hypoxia have shown a lower metabolic rate during brooding, and a shorter brooding period by an average of 5 days (Reardon and Chapman 2010). This indicates that they are compensating for reduced oxygen

availability by limiting their metabolic needs and by spending less time doing energetically expensive activities (Reardon and Chapman 2010). Despite the great importance of male reproductive capacity in the success of a species, few efforts have explored a correlation between oxygen regimes and reproductive fitness in male *P. multicolor*. Additionally, there are no studies that have assessed the relationship between sperm morphometry and motility in this species. To my knowledge, only one study has assessed the performance of sperm under hypoxic conditions from a fish species that has naturally adapted to long-term hypoxia (Fitzpatrick *et al.*, 2009). It was found that sperm velocity of the hypoxia tolerant Plainfin Midshipman (*Porichthys notatus*) was higher when measured under hypoxic conditions in the lab relative to normoxic conditions (Fitzpatrick *et al.*, 2009). This will be the first study to assess differences in testes size, asymmetry, sperm morphometry and motility of a single species across a wide range of habitat types.

1.8 STUDY QUESTION

This thesis focuses on the reproductive characteristics of male *Pseudocrenilabrus multicolor victoriae* between two regions and across nine sites with different oxygen regimes. My main question is: Do male *P. multicolor* show measurable population-level differences in fitness correlates: body length and mass, testes size and asymmetry, and sperm morphometry and velocity?

1.9 HYPOTHESIS AND PREDICTIONS

I hypothesized that male *P. multicolor* adapted to different habitats over many generations would exhibit variation in reproductive traits. The following predictions were tested:

(1) If hypoxia results in energetic stress, males from hypoxic sites should display lower testes mass and greater testes asymmetry than males from normoxic sites.

(2a) If sperm head shape affects sperm motility, sperm with more hydrodynamic heads should also have a higher swimming velocity.

(2b) If sperm flagellum length affects sperm motility, males that have sperm with longer flagella should also have sperm with a higher swimming velocity.

(3) If trade-offs between sperm swimming velocity and sperm swimming longevity exist for this species, males with initially faster sperm performance should display a more rapid decrease in sperm swimming velocity than sperm with initially slower swimming velocities.

MATERIALS AND METHODS

2.1 Study sites

Pseudocrenilabrus multicolor victoriae were collected in June 2011 and June 2013 from nine sites in two regions of the Lake Victoria basin of Western Uganda: Lake Nabugabo and the Mpanga River. Sites from the Mpanga region included Bunoga and Bwera. Sites from the Nabugabo region included Lwamunda Swamp, Lwamunda Pump House, Dead Duck Bay, Kazzi Lagoons, JJ Bay, Lake Kayanja, and Ndyabusole stream (Figure 1). There is a great variation in dissolved oxygen across sites (Table 1).

2.2 Data collection

Based long-term data from other studies, the above sites have been categorized as normoxic, hypoxic, or seasonally fluctuating (Table 1). Bunoga has monthly DO values ranging from 1.7 to 9.05 mg O_2 L^{-1} (McNeil 2012; Crocker *et al.,* 2013a). This large variation in dissolved oxygen is associated with seasonal water levels changes, where the lowest values are found during the rainy season, which causes extensive runoff. For this reason, it has been categorized as seasonally fluctuating. Dissolved oxygen content was also measured with a YSI multimeter (YSI Inc., Yellow Springs, OH, USA) at each site during each day of fish collection. Baited minnow traps were set for 3 to 4 hours at each site and checked every 20 to 30 minutes. Males were transported to the field station, where they were euthanized with an overdose of clove oil (1:10 clove oil: ethanol) and measured for total length ($L_T \pm$ 1 mm), standard length ($L_S \pm$ 1mm), and total mass ($M_T \pm$ 0.1g). Testes were

removed and the mass of left and right testes were taken separately ($Test_L$ and $Test_R$ ± 0.001g). From total fish mass and the mass of the testes, somatic mass (M_S ± 0.10 g) and testes asymmetry (T_A) was calculated.

Somatic mass was calculated as follows:

$M_S = M_T - G_T$

where M_S represents somatic fish mass, M_T represents total fish mass, and G_T represents total testes mass (g).

Testis asymmetry (T_A) was calculated as follows:

$T_A = (Test_L * Test_R^{-1})$

where $Test_R$ and $Test_L$ represents right and left testis mass (g), respectively.

Although Fulton's condition factor (K) is widely used as a fitness proxy for overall fish health (McPherson 2010), it was not used in this study for several reasons. First, the formula is based on the assumption that isometric growth occurs, which is not documented in all species (Jones *et al.*, 1999). Additionally, some authors have argued that morphometric condition factors measure the shape of fish, rather than their condition (McPherson 2010). *P. multicolor* show variation in body shape and size across sites in response to hypoxia, and this variation reflects an increased head and gill size in fish from hypoxic sites, rather than better condition (Chapman *et al.*, 2000; 2008). Finally, for some fish species, fish length alone has been the strongest predictor of sperm velocity (Blukacz *et al.*, 2010). Therefore, conclusions about Fulton's condition are not made.

2.2 Sperm swimming capacity and sperm morphometry

Due to the size of the testes, milt could not be obtained by abdominal pressure. Instead, after the testes were weighed, approximately 2µL of milt (sperm and seminal plasma) was obtained and placed in a small weighing boat and rapidly diluted with rain water. Rain water was used to activate the sperm as standard protocol, since having constant electric power can be difficult for the maintenance of buffer solutions. A subsample of diluted sperm (4 µL) was placed on a pre-focused 1 mm deep-welled Leja slide (Leja, The Netherlands). Sperm motility was recorded initially for 1.5 minutes. However, after reviewing the initial videos, it was decided to record all videos for a total of 45 seconds, since after this time there was a dramatic reduction in sperm motility.

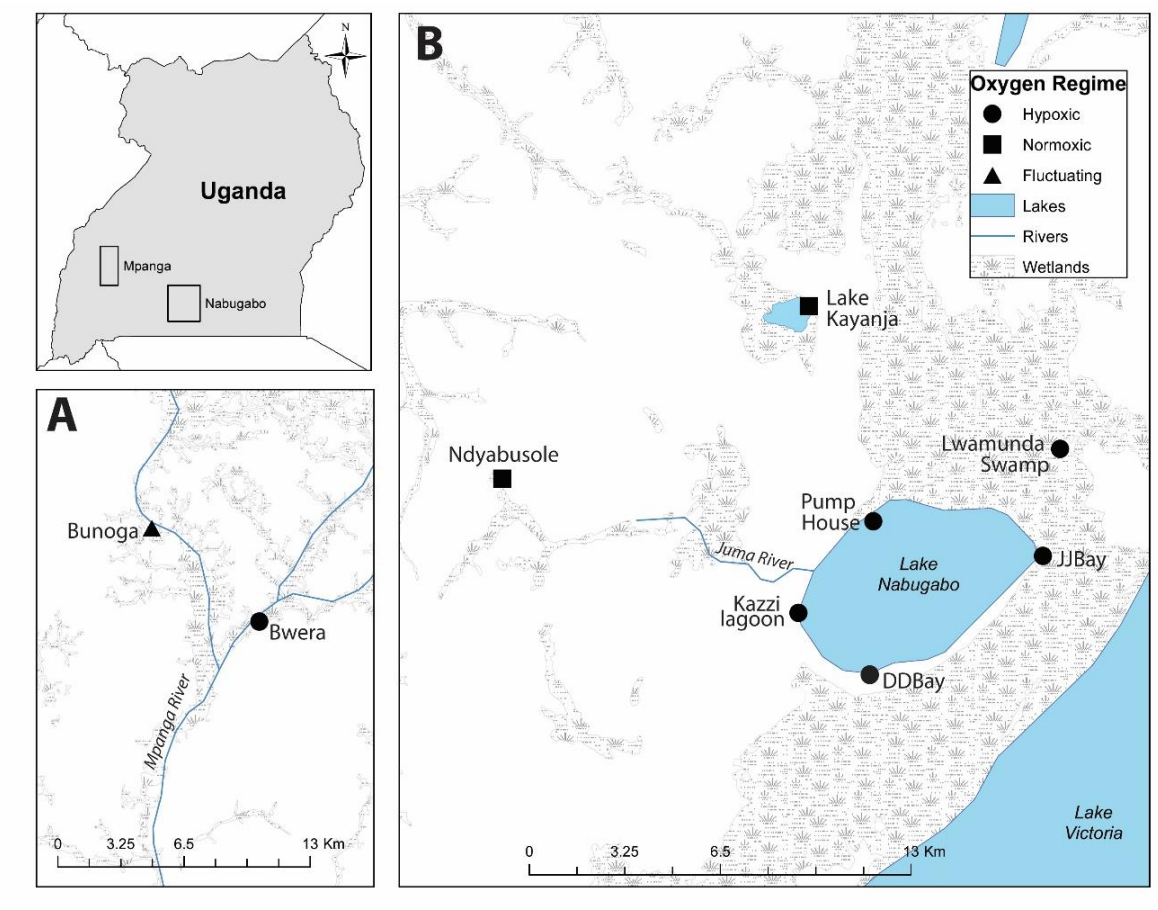

Figure 1. Sampling sites in Uganda, Africa where *Pseudocrenilabrus multicolor victoriae* were collected. (A) Sites in Mpanga River Region. (B) Sites within the Nabugabo Region.

Table 1. Dissolved oxygen data of sampling sites in Uganda, Africa.

Site	Characterization	DO (mg O^2 L^{-1} ± 0.01)	Type of reading
Mpanga Region			
Bunoga[1]	fluctuating	7.53 ± 0.04	point in time average
Bwera[2]	hypoxic	0.6 ± 0.2	seasonal average
Nabugabo Region			
Lwamunda swamp[3]	hypoxic	0.55 ± 0.03	point in time average
Pump House	hypoxic	4.27 - 5.00 surface 0.16 - 1.63 bottom	point in time range
Dead Duck Bay	hypoxic	0.19 - 0.79 surface 0.39 - 0.49 bottom	point in time range
Kazzi lagoons[4]	hypoxic	1.35 ± 0.75	monthly average
JJ Bay	hypoxic	2.92 - 3.21 surface 3.02 - 3.34 bottom	point in time range
Lake Kayanja[5]	normoxic	7.43 ± 0.12	point in time average
Ndyabusole stream	normoxic	7.3 - 9.11	point in time range

1,3. (Crocker *et al.*, 2013*a*). 2. (Friesen *et al.*, 2012). 4. (Reardon and Chapman 2009) 5. (Crispo and Chapman 2008).

All videos were captured at 100 X magnification with an Infinity 1 USB digital camera (resolution 800 x 600, an exposure of 8.3, 60 frames per second, and a gain of 1.43) mounted on a Leica light microscope (Leica Microsystems, Wetzler, Germany). Sperm swimming parameters (velocity and linearity) were analyzed using a Ceros Animal Motility sperm analysis system (Version 12.3, Hamilton-Thorne Biosciences, Massachusetts). The system was set as follows: number of frames = 60, minimum contrast = 32, photometer = 1, and minimum cell size = 5 pixels. Sperm velocity and linearity per individual were analyzed for 10 sperm per fish at three different post-activation times: 15, 25, and 30 seconds. The velocity parameters recorded were curvilinear velocity (VCL), smooth-path velocity (VAP) and straight-line velocity (VSL). VCL is a measure of the points along the sperm's full path over time, while VAP is a measure of an averaged path over time. VSL is a measure of the straight line distance from the starting point and ending point over time. Linearity is calculated as the percent of straight-line velocity (straight line distance travelled over time) to curvilinear velocity (actual path travelled over time). The higher the linearity value, the straighter the path travelled by the sperm.

Sperm longevity was calculated as final velocity as a percent of initial velocity for each male as follows:

$$\text{Longevity} = (\text{VAP15} * \text{VAP30}^{-1})$$

A second subsample (2 µL) of the sperm was collected directly from the testes using a micropipette and diluted into 200 µL of 30% formaldehyde solution (Fisher Scientific, Ottawa, Ontario, Canada). Immediately after dilution, a sperm smear was prepared. After 24 hours of drying, slides were stained by 10 minute

immersion in methanol, followed by air drying, followed by 7 minute immersion in Eosin (Bonanno and Schulte-Hostedde 2009). Slides were then mounted with Permount and coverslips (Fischer Scientific, Nepean, Ontario, Canada). Photo records were taken from the smears using an Olympus CX41 light microscope (Olympus Corporation of the Americas Inc., Center Valley Pennsylvania), using the same camera described above. A minimum of 10 fully visible spermatozoa per individual were photographed and measured using Image J software (National Institutes of Health, Bethseda Maryland). I measured the following traits for each spermatozoa: the length of the spermatozoa head from flagellum insertion point to anterior tip ($H_L \pm 0.01$ µm), the width of the head perpendicular to length and from the widest point ($H_W \pm 0.01$ µm) of the head, and the length of the flagellum ($F_L \pm 0.01$ µm). These traits were measured three times independently, and then averaged. The midpiece was often not visible at this magnification and was not measured.

Additionally, sperm head length to head width (hydrodynamic) ratio, and percent of head length to flagellum length were calculated to assess relationships between morphometry and velocity (Humphries *et al.*, 2008) as follows:.

Hydrodynamic ratio = $H_W : H_L$

Head length to flagellum length ratio = $(H_L * F_L^{-1}) * 100$

Where H_W is head width, H_L is head length, and F_L is flagellum length (µm).

2.3 Statistical analyses

Statistical analyses were performed with Statistica (Statsoft Inc., 2013, Tulsa Oklahoma, Version 12) and SPSS (IBM Corp., 2015, Armonk, NY, Version 23).

Histograms of variables were plotted to assess normality, and homogeneity of variance was assessed by plotting residuals. If variables did not satisfy parametric assumptions, they were transformed. Mass and sperm morphometry parameters (head length, head width, and flagellum length) were box-cox transformed, while testes mass, and smooth path velocity were log transformed to achieve normality for parametric tests. Finally, proportion variables such as sperm head length to flagellum length and testes asymmetry were arc sin square root transformed. Three individuals were removed from all analysis because their mass, length, and testes mass measurements were more than two standard deviations from the mean. Each velocity type (VAP, VCL, and VSL) gave qualitatively similar results, so only analyses of smooth path velocity (VAP) are reported in this thesis.

Significant genetic (Crispo and Chapman 2008) and metabolic enzyme activity (Crocker *et al.,* 2013a) differences have been reported in *P. multicolor* based on region and dissolved oxygen regime. Therefore, fitness correlates were analyzed using two sets of nested analysis of covariance (ANCOVA) for site nested within region, and dissolved oxygen regime nested within region, with standard length as a covariate. Total testes mass was assessed with nested analysis of covariance with somatic mass as a covariate. Tukey post-hoc analysis was used to assess which sites or regimes differed from each other. For analyses by dissolved oxygen regime, only variables that had significant results are reported in tables. A dependent t-test was used to assess if a significant difference existed between left and right testis mass at the population level. A principal component analysis was performed on sperm traits of head width, head length, and flagellum length. The factor scores for each male

from principal component one were used in nested ANCOVAs. Repeated measures ANOVA with standard length as a covariate, and Bonferroni post-hoc tests were used to assess significant differences in velocity at each time post-activation by site. In order to test differences in longevity, velocity at 30 seconds were calculated as a percent of velocity at 15 seconds for each male, and an ANCOVA with standard length as a covariate was run by site. Hierarchical regression was used to investigate the relationship between fish body traits, sperm morphometry traits, and sperm motility. In order to control for body size, the parameters in the first level of the model were standard length and somatic mass. The second level parameter was total testes mass, and the third level included all three sperm morphometry measurements: head length, head width, and flagellum length. The assumptions of multiple linear regressions were tested by doing the following: plotting all parameters in scatterplots to ensure that a linear relationship existed. In SPSS, the Durbin-Watson value was attained to test the independence of residuals. Homoscedasticity was assessed by plotting the studentized residuals against unstandardized predicted values. Data were also analyzed for multicollinearity by ensuring that the tolerance value in the multicollinearity statistics was below 0.9 for each trait. Testes asymmetry was not used in this model because it expressed multicollinearity. Finally, a probability plot was used to assess normality.

RESULTS

Analyses by site

3.1 General body morphometry

Male *P. multicolor* display variation in body morphometry, sperm morphometry, and sperm motility across sites (Table 2). When controlling for fish size, each trait was significantly different across sites (Table 3). There was an interaction between standard length and site when assessing total mass. Pearson's correlation for standard length and total mass by site indicated that mass and length were highly correlated ($r = 0.9$) at all sites except for Lwamunda Swamp, where it was much lower ($r = 0.86$), indicating that fish were shorter in body length per unit mass in Lwamunda swamp than at any other site (Appendix 1). There was also interaction between site and standard length when assessing the H_L:F_L ratio ($P=0.03$, Table 3). Pearson's correlation for standard length and the H_L:F_L ratio by site indicated that males from Bwera, Kazzi Lagoon, and Lwamunda Swamp had a positive relationship between the ratio and standard length, whereas the rest of the sites had a negative relationship (Appendix 1). This means that for most sites, as fish length increased, sperm length also increased.

Pearson's correlation between testes mass and somatic mass for all males indicated a positive relationship between fish somatic mass and testes mass for this species ($r = 0.656$, $P<0.001$; Appendix 2). Total testes mass of males from Bunoga was higher than testes mass of males from Dead Duck Bay ($P=0.005$), Kayanja ($P=0.004$), and Ndyabusole ($P=0.0003$). Similarly, testes mass of males from Bwera

was higher than those of Dead Duck Bay (P=0.01), Kayanja (P=0.01), and Ndyabusole (P=0.001). Males from Lwamunda had higher testes mass than males from Dead Duck Bay (P<0.0001), JJ Bay (P=0.02), Kayanja (P<0.0001), Kazzi Lagoon (P=0.002), and Pump House (P=0.02). Ndyabusole males had lower testes mass than males from Pump House (P=0.009) and Kazzi Lagoon (P=0.008). .

Males from JJ Bay had the lowest testes asymmetry and were different than males from Bunoga (P=0.019), Bwera (P=0.003), and Pump House (P=0.003). Lwamunda Swamp males had lower testes asymmetry than males from Pump House (P=0.031) and Bwera (P=0.035). Ndyabusole fish had lower testes asymmetry than males from Pump House (P<0.0001). Dependent t-tests on left and right testes mass were carried out for each site, and right testis mass was significantly larger than the left testis within a site and across populations (P < 0.0001 for each site; Figure 2). Pearson's correlation was carried out on testes mass and asymmetry. As the difference in mass between the left and right testis increased, so did total mass of the testes (r = 0.73, P<0.0001).

Table 2. General morphological traits, sperm morphometry traits and sperm motility of *P. multicolor* sampled at nine sites in Uganda, Africa. Variables are total length (TL), standard length (SL), total mass (mass), somatic mass (SM), left testis mass (LT), right testis mass (RT), total testes mass (GT), testes asymmetry (ASY), sperm head length (HL), head width (HW), flagellum length (FL), hydrodynamic ratio (HR), head length to flagellum length ratio (HL:FL), sperm velocity (VAP), and linearity of trajectory (LIN). Values are mean ± S.E.

Body Morphology	**Bunoga**	**Bwera**	**Dead Duck Bay**	**JJ Bay**	**Kayanja**	**Kazzi**	**Lwamunda**	**Nyabusole**	**P House**
TL (cm)	7.3 ± 0.309	6.3 ± 0.157	5.99 ± 0.160	5.32 ± 0.104	6.01 ± 0.195	6.12 ± 0.112	6.73 ± 0.146[ae]	7.07 ± 0.151	5.84 ± 0.145
SL (cm)	5.9 ± 0.249	5.1 ± 0.131	4.83 ± 0.134	4.25 ± 0.084	4.8 ± 0.162	4.95 ± 0.098	5.45 ± 0.116	5.65 ± 0.124	4.72 ± 0.125
Mass (g)	7.1 ± 0.730	4.2 ± 0.350	3.61 ± 0.281	2.45 ± 0.147	3.67 ± 0.335	3.82 ± 0.210	4.66 ± 0.316	5.6 ± 0.356	3.56 ± 0.297
SM (g)	7.0 ± 0.726	4.2 ± 0.360	4.08 ± 0.263	2.83 ± 0.178	4.26 ± 0.329	4.07 ± 0.204	4.39 ± 0.244	5.83 ± 0.355	4.23 ± 0.361
LT (g)	0.008 ± 0.001	0.004 ± 0.0006	0.003 ± 0.0002	0.003 ± 0.0005	0.004 ± 0.0004	0.004 ± 0.0005	0.008 ± 0.001	0.005 ± 0.0008	0.004 ± 0.0007
RT (g)	0.027 ± 0.004	0.012 ± 0.002	0.008 ± 0.0006	0.005 ± 0.0004	0.009 ± 0.0009	0.01 ± 0.001	0.015 ± 0.001	0.012 ± 0.001	0.012 ± 0.002
GT (g)	0.031 ± 0.005	0.016 ± 0.002	0.009 ± 0.0008	0.007 ± 0.0006	0.011 ± 0.001	0.013 ± 0.001	0.021 ± 0.002	0.015 ± 0.002	0.014 ± 0.002
ASY	0.369 ± 0.138	0.378 ± 0.214	0.492 ± 0.221	0.622 ± 0.238	0.475 ± 0.252	0.466 ± 0.195	0.513 ± 0.252	0.394 ± 0.190	0.336 ± 0.216
Sperm Morphology									
HL	1.92 ± 0.048	2.01 ± 0.030	1.98 ± 0.030	1.9 ± 0.032	1.91 ± 0.042	1.98 ± 0.223	1.84 ± 0.034	1.91 ± 0.037	1.81 ± 0.020
HW	2.01 ± 0.035	2.18 ± 0.024	2.19 ± 0.037	2.11 ± 0.025	2.19 ± 0.050	2.22 ± 0.027	2.07 ± 0.034	2.15 ± 0.039	2.06 ± 0.023
FL	22.45 ± 0.344	21.74 ± 0.250	20.2 ± 0.187	20 ± 0.191	19.96 ± 0.201	20.26 ± 0.232	19.77 ± 0.233	20.78 ± 0.238	19.85 ± 0.155
HR	1.049 ± 0.019	1.087 ± 0.010	1.106 ± 0.011	1.124 ± 0.011	1.151 ± 0.02	1.125 ± 0.014	1.129 ± 0.007	1.124 ± 0.009	1.134 ± 0.008
HL:FL	0.086 ± 0.003	0.092 ± 0.002	0.099 ± 0.00	0.094 ± 0.002	0.096 ± 0.002	0.098 ± 0.002	0.093 ± 0.002	0.092 ± 0.002	0.092 ± 0.001
Sperm Motility									
VAP15 (μm s^{-1})	37 ± 1.271	47.99 ± 2.833	55.37 ± 2.306	46.87 ± 3.759	47.79 ± 3.141	56.76 ± 1.875	43.89 ± 2.227	43.64 ± 2.166	62.6 ± 4.711
VAP25 (μm s^{-1})	34.09 ± 1.350	47.18 ± 2.735	55.72 ± 2.382	39.67 ± 2.637	47.72 ± 2.394	55.02 ± 2.111	42.59 ± 2.163	40.96 ± 1.926	63.29 ± 3.113
VAP30 (μm s^{-1})	34.13 ± 2.104	46.25 ± 2.658	53.57 ± 2.752	41.53 ± 3.047	47.17 ± 2.405	55.01 ± 2.340	44.32 ± 2.588	39.8 ± 1.957	55.28 ± 3.778
LIN 15	41.08 ± 1.254	48.03± 1.523	49.24 ± 1.29	46.46 ± 1.998	46.59 ± 1.435	52.5 ± 0.79	46.71 ± 1.293	47.38 ± 1.301	54.25 ± 2.553
LIN 25	39.47 ± 1.046	46.29 ± 1.558	51.20 ± 1.091	41.15 ± 1.338	47.24 ± 1.602	51.26 ± 1.007	47.04 ± 1.27	45.84 ± 1.02	55.83 ± 1.691
LIN 30	40.09 ± 1.214	48.18 ± 1.368	48.5 ± 1.767	43.22 ± 1.903	48.76 ± 1.358	50.79 ± 1.369	46.33 ± 1.466	46.03 ± 1.054	52.6 ± 2.732

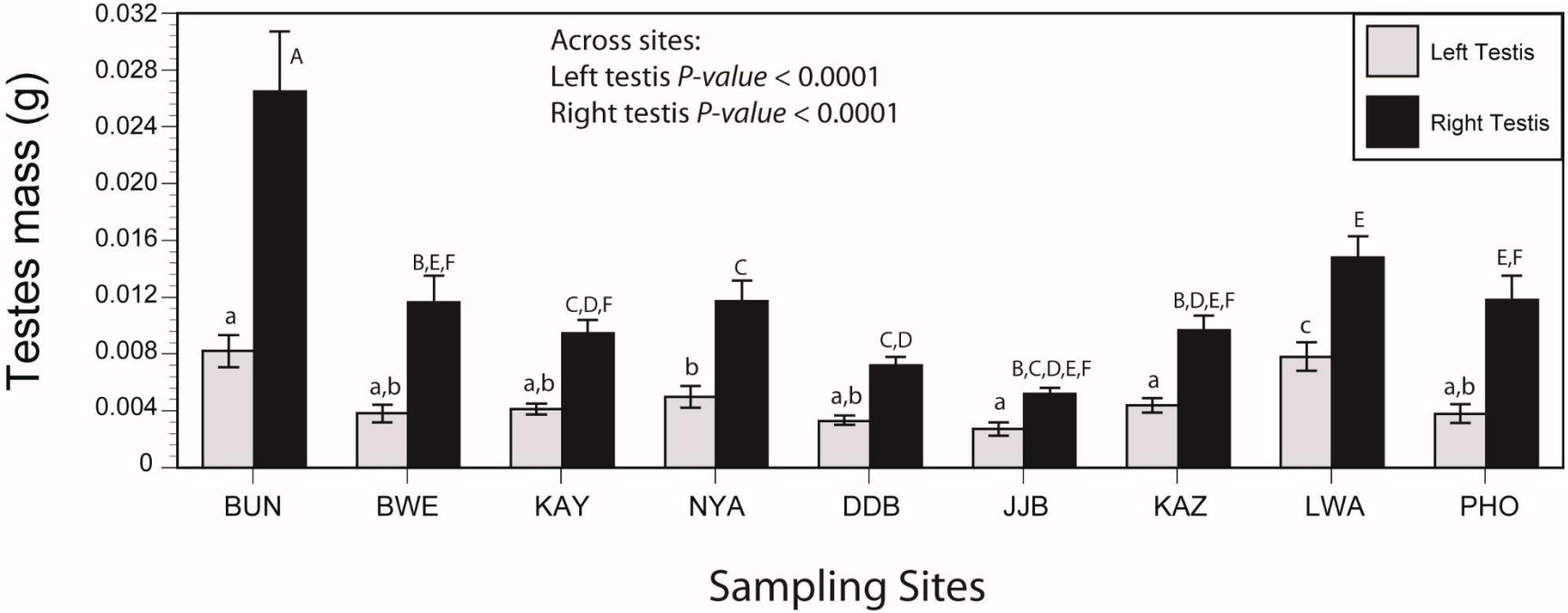

Figure 2. Left testis mass (LT) and right testis mass (RT) across sampling sites. Sites are Bunoga (BUN), Bwera (BWE), Lake Kayanja (KAY), Ndyabusole (NYA), Dead Duck Bay (DDB), JJ Bay (JJB), Kazzi Lagoon (KAZ). Lwamunda Swamp (LWA) and Pump House (PHO). Letters indicate significant differences among sites. Bars with the same letter do not differ (Tukey post-hoc comparisons, P>0.05). Values are shown as mean ± S.E. .

Table 3. Results of nested ANCOVAs by site within region and with standard length (SL) or somatic mass (SM) as a covariate for traits of *P. multicolor* including total mass (M), testes mass (GT), testis asymmetry (ASY), sperm morphometry (Sperm PC1), Head ratio (HR), Head length to flagellum length ratio (H_L:F_L), velocity (VAP), and sperm linearity (LIN).

Trait	ANCOVA	df	F	P-value
M	Site (Region) + SL	8	0.493	0.861
	*SL*Site*			**<0.0001**
GT	Site (Region) + SM	8	5.231	**<0.0001**
	*SM*Site*			0.693
ASY	Site (Region) + SL	8	2.595	**0.011**
	*SL*Site*			0.355
PC1	Site (Region) + SL	8	5.656	**<0.0001**
	*SL*Site*			0.104
HR	Site (Region) + SL	8	4.036	**<0.0001**
	*SL*Site*			0.915
HL:FL	Site (Region) + SL	8	1.039	0.403
	*SL*Site*			**0.037**
VAP15	Site (Region) + SL	8	5.387	**<0.0001**
	*SL*Site*			0.720
VAP25	Site (Region) + SL	8	11.286	**<0.0001**
	*SL*Site*			0.375
VAP30	Site (Region) + SL	8	5.911	**<0.0001**
	*SL*Site*			0.075
LIN15	Site (Region) + SL	8	4.727	**<0.0001**
	*SL*Site*			0.231
LIN25	Site (Region) + SL	8	12.231	**<0.0001**
	*SL*Site*			0.436
LIN30	Site (Region) + SL	8	4.421	**<0.0001**
	*SL*Site*			0.248

3.2 Sperm morphometrics

P. multicolor sperm total length (the sum of head length and flagellum length) ranged from 18.4 µm (min) to 26.4 µm (max), with an average of 22.4 µm (Figure 3). The first component (PC1) of the principal component analysis (PCA) explained sixty percent of the total variance (Table 4). An ANCOVA of PC1 scores across sites showed variation in sperm shape across sites (Figure 4). Specifically, sperm from males in Bunoga was similar to sperm from males in all other sites except Pump House ($P = 0.041$); Bwera differed from JJBay ($P=0.001$), Lake Kayanja ($P = 0.011$), Lwamunda Swamp and Pump House ($P < 0.0001$ respectively); Dead Duck Bay sperm differed from JJBay ($P=0.016$), Lwamunda Swamp ($P = 0.004$) and Pump House ($P < 0.0001$); JJBay differed from Kazzi Lagoon ($P=0.002$); Lake Kayanja sperm differed from Kazzi lagoon ($P=0.021$) and Pump House ($P=0.022$); Kazzi Lagoon differed from Lwamunda Swamp and Pump House ($P<0.0001$ respectively); Lwamunda Swamp significantly differed from Ndysbusole ($P=0.014$) and finally, Ndyabusole significantly differed from Pump House ($P=0.002$).

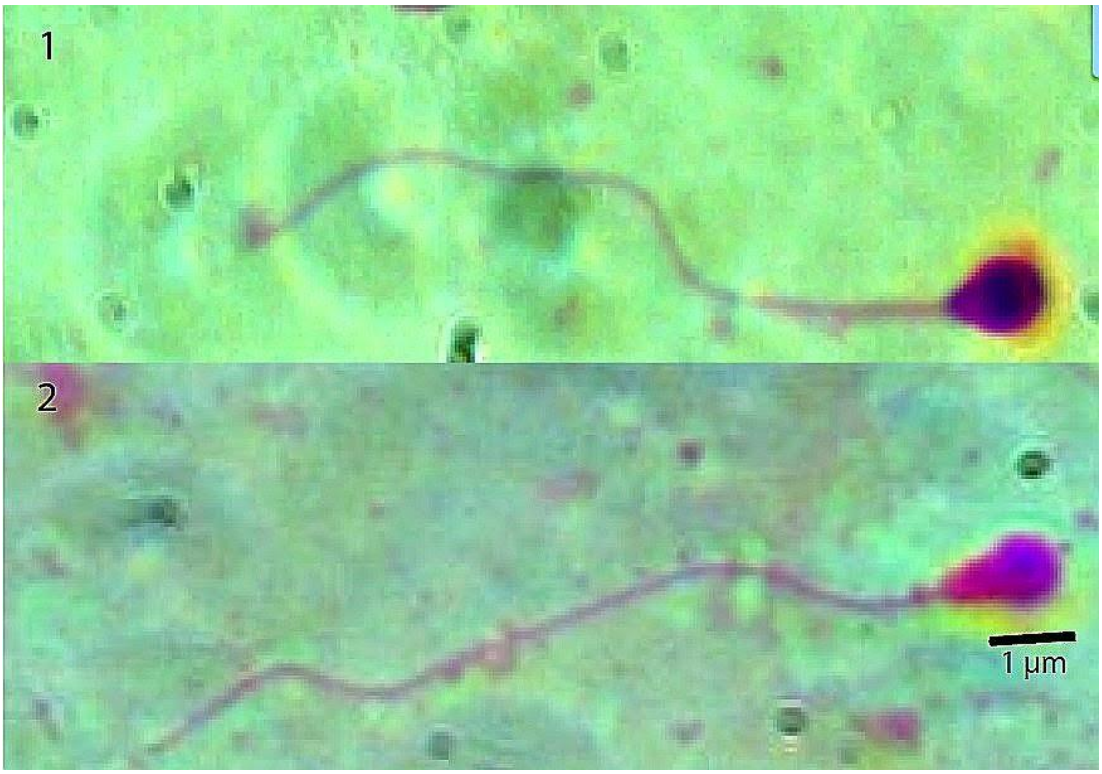

Figure 3. (1): Spermatozoa from Pump House, where the highest sperm velocity was recorded, Head width = 1.8 µm, Flagellum length = 20.5 µm; and (2): Spermatozoa from Bunoga, which had more hydrodynamic shape and the lowest velocity of all sites. Head width = 1.5 µm, Flagellum length = 22.6 µm.

Table 4. Results of principal component analysis of sperm morphometry traits of *P. multicolor victoriae* across sites.

Sperm trait	PC1 Matrix	PC1Scores
Head length	0.947	0.527
Head width	0.929	0.517
Flagellum length	0.019	0.107
Eigen value	1.795	
Prp total var	59.84	

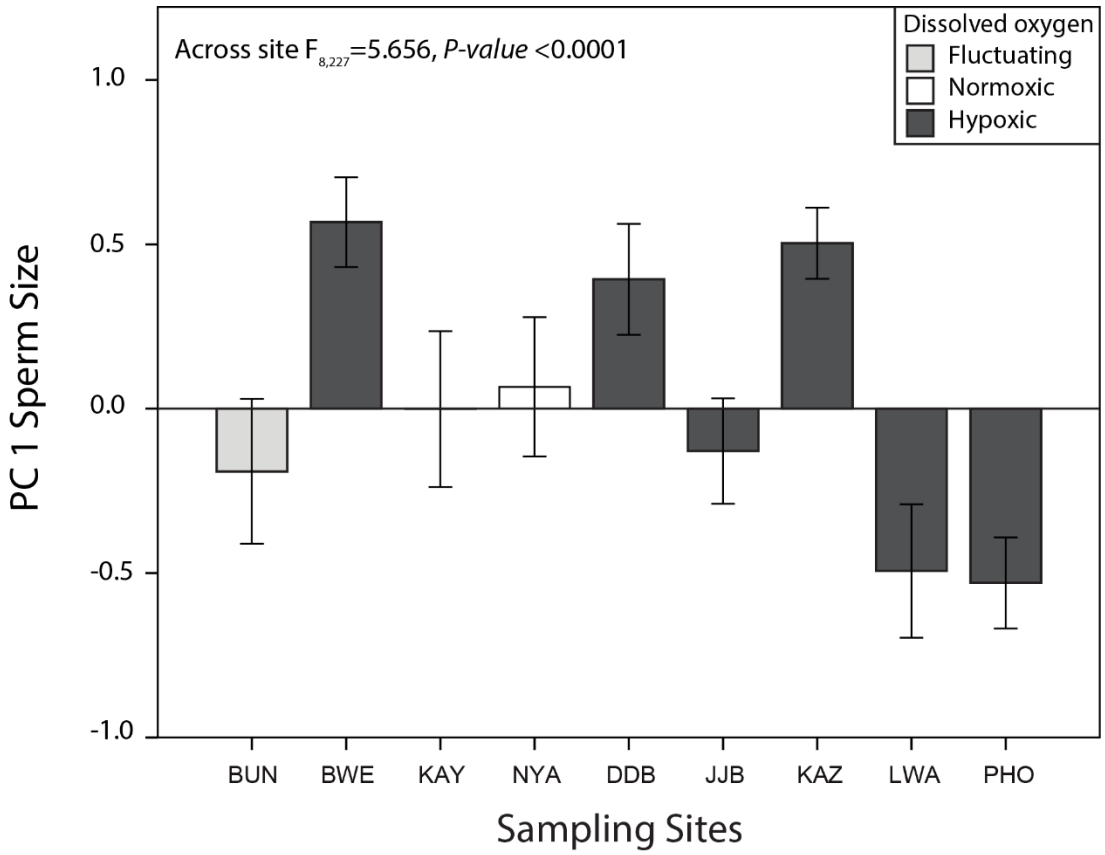

Figure 4. Sperm PC1 scores for *P. multicolor victoriae* across sites. Bunoga (BUN), Bwera (BWE), Lake Kayanja (KAY), Ndyabusole (NYA), Dead Duck Bay (DDB), JJ Bay (JJB), Kazzi Lagoon (KAZ). Lwamunda Swamp (LWA) and Pump House (PHO). Values are show as Mean ± S.E.

The hydrodynamic ratio (head length to head width), as well as the H_L:F_L ratio (head length to flagellum length) also differed across sites (Table 3, Figure 5). Fish with the lowest sperm head ratio (narrowest heads) were from Bunoga, and this site was different from Dead Duck Bay (P=0.013), JJ Bay (P=0.002), Lake Kayanja (P<0.0001), Kazzi Lagoon (P=0.005), Lwamunda Swamp (P=0.0001), Ndyabusole (P=0.005) and Pump House (P=0.001). Bwera was not different from Bunoga, but was different from Kayanja (P=0.0005), Kazzi Lagoon (P=0.036), Lwamunda

Swamp (P=0.015), Ndyabusole (P=0.044), and Pump House (P=0.009). Sperm heads from males in Lake Kayanja were wider than sperm from males in Dead Duck Bay (P=0.015). Fish with the highest ratio of sperm head length to flagellum length (or the shortest sperm) were from Dead Duck Bay (Table 2). Fish with the lowest ratio of head length to flagellum length (or the longest sperm) were from Bunoga (Table 2).

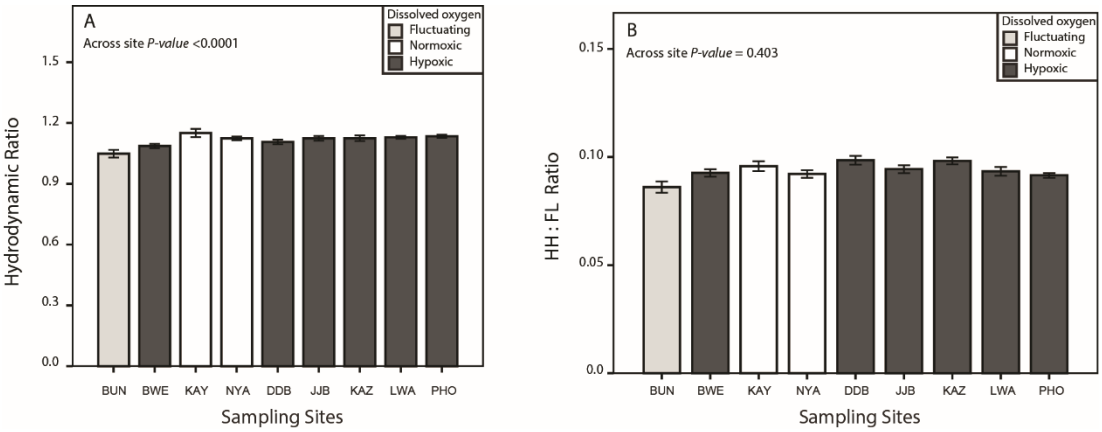

Figure 5. Sperm morphometry variation of *P. multicolor victoriae* across sites. (A) Hydrodynamic ratio and (B) Head length to flagellum length ratio (HH:FL). Bunoga (BUN), Bwera (BWE), Lake Kayanja (KAY), Ndyabusole (NYA), Dead Duck Bay (DDB), JJ Bay (JJB), Kazzi Lagoon (KAZ). Lwamunda Swamp (LWA) and Pump House (PHO). Values are show as Mean ± S.E. .

3.3 Sperm motility

Sperm velocity at each post-activation time differed by site (Table 3). At 15 seconds post-activation, males from Bunoga had slower sperm than males from Bwera (P=0.012), Dead Duck Bay (P<0.0001), Kayanja (P=0.024), Kazzi Lagoon (P<0.0001), and Pump House (P<0.0001). Males from Bwera had slower sperm than males from Dead Duck Bay (P=0.047), Kazzi Lagoon (P=0.018), and Pump House

(P=0.007). Sperm from males in Dead Duck Bay were also faster than males from JJ Bay (P=0.007), Lake Kayanja (P=0.044), Lwamunda Swamp (P=0.01), and Ndyabusole (P=0.001). Sperm from JJ Bay males were slower than sperm from Kazzi Lagoon males (P=0.002). Sperm from Lake Kayanja males were slower than sperm from Kazzi Lagoon males (P=0.018). Males from Kazzi Lagoon had faster sperm than males from Lwamunda Swamp (P=0.003) and Ndyabusole (P=0.0003). Finally, Pump House sperm was faster than sperm from all other sites except for Kazzi Lagoon and Dead Duck Bay: Bunoga (P<0.0001), Bwera (P=0.007), JJ Bay (P=0.0008), Kayanja (P=0.006), Lwamunda (P=0.002), Ndyabusole (P=0.0003).

Some sperm velocity relationships changed from 15 to 25 seconds between sites. At 25 seconds post-activation, sperm from males in Bunoga became slower than sperm from Lwamunda (P=0.011) and Nyabusole males (P=0.028), leaving it only not different from JJ Bay. Sperm from Bwera males became faster than sperm from JJ Bay (P=0.019), and Nyabusole males (P=0.042). Sperm from Lake Kayanja males also became faster than JJ Bay (P=0.006) and Ndyabusole (P=0.025). Finally, at 30 seconds post-activation, sperm swimming velocities from most sites were no longer different from one another. However, sperm from Bunoga males were slower than all other sites except for JJ Bay (P=0.256) and Ndyabusole (P=0.108), and Kazzi Lagoon sperm was significantly faster than Bwera (P=0.032), JJ Bay (P<0.0001), and Kayanja (P=0.042); Dead Duck Bay sperm was faster than JJ Bay (P=0.0003) and Ndyabusole (P=0.0009); and finally, Pump House sperm was significantly faster than JJ Bay (P=0.001) and Ndyabusole sperm (P=0.004).

Sperm swimming velocity in all males decayed significantly over time (within subjects repeated measures ANCOVA, $F_{2,164}$=3.068, P=0.049). Bonferroni post-hoc analysis showed that velocity at 15 seconds post-activation was significantly different from velocity at both 25 (P=0.003) and 30 seconds (P=<0.0001), and velocity at 25 seconds post-activation was different from 30 seconds post-activation (P=0.048). Sperm velocity decay also differed significantly by site (Between subjects repeated measures ANCOVA, time $F_{2,332}$=11.23, P=0.00002). There was an interaction between sperm swimming velocity and fish standard length ($F_{2, 164}$ =4.054, P= 0.019), and between sperm swimming velocity and site ($F_{2, 328}$=2.085, P=0.009), indicating that fish from different sites and of different sizes had different sperm swimming decay patterns (Figure 6). Longevity (the difference in final velocity compared to initial velocity) did not differ by site ($F_{8,174}$ =1.37, P=0.213).

Linearity was positively correlated with sperm swimming velocity at all post-activation times (15 seconds r=0.89, P=<0.0001, 25 and 30 seconds r=0.86, P=<0.0001), indicating that faster sperm travelled in a more linear trajectory. Linearity of sperm trajectory differed across sites, with differences between sites being qualitatively the same as velocity (Table 3). For example, the lowest linearity was found in Bunoga males' sperm, and the highest linearity was found in Pump House males' sperm at 15 seconds post-activation (Table 2). Linearity did not change across sperm swimming decay times (Repeated measures ANCOVA, $F_{2,164}$=0.549, P=0.579), indicating that as sperm velocity decayed, their trajectory patterns did not change. The H_L:F_L ratio was also positively correlated with sperm

swimming velocity at each time post-activation (VAP15 r=0.354 P=<0.0001, VAP25 r=0.402, P=<0.0001, VAP30 r=0.403, P=<0.0001). This indicates that as sperm flagellum length decreases, velocity increases.

Results of hierarchical regression showed that Model 3 (which included standard length, somatic mass, testes mass, sperm head length, head width, and flagellum length) best explained sperm velocity and linearity (P=<0.0001; Table 5). The individual variables that most strongly predicted both sperm velocity and linearity at all times post-activation were sperm head width and sperm flagellum length (Table 6). Sperm head width had a positive relationship with sperm velocity and linearity, while sperm flagellum length had a negative relationship with sperm velocity and linearity (Table 6). Additionally, fish somatic mass was a significant and positive predictor for both velocity and linearity at 15 seconds post-activation. Linearity at 25 seconds post-activation was also marginally significantly explained by model 2 (total testes mass; Table 5).

Summary of results by site

In summary, total gonad mass differed by site. Males from Bunoga had the highest testes mass, while males from JJ Bay had the lowest. The sperm head length to flagellum length ratio (H_L:F_L) had a different relationship with fish size across sites, indicating that smaller fish had a higher ratio, or smaller sperm. Furthermore, testes asymmetry varied by site, with males from Bunoga having the highest asymmetry, and males from JJ Bay having the lowest. Testes mass and testes asymmetry were highly correlated, and as testes mass increased, so did testes asymmetry.

Sperm head size differed by site, with hypoxic sites having the widest heads, and the fluctuating oxygen site Bunoga having the narrowest sperm heads. Velocity of sperm at each postactivation time was lowest in males from Bunoga, and highest in Pump House. Overall, sperm velocity decayed significantly over time. However, there was no difference in longevity of sperm across sites. Faster sperm had a more linear trajectory than slower sperm, and this relationship did not change over the 15 second postactivation period. The morphometric traits that best predicted velocity and linearity were (in order of highest p-value to lowest p-value): sperm head width, sperm flagellum length, somatic mass, and standard length.

Analyses by dissolved oxygen regime

Male traits showed variation across dissolved oxygen regimes (Table 7). There was a significant interaction between fish somatic mass and oxygen regime when assessing total testes mass across dissolved oxygen regimes (Table 8). Pearson's correlation showed that the fluctuating site had a much stronger relationship between somatic mass and total testes mass than did the hypoxic and normoxic sites (Appendix 1). Head ratio was highest in males from normoxic sites and was significantly different from both fluctuating ($P<0.0001$) and normoxic ($P=0.004$) regimes. Head ratio in males from fluctuating regimes was also significantly lower than males from the hypoxic regime ($P=0.004$). Although the H_L:F_L ratio did not differ across regimes, flagellum length was significantly different between all 3 regimes: fluctuating vs. hypoxic and normoxic ($P<0.0001$, respectively); and hypoxic vs. normoxic ($P=0.01$).

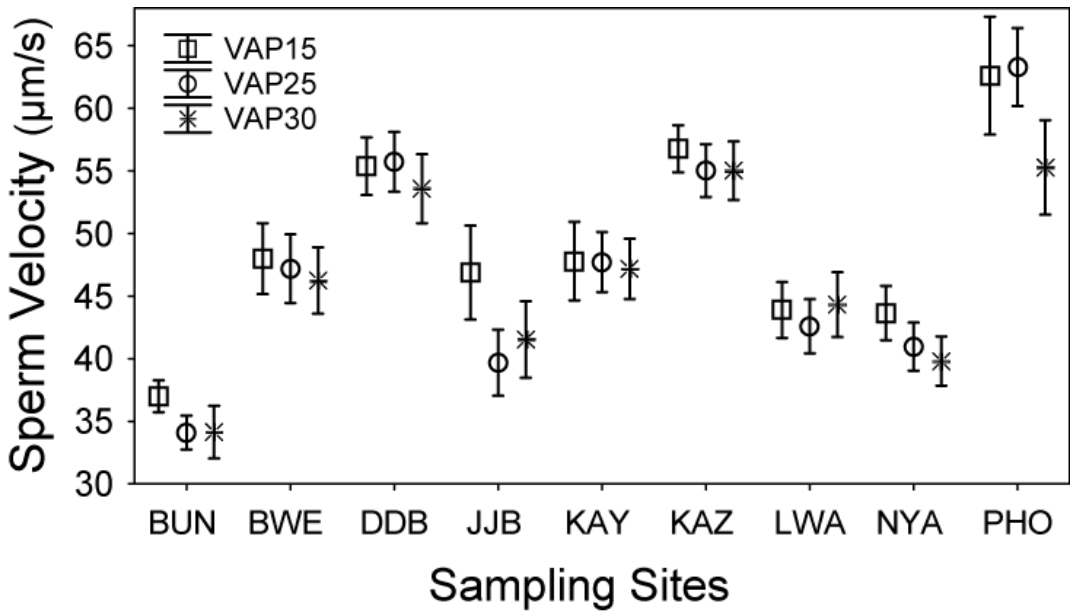

Figure 6. Smooth path velocity of male *P. multicolor* sperm at different post-activations times across sites. Bunoga (BUN), Bwera (BWE), Dead Duck Bay (DDB), JJ Bay (JJB), Kayanja (KAY), Kazzi Lagoon (KAZ). Lwamunda (LWA), Nyabusole (NYA), and Pump House (PHO). Values are shown as mean ± S.E. See text for P-values of post-hoc tests.

Table. 5. Results of hierarchical linear regression of 3 models with velocity and linearity at each post-activation time. Model 1 contained only somatic mass and standard length, model 2 added total testes mass, and model 3 added sperm head length, head width, and flagellum length. Table shows R^2, adjusted R^2, R square change (or the total proportion explained by that model alone), and the significance of each model. Model 1 included somatic mass and standard length, model 2 added total testes mass, and model 3 added sperm morphometry: head length, head width, and flagellum length

VAP15	**R^2**	**Adj R^2**	**R^2 Ch**	***P-value***	**LIN15**	**R2**	**Adj R^2**	**R^2 Ch**	***P-value***
Model 1	0.046	0.032	0.046	**0.039**	Model 1	0.046	0.033	0.046	**0.038**
Model 2	0.048	0.027	0.002	0.577	Model 2	0.05	0.029	0.003	0.483
Model 3	0.289	0.257	0.241	**<0.0001**	Model 3	0.231	0.197	0.182	**<0.0001**
VAP25	**R^2**	**Adj R^2**	**R^2 Ch**	***P-value***	**LIN25**	**R^2**	**Adj R^2**	**R^2 Ch**	***P-value***
Model 1	0.019	0.005	0.019	0.269	Model 1	0.008	-0.007	0.008	0.585
Model 2	0.038	0.017	0.019	0.102	Model 2	0.035	0.014	0.028	**0.049**
Model 3	0.379	0.351	0.341	**<0.0001**	Model 3	0.297	0.266	0.262	**<0.0001**
VAP30	**R^2**	**Adj R^2**	**R^2 Ch**	***P-value***	**LIN30**	**R^2**	**Adj R^2**	**R^2 Ch**	***P-value***
Model 1	0.049	0.035	0.049	**0.032**	Model 1	0.035	0.021	0.035	0.086
Model 2	0.05	0.03	0.002	0.598	Model 2	0.035	0.014	0	0.926
Model 3	0.382	0.354	0.331	**<0.0001**	Model 3	0.261	0.228	0.226	**<0.0001**

Table 6. Beta coefficients and *P-value* of each variable from Model 3 for velocity and linearity at each post-activation time.

VAP15			**VAP25**		**VAP30**	
Var	β Coeff	*P-value*	β Coeff	*P-value*	β Coeff	*P-value*
SL	-0.455	0.062	0.058	0.8	-0.196	0.386
SM	0.495	**0.032**	0.091	0.671	0.163	0.445
GT	0.013	0.906	-0.11	0.28	0.027	0.789
HH	-0.027	0.824	-0.125	0.27	-0.126	0.263
HW	0.458	**0.0002**	0.637	<0.0001	0.645	<0.0001
FL	-0.282	**0.0004**	-0.277	0.0002	-0.239	0.001
LIN15			**LIN25**		**LIN30**	
Var	β Coeff	*P-value*	β Coeff	*P-value*	β Coeff	*P-value*
SL	-0.525	0.039	0.346	0.153	-0.391	0.115
SM	0.65	**0.007**	-0.129	0.571	0.406	0.082
GT	-0.017	0.877	-0.168	0.122	0.063	0.57
HH	-0.045	0.719	-0.164	0.174	-0.151	0.221
HW	0.382	**0.003**	0.527	**<0.0001**	0.549	**<0.0001**
FL	-0.287	**0.001**	-0.333	**<0.0001**	-0.225	**0.006**

There was a significant interaction between fish standard length and oxygen regime when assessing velocity and linearity at 30 seconds post-activation (Table 8). Correlation between standard length and velocity at 30s post-activation showed that males in hypoxic sites had a slight positive relationship between velocity and standard length, whereas males in normoxic and fluctuating regimes had a very strong negative relationship between velocity and standard length (Appendix 1). Qualitatively similar results were shown for linearity of sperm trajectory (Appendix 1). Velocity at 15 seconds post-activation was highest in males from hypoxic sites and significantly different from sperm velocity of males from fluctuating regime (P=0.002) but was not different from sperm velocity of males in normoxic sites

(P=0.06). Velocity of sperm from males from normoxic sites was also not significantly different from sperm velocity in the fluctuating regime (P=0.06). At 25 seconds post-activation, males from the fluctuating regime had a sperm velocity that was significantly slower than both hypoxic (P<0.0001) and normoxic (P=0.007) males. Males from hypoxic and normoxic regimes still did not differ significantly in sperm velocity (P=0.05). Longevity (the difference in final velocity compared to initial velocity) did not differ by site (P=0.175). Longevity did not differ by dissolved oxygen regime ($F_{3,174} = 0.337$, P=0.779). Linearity at 15 seconds post-activation was highest in males from hypoxic sites, differing significantly from males in fluctuating regimes (P<0.0001) but not from males in normoxic regime (P=0.186). Males from fluctuating regimes were also significantly less linear than males in normoxic regimes (P=0.005). At 25 seconds post-activation, the relationship between regimes remained qualitatively the same.

In summary, total testes mass and somatic mass had a stronger relationship in the fluctuating regime versus the normoxic and hypoxic regimes. Head width of sperm was highest in hypoxic sites, and flagellum length was lowest in hypoxic sites. Although sperm from hypoxic sites had the highest mean velocity, sperm velocity and linearity did not differ between hypoxic and normoxic regimes. However, males from the fluctuating regime had significantly slower sperm than males from the hypoxic regime. Longevity did not differ across regimes.

Table 7. General morphological traits, sperm morphometry traits and sperm motility of *P. multicolor* by oxygen regime. Variables are total length (TL), standard length (SL), total mass (mass), somatic mass (SM), left testis mass (LT), right testis mass (RT), total testes mass (GT), testes asymmetry (ASY), sperm head length (HL), head width (HW), flagellum length (FL), hydrodynamic ratio (HR), head length to flagellum length ratio (HL:FL), sperm velocity (VAP), and linearity of trajectory (LIN). Values are mean ± S.E

Trait	Fluctuatin	Hypoxic	Normoxic
	g		
Body morphometry			
TL (cm)	7.319 ± 0.309	6.065± 0.064	6.505 ± 0.142
SL (cm)	5.9 ± 0.249	4.894 ± 0.054	5.191± 0.116
Mass (g)	7.048± 0.73	3.728 ± 0.121	4.561± 0.27
SM	7.017 ± 0.726	4.036 ± 0.119	5.032± 0.262
LT	0.008 ± 0.001	0.005 ± 0.0003	0.005± 0.0004
RT	0.027 ± 0.004	0.011 ± 0.0006	0.011± 0.0009
GT	0.031 ± 0.005	0.014 ± 0.0008	0.013± 0.001
ASY	0.369 ± 0.037	0.457 ± 0.02	0.434 ± 0.0333
Sperm morphometry			
HL	1.924 ± 0.048	1.911 ± 0.013	1.909 ± 0.0282
HW	2.008 ± 0.035	2.134 ± 0.013	2.168 ± 0.032
FL	22.453 ± 0.344	20.253 ± 0.099	20.334 ± 0.162
HR	1.049 ± 0.019	1.119 ± 0.004	1.139 ± 0.011
H_L :F_L	0.0861 ± 0.003	0.095 ± 0.0007	0.094 ± 0.001
Sperm motility			
VAP15	36.999 ± 1.271	51.682 ± 1.301	45.355 ± 1.821
VAP25	34.094 ± 1.350	49.715 ± 1.242	43.751 ± 1.566
VAP30	34.134 ± 2.104	48.977 ± 1.246	42.848 ± 1.596
LIN15	41.079 ± 1.254	49.213 ± 0.676	47.051 ± 0.959
LIN25	39.471 ± 1.046	48.169 ± 0.673	46.419 ± 0.888
LIN30	40.092 ± 1.214	47.912 ± 0.743	47.161 ± 0.850

Table 8. Results of nested ANCOVAs by dissolved oxygen regime within region and with standard length as a covariate for traits of *P. multicolor* including testes mass (GT), flagellum length (FL), sperm head ratio (HR), velocity (VAP), and sperm linearity (LIN).

Trait	**ANCOVA**	**df**	**F**	**P-value**
GT	D.O. Regime (Region) + SM	3	0.138	0.670
	*SM*Site*			**<0.0001**
FL	D.O. Regime (Region) + SL	3	24.9	**<0.0001**
	*SL*Site*			0.084
HR	D.O. Regime (Region) + SL	3	9.222	**<0.0001**
	*SL*Site*			0.937
VAP15	D.O. Regime (Region) + SL	3	5.686	**0.001**
	*SL*Site*			0.601
VAP25	D.O. Regime (Region) + SL	3	7.267	**0.0001**
	*SL*Site*			0.060
VAP30	D.O. Regime (Region) + SL	3	2.761	**0.044**
	*SL*Site*			**0.002**
LIN15	D.O. Regime (Region) + SL	3	6.167	**0.001**
	*SL*Site*			0.754
LIN25	D.O. Regime (Region) + SL	3	7.587	**0.001**
	*SL*Site*			0.206
LIN30	D.O. Regime (Region) + SL	3	2.199	0.09
	*SL*Site*			**0.031**

DISCUSSION

There is a paucity of studies on the natural variation in sperm morphometry and motility in a single species across different habitat conditions. *Pseudocrenilabrus multicolor victoriae* is a widespread African cichlid that exploits habitats ranging from chronically hypoxic swamps to flowing normoxic rivers and streams, making it a model species for studying adaptation. This study was the first to investigate whether male *P. multicolor* displayed variation in testes asymmetry, sperm morphometry, and sperm swimming performance across a wide variety of habitats, including three different oxygen regimes.

4.1 General body morphometry

Harsh environments, such as those with low dissolved oxygen, may induce energetic trade-offs between growth and reproduction, as both are energetically costly (Roff 1992; Taborsky 1998; Wu 2002; Awata *et al.,* 2008). *P. multicolor* display variation in body size and shape across oxygen regimes due to direct and indirect effects of hypoxia (Crispo and Chapman 2011). In split-brood studies, *P. multicolor* raised under hypoxia had shorter body lengths, and deeper heads due to larger gills (Crispo and Chapman 2011). In this study, males sampled from hypoxic sites had the lowest average standard length and mass, indicating that hypoxia does affect overall fish size in this species. However, whether or not these size and shape trade-offs in hypoxia affect reproductive output is unknown.

Testes mass relative to body mass is a measure of reproductive investment in males because larger testes are often correlated with higher sperm production (Møller and Birkhead 1989; Stockley *et al.*, 1997). Fish species that are adapted to hypoxia can face reproductive stress that reduces testes mass under low dissolved oxygen levels (Wu *et al.,* 2003, Thomas *et al.,* 2006; Landry *et al.,* 2007; Thomas and Rahman 2009). For example, male Atlantic Croaker collected from hypoxic and normoxic estuaries were compared, and males from hypoxic sites had reduced testosterone levels, reduced testes development, and a reduced production of sperm relative to males from normoxia (Thomas *et al.,* 2007). Additionally, males from the species *Barbus neumayeri* living in the same drainage in Africa as *P. multicolor* displayed the same pattern, with males in hypoxic regimes having a reduced GSI relative to males from normoxic regimes (Martínez *et al.,* 2015). Therefore, I expected *P. multicolor* to have relatively lower testes mass under hypoxia. However, there was no clear difference in the testes mass to somatic mass relationship across sites or oxygen regimes, and larger fish had larger testes regardless of site of origin.

This study was the first to show that *P. multicolor* display directional testes asymmetry on a population scale, where the right testis was approximately double the size of the left across all populations sampled. Testes asymmetry has been seen in other species and groups, including birds (reviewed in Møller 1994), amphibians, reptiles, and mammals (reviewed in Yu 1998), although it is usually the left testis that is larger than the right (Jamieson *et al.,* 2007). In birds, testes asymmetry has been correlated with better male condition (Møller 1994). However, in fish, the degree of testes asymmetry has been shown to be related to environmental stressors

(Clarke 1995; Sopinka *et al.,* 2012). For example, testes asymmetry in the Plainfin Midshipman was shown to be greatest in individuals exposed to dissolved contaminants (Sopinka *et al.,* 2012). I predicted that testes asymmetry would be higher in the hypoxic regime due to energetic stress caused by hypoxia. However, the level of asymmetry was not significantly different across oxygen regimes, indicating that hypoxia does not affect asymmetry in this species. Another reason for increased asymmetry in fish can be mating competition, as was seen in whitefish (Burness *et al.,* 2008). In this study, the level of asymmetry was not significantly correlated with sperm morphometry or velocity, but was strongly correlated to total testes mass. In this species, larger males had larger testes. This relationship between size and asymmetry may indicate that larger males face a trade-off between growth and the production of viable or competitive sperm in both testes (Burness *et al.,* 2008). Additionally, the highest asymmetry was seen in males from Bunoga, and these males had the slowest sperm velocity of all sites, indicating that asymmetry may also be related to energetic stress in this species. Future studies should directly assess whether testes asymmetry is associated with energetic trade-offs, and whether the level of asymmetry is related to sperm performance.

4.2 Sperm morphometry and motility

Sperm morphometry varied across sites and dissolved oxygen regimes, and there was a trend in sperm size and shape based on regime. Sperm from hypoxic sites had the widest and roundest sperm heads and the shortest flagella. On average, sperm velocity was also highest in the hypoxic regime. It was noted in this study that the

wider heads of the sperm in hypoxic sites often allowed for a larger midpiece vesicle, which became visually discernible from the head and flagellum in many sperm from hypoxic sites (results not shown). Therefore, this author hypothesizes that the strong positive relationship between sperm head width and sperm velocity in this study may be due to a higher ATP content in larger midpieces. Some studies have linked sperm ATP content or the size of the midpiece (which contains ATP) to sperm velocity or sperm fertilization success (Vladic *et al.,* 2002). Atlantic Salmon sperm fertilization success was positively correlated with ATP stores (Vladic *et al.,* 2002). In Sticklebacks, both the flagellum length and the size of the midpiece were positively correlated with fertilization success (Bakker *et al.,* 2014). Future studies should assess midpiece size and its relationship with ATP content, as well as the relationships between sperm velocity, fertilization success, and ATP content in this species. If ATP content is positively correlated with sperm velocity, variation in sperm swimming performance may be explained by variation in ATP content across populations. Another possibility is that sperm are facing a trade-off between morphometry and energy storage ability, where the more narrow headed sperm have less room for ATP stores.

Sperm from the fluctuating regime had a more hydrodynamic (narrow) head shape than all other sites. Humphries *et al.,* (2008) hypothesized that a more hydrodynamic head shape (narrower) would be beneficial to reduce drag for swimming sperm. The fluctuating site is also a river, and previous studies have shown that sperm performance and fertilization success can be negatively affected by turbulent or flowing water (Petersen *et al.,* 2001). For example, in observations of

natural spawning activities of coral species, the levels of water flow had a dramatic effect on fertilization success, with higher flow reducing fertilization success (Coma and Lasker 1997). In a study on Bluehead Wrasse, sperm performance under increased water velocity was also negatively correlated with fertilization success (Petersen *et al.,* 2001). If males in flowing water have adapted to have narrower sperm heads to better swim through flowing water, there may be a trade-off between hydrodynamic shape and ATP stores in these sperm. Future studies should assess the effect of water flow on sperm morphometry and performance in this species. Furthermore, males from the fluctuating site had the longest flagella. Males in the Bunoga and Bwera sites, which are close to each other in the Mpanga region had similar sperm flagellum lengths, but Bwera males had wider sperm heads like all other hypoxic sites, and a significantly higher sperm swimming velocity than Bunoga males. This suggests that sperm head width is a better indicator than flagellum length of sperm swimming performance in this species (Roff 1992; Taborsky 1998; Wu 2002; Awata *et al.,* 2008).

Humphries *et al.,* (2008) hypothesized that the ratio of head size to flagellum length would be an important factor to analyze in studies attempting to link sperm morphometry to sperm velocity, reasoning that the thrust force of the flagellum would need to overcome the drag forces of the head. Interestingly, the head length of sperm was not related to sperm velocity, and flagellum length alone was negatively correlated with velocity. However, the head length to flagellum length ratio was positively correlated with velocity. This may indicate that a ratio of head and

flagellum size may be a better indicator of sperm swimming performance than singular morphometry measures in this species (Humphries *et al.*, 2008).

Additionally, sperm performance may be affected by the mating system of this fish. The occurrence of sneakers, or males with competitively better sperm, is also a possibility. It is now known that genetic monogamy is scarce in fish mating systems (reviewed in Rocha *et al.*, 2008), indicating that competition induced sexual selection of sperm traits may be acting on males in socially monogamous systems such as *P. multicolor'*s. It should be noted that some males collected in this study resembled females in body colouration and size (results not shown), meaning that some of the smaller males lacked the pronounced red spot on their anal fin and yellow ventral colouration, yet they had viable sperm. Future studies should assess the mating system of this species in a natural setting, and assess whether sneakers or female mimics exist.

Sperm velocity may be just as important in non-competitive or less competitive mating systems if overall sperm longevity is low (Gomendio and Roldan 1993; Ball and Parker 1996). Indeed, most externally fertilizing species have a very short period of progressive motility after activation (Ishijima *et al.*, 1998). If faster sperm require more ATP than slower sperm, it may be beneficial to maintain intraspecific variation in sperm fertilization tactics to increase longevity of some sperm, as was seen in Rainbowfish (Simpson *et al.*, 2014), and Rainbow Trout (Tuset *et al.*, 2008).

Sperm swimming velocities at each post-activation time were variable across sites. The males with the fastest sperm were from three hypoxic sites: Pump House,

Dead Duck Bay, and Kazzi Lagoon. The sperm from these sites remained the fastest across all three post-activation times, indicating that sperm from hypoxic sites have higher motility in this species. Additionally, decay did not occur more quickly in faster sperm. Different authors suggest that faster swimming sperm may face a trade-off with longevity, having a shorter time to find and fertilize an egg than slower sperm (Gomendio and Roldan 1993; Ball and Parker 1996), nevertheless, the pattern does not seem to be widespread across species. In Bluegill Sunfish (*Lepomis macrochirus*), it was found that sneaker males had faster sperm with a reduced longevity than parental male sperm (Burness *et al.,* 2004). Several studies of Atlantic Salmon did not find a link between flagellum length and velocity, or a trade-off between velocity and longevity (Vladic *et al.,* 2002; Gage *et al.,* 2004). In the Redside Dace (*Clinostomus elongatus*), a promiscuous species, there was a significant positive relationship between flagellum length and sperm velocity, but no trade-off between velocity and longevity was found (Pitcher *et al.,* 2009a). Finally, Fitzpatrick and collaborators (2009) did not find a common pattern between sperm swimming velocity and sperm longevity across 25 different cichlid species.

Another alternative explanation for why males in hypoxic sites have relatively faster sperm than males from other habitats may be that males from hypoxic sites face a reproduction and life span trade-off as an adaptation to hypoxia. In general, costs to fish under hypoxia increase as body size increases, because there is less gill surface area to absorb oxygen and more internal volume that requires it (Pauly 1981; Reardon and Chapman 2012). If a hypoxic environment favours smaller fish that are able to mature faster, there may be more energy available in these males

to invest in sperm performance over larger or slower growing fish (Enberg *et al.,* 2012). However, this is a difficult hypothesis to confirm because of so many confounding variables, such as nutrition and predation rates in natural settings (Enberg *et al.,* 2012). Furthermore, no work has been done on size and age at maturity in this species to date. Future studies should assess whether any maturity vs. lifespan trade-offs exist, and whether or not sperm velocity is related to age in this species.

Finally, some logistic limitations of studying sperm swimming performance outside of a more controlled lab setting must be addressed in future studies. As mentioned in the methods section, sperm were assessed for swimming performance using water taken from a rain barrel, and dissolved oxygen levels were the same for all sperm swimming trials, despite site of origin. Future studies should conduct the same sperm performance assessments under both hypoxic and normoxic dissolved oxygen levels to assess if a difference in velocity results from the immediate oxygen levels available to the sperm. For example, Fitzpatrick *et al.,* (2009) found that Plainfin midshipman sperm from hypoxic sites performed better under hypoxic conditions, although the exact reason for that was not found.

4.3 Other effects on energy in fishes in natural settings

Aside from dissolved oxygen levels, there are multiple environmental conditions that may cause variation in body size and gonad mass across and within regimes, including food availability (Collins and Anderson 1999; Donelson *et al.,* 2010), predation risk (Pavlová *et al.,* 2010), and the level of mating competition (Gage

1995; Long and Montgomerie 2006). The effect of food availability on reproductive output has been studied more often in female fish (Collins and Anderson 1999; Donelson et al., 2010), as eggs require more energy to produce than sperm (Jonsson *et al.,* 1991). In some species such as the White Crappie (*Pomoxis annularis*), a lowered diet negatively affected egg production in females but did not affect sperm production in males (Bunnell *et al.,* 2007). Predation risk may also vary across habitats for *P. multicolor,* with less risk for fish in the hypoxic sites, as most predator species are larger and not as hypoxia tolerant as smaller species (Chapman *et al.,* 2002; Reid *et al.,* 2013). Lwamunda Swamp has some of the most consistent and chronic hypoxia of all sites sampled, and males were also relatively larger in this site than males in other hypoxic sites. Fish may benefit from a lack of predators in this swamp by an increase in foraging ability, allowing them to invest more energy in growth and reproduction (Cerri and Fraser 1983; Pavlová *et al.,* 2010; Martínez *et al.,* 2015). A lack of predators in a more hypoxic environment may also mean that there is a higher density of *P. multicolor* in those sites, increasing the level of mating competition for males as observed in other fish species (Gage *et al.,* 1995; Long and Montgomerie 2006). Little is known about reproduction in *P. multicolor* outside of laboratory studies and observational data from many decades ago. Variation in testes mass between and within species often reflects the level of mating competition (Gage 1994; Stockley *et al.,* 1997). For example, in a laboratory study assessing whether male cichlids of the species *Julidochromis transcriptus* adjust testes size in relation to perceived competition levels, it was found that males placed in competition with other males developed relatively heavier testes than males placed in a monogamous

system (Awata *et al.,* 2008). Future studies should assess whether competition for mates varies across habitats for this species, and whether relative testes mass varies with competition.

CONCLUSION

Pseudocrenilabrus multicolor victoriae is a hypoxia tolerant species that displays morphological and physiological differences across sites with different dissolved oxygen levels (Chapman *et al.,* 2000; Chapman *et al.,* 2008; Crispo and Chapman 2008; Martínez *et al.,* 2009; Crispo and Chapman 2010; 2011; Crocker *et al.,* 2013 *a, b*). Similar to other traits in *P. multicolor*, it was found that sperm morphometry and velocity varied across habitats. An overall pattern was observed, where males from Bunoga, which is a seasonally fluctuating river, were the largest, and had the slowest swimming sperm of all sites. Males investing more energy into overall growth may face a trade-off between body size and sperm performance in this species. For example, some fish species, such as Coho Salmon (*Oncorhynchus kisutch*), face energetic trade-offs between secondary sexual characteristics and sperm performance (Pitcher *et al.,* 2009b), where those investing more in body traits had sperm with a slower velocity. Males from Bunoga were also unique in that they displayed the greatest inter-individual variation of traits, as seen in the standard error of each trait mean. Similar trends were seen in enzyme levels for *P. multicolor* from the Mpanga region, where there was a greater amount of variation in enzyme levels between individuals in Bunoga than between individuals from sites in Nabugabo (Crocker *et al.,* 2012a). The seasonally fluctuating oxygen levels in this river site may induce more variation in male phenotypes, as physiological needs change on a seasonal basis (Crocker *et al.,* 2012*a*). Finally, male *P. multicolor* invests energy into carotenoid body colouration to attract females during courtship (Fernö 1986). Hence, future studies should assess if males are facing a trade-off between

investment in colouration and investment in sperm traits. A split-brood study that assesses testes mass, testes asymmetry, and sperm morphometry and motility in a range of dissolved oxygen levels and levels of water flow is recommended.

Hypoxia's effects on neuroendocrine reproductive pathways in fish are becoming clearer as research progresses (Wu *et al.,* 2003; Martínez *et al.,* 2004; 2006; Thomas and Rahman 2009). It is important to collect baseline information on the physiological adaptations of fish species currently thriving in hypoxic habitats, as they could become threatened in the future (Wikelski and Cooke 2006). Additionally, the negative effects of globally rising hypoxia may be harder to detect in hypoxia tolerant species without baseline information, as they do not always display obvious or significant physiological changes (Zhou *et al.,* 2000; Martínez *et al.,* 2006; Crocker *et al.,* 2013*a*). Alternatively, because this species is living at much lower levels of oxygen than most freshwater species can survive in (0.6 versus 5 -6 mg O_2 L^{-1}), smaller changes in temperature and dissolved oxygen levels may have more of a detrimental effect on this and other tropical freshwater species living in similar conditions. Due to their habitat distribution, *P. multicolor* is a great model to study the effects of hypoxia, which can be extrapolated to other freshwater species worldwide. By looking at the fish general morphometry, sperm shape and size and sperm motility from a variety of sites with different habitat characteristics, researchers can find relationships between habitat and reproductive characteristics that engender further important research.

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APPENDIX 1

CORRELATIONS FOR VARIABLES THAT INTERACTED WITH THE COVARIATE IN ANCOVA ANALYSIS

Site	**SL x M**		**SL x H_LF_L**	
	r	**P**	**r**	**P**
BUN	0.989	**<0.0001**	-0.591	**0.016**
BWE	0.977	**<0.0001**	0.2	0.347
DDB	0.957	**<0.0001**	-0.283	0.191
JJB	0.950	**<0.0001**	-0.171	0.424
KAY	0.988	**<0.0001**	-0.337	0.06
KAZ	0.979	**<0.0001**	0.112	0.554
LWA	0.868	**<0.0001**	0.026	0.884
NYA	0.974	**<0.0001**	-0.15	0.473
PH	0.925	**<0.0001**	-0.416	**0.018**

D.O. Regime	SL xVAP30		SL x LIN 30	
	r	p	r	p
FLU	-0.853	**0.0001**	-0.624	**0.017**
HYP	0.1	0.284	0.14	0.136
NOR	-0.416	**0.004**	-0.379	**0.009**

D.O. Regime	SM x GT	
	r	p
FLU	0.870	<**0.0001**
HYP	0.714	<**0.0001**
NOR	0.624	<**0.0001**

APPENDIX 2

RELATIONSHIP BETWEEN SOMATIC MASS AND TESTES MASS IN MALE *P. MULTICOLOR*

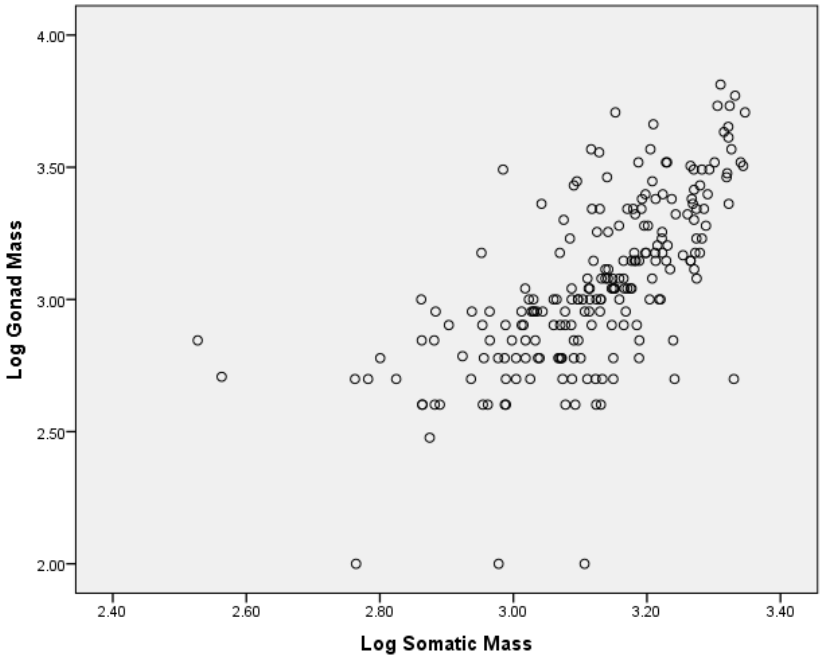

Figure 7. Scatter plot of the log of somatic mass and the log of total testes mass to demonstrate the approximate linear relationship between size and gonad mass in this species.