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Variation in sperm morphometry of the African cyprinid *Barbus neumayeri* (Neumayer's barb)

M.L. Martínez, V. Mullin, and A. Schulte-Hostedde

Abstract: In this study, we explored variation in sperm morphometry of the African cyprinid *Barbus neumayeri* Fischer, 1884 (Neumayer's barb) across seven sites with a wide range in dissolved oxygen, from hypoxic swamps to intermittent normoxic streams to well-oxygenated rivers. We explore whether fish physiological condition (K) or hypoxia can affect the reproductive traits, and whether condition–hypoxia dependence of sperm traits including head length (L_H), head width (W_H), flagellum length (L_F), and hydrodynamic ratio (HR) vary across sampling sites. Significant differences were found in fish total length ($P = 0.0212$), as well as in K, left and right testis masses, total gonad mass, and gonadosomatic index ($P < 0.0001$ for all traits). Total gonad mass was lower in hypoxic sites than in well-oxygenated sites. Interestingly, the left and right testes from normoxic environments were double the size of testes from hypoxic environments. Despite little variation in sperm flagellum length, sperm heads were longer in swamps than in streams or rivers, giving the sperm head a more hydrodynamic shape. This variation in HR may be beneficial in the more stagnant waters of the swamp compared with the other environments. Future studies are necessary to understand whether variation in sperm morphology correlates with sperm swimming performance and male reproductive capacity.

Key words: *Barbus neumayeri*, Neumayer's barb, reproductive capacity, sperm morphometry, hydrodynamic ratio, anthropogenic stressors.

Résumé : Nous explorons les variations de la morphométrie des spermatozoïdes chez le cyprinidé africain *Barbus neumayeri* Fischer, 1884 entre sept sites représentant une vaste diversitée de teneurs en oxygène dissous, allant de marécages hypoxiques à des cours d'eau au niveaux d'oxygène variable et des rivières bien oxygénées. Nous examinons si la condition physiologique (K) des poissons ou l'hypoxie peuvent avoir une incidence sur les caractères associés à la reproduction, et si la dépendance entre embonpoint et hypoxie des caractères des spermatozoïdes, dont la longueur de la tête (L_H), la largeur de la tête (W_H), la longueur du flagelle (L_F) et le rapport hydrodynamique (HR), varie d'un site à l'autre. Des différences significatives sont notées en ce qui concerne la longueur totale des poissons ($P = 0,0212$) ainsi que le K, les masses des testicules gauche et droit, la masse totale des gonades et l'indice gonadosomatique ($P < 0,0001$ pour tous les caractères). La masse totale des gonades est plus faible dans les sites hypoxiques que dans les sites bien oxygénés. Fait à noter, la taille des testicules gauche et droit est deux fois plus grande dans les milieux normoxiques que dans les milieux hypoxiques. Bien que la longueur du flagelle des spermatozoïdes varie peu, leurs têtes sont plus longues dans les marécages que dans les cours d'eau et rivières, ce qui leur confère une forme plus hydrodynamique. Ces variations du HR peuvent être bénéfiques dans les eaux plus stagnantes des marécages comparativement aux autres milieux. D'autres études sont nécessaires pour comprendre si les variations de la morphologie des spermatozoïdes sont corrélées à la performance natatoire de ces derniers et la capacité de reproduction des mâles. [Traduit par la Rédaction]

Mots-clés : *Barbus neumayeri*, capacité de reproduction, morphométrie des spermatozoïdes, rapport hydrodynamique, facteurs de stress anthropiques.

Introduction

The widespread African cyprinid *Barbus neumayeri* Fisher, 1884 (Neumayer's barb) inhabits a diverse array of environment types such as swamps, streams, and open rivers. Very few studies have examined the reproductive behaviour and capacity of *B. neumayeri*, but it has been reported that *B. neumayeri* mature in synchrony, reproduce throughout the year, while displaying bimodal peaks in reproduction that correlates with the rainy seasons (Chapman et al. 2000). Although it is not clear whether postcopulatory sexual selection in the form of sperm competition occurs in this species, it does seem likely given that the species displays group-spawning behaviour that produce clouds of sperm where the ova of a female is exposed to the ejaculate of more than one male (Chapman and Frank 2000). Similar behaviour occurs in lake whitefish (*Coregonus clupeaformis* (Mitchill, 1818)), which engage in sperm competition (Burness et al. 2008).

Sperm production can be costly, especially under sperm competition (Olsson et al. 1997; Wedell et al. 2002; Rubolini et al. 2007; Hayward and Gillooly 2011). Over the last 15 years, several studies have demonstrated that low levels of dissolved oxygen (DO), or hypoxia, can greatly impair fitness in fish (Wu 2002; Wu et al. 2003; Cheek et al. 2009; Stierhoff et al. 2009; Schulte 2014). Under hypoxic conditions, fish normally display lower growth rates and reduced metabolism (Chabot and Dutil 1999; Zhou et al. 2000; Kolding et al. 2008; Stierhoff et al. 2009; Martínez et al. 2011). In addition, various studies have shown a decrease in energy allocation toward the display of secondary sexual traits and gonad development, resulting in a reduced gonadosomatic index (Chabot and Dutil 1999; Wu et al. 2003; Fitzpatrick et al. 2009; Thomas and Rahman 2009). Furthermore, in many fish species, low DO levels result in changes to energy allocation, leading to endocrine dis-

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Table 1. Classification of the sampling sites from which male Neumayer's barbs (*Barbus neumayeri*) were collected.

Sampling site	Site ID	Environment type	O$_2$ availability	Annual mean DO level (mg O$_2$·L^{-1})	Reference
Rwembaita Swamp	RWS	In-forest swamp	Hypoxic	1.35	Martínez et al. 2011
Njuguta Swamp	NJS	In-forest swamp	Hypoxic	4.06	Chapman and Frank 2000
Inlet Stream East	ISE	In-forest stream	Normoxic	5.15	Langerhans et al. 2007
Inlet Stream West	ISW	In-forest stream	Normoxic	5.58	Martínez et al. 2011
Mikana Stream	Mik	In-forest stream	Normoxic	6.74	Langerhans et al. 2007
Njuguta River	NJR	In-forest river	Normoxic	6.81	Chapman et al. 1999
Middle of the Dura River	Dmid	In-forest river	Normoxic	7.32	Langerhans et al. 2007

Note: Seven divergent environments were sampled for this study (streams, swamps, or rivers). These sites also displayed variation in dissolved oxygen (DO) availability (hypoxic or normoxic).

ruption and the impairment of male reproductive processes (Wu et al. 2003; Landry et al. 2007; Thomas et al. 2007; Fitzpatrick et al. 2009; Martinovic et al. 2009). These effects include changes in the concentration and ratios of sex steroid hormones (Landry et al. 2007; Friesen et al. 2012), and disruption in gonad formation, fertilization, and spawning behaviour (Wu et al. 2003; Thomas et al. 2007; Kolding et al. 2008; Thomas and Rahman 2012).

In this study, we examine variation in traits presumed to be related to the reproductive success of *B. neumayeri* across seven different sites, characterized by divergent DO levels (Greenwood 1962; Chapman et al. 1999; Harniman et al. 2013). If trade-offs exist relative to somatic growth and development of reproductive tissue (gonads) under continuous hypoxic conditions, we predict that fish living in hypoxic sites should exhibit lower measures of fitness (condition factor, gonad size), as observed in *C. clupeaformis* (Burness et al. 2008), the Nile tilapia (*Oreochromis niloticus* (L., 1758)) (Kolding et al. 2008), and the Gulf killifish (*Fundulus grandis* Baird and Girard, 1853) (Cheek 2011) among others. Furthermore, we also predict that gonad development will be dependent on body condition, where individuals in poor condition should have smaller gonads (Burness et al. 2008).

In various animal taxa (birds, fish, mammals, and reptiles), there is an increased tendency for males to display directional asymmetry relative to testes size (Møller 1994; Yu 1998; Burness et al. 2008; Liu et al. 2012). Møller (1994) outlined three different types of asymmetry (directional asymmetry, antisymmetry, and fluctuating asymmetry), depending on how the mean of the values of the left-minus-right traits deviate from zero. The most prevalent pattern is a left testis being bigger and heavier than the right one, although the opposite is also possible (Yu 1998). The testes asymmetry theory predicts that in mature individuals, male body condition should be positively related to testis asymmetry (Møller 1994; Burness et al. 2008; Liu et al. 2012; but see Birkhead et al. (1998)). Alternatively, stress has been associated with testis asymmetry in birds and fish (Birkhead et al. 1998; Allenbach 2011), and so, if hypoxia acts as a strong directional force causing an overall reduction on the physiological condition of *B. neumayeri*, then testis asymmetry should be present.

There is substantial variation in reproductive investment under stress exposure among fish. In some fish, the direct effect of stress in fish physiological condition seems to play an important role in gonad development, sperm production, and sperm swimming speed (Schreck et al. 2001; Landry et al. 2007; Burness et al. 2008; Thomas and Rahman 2009; but see Fitzpatrick et al. 2009). Males are required to invest more energy into spermatogenesis under sperm competition to maximize fitness (Parker 1970, 1998), and this cost should be larger under hypoxic conditions as shown in the intertidal fish, the plainfin midshipman (*Porichthys notatus* Girard, 1854) (Fitzpatrick et al. 2009). Some models suggest that trade-offs between sperm size and number is important under sperm competition conditions (Parker 1998, Immler et al. 2011). Nevertheless, there is no consensus about whether a positive relationship exists between the level of sperm competition and the size of sperm (Gage 1994; Snook 2005; Parker et al. 2010; Parker

and Pizzari 2010). If there is variation in energy allocation for reproduction across sites and sperm competition is present in this species, then we predict that there will also be variation in sperm traits such as size across sites.

Materials and methods

Fish species and study system

Barbus neumayeri is a cyprinid that is widely distributed and abundant in the mosaic of hypoxic swamps, normoxic streams, and rivers in east and central Africa (Chapman et al. 2000). We sampled fish at seven different locations across Kibale National Park, western Uganda (00°13′N–00°41′N and 30°19′E–30°32′E; Table 1; for details see Harniman et al. 2013), between June and July 2011. Sampling sites are characterized by equatorial moist forest but also include an assortment of wetland, grassland, pine plantation, thicket, swamp, and colonizing forest (Schaack and Chapman 2003). Papyrus plant (*Cyperus papyrus* L.) dominates the region, which reaches up to 5 m in height and forms a closed canopy around the swamps (Greenwood 2010). Minimal exposure to light, low levels of water mixing, and high rates of organic decomposition produce extremely hypoxic conditions in the swamps (Chapman et al. 1999). Water current is extremely slow in the swamp and sometimes is undetectable during the dry season, compared with the numerous streams that drain into each swamp (Langerhans et al. 2007). In contrast, inlet streams and surrounding rivers are characterized by flowing water and display normoxic conditions (Schaack and Chapman 2003; Langerhans et al. 2007).

Fish sampling

Seventy-five males were collected with standard minnow traps. Traps, baited with bread, were placed at each sampling site in water (approximately 20–30 cm in depth). The traps were left over night and collected the following morning. Fish were bagged and transported to the field station in Kibale National Park and placed into aquaria with aerators for 24 h. One day after capture, individuals were euthanized with clove oil (1:4 ratio of clove oil to ethanol). Each individual was measured for total length (L_T; ±1 mm), standard length (L_S; ±1 mm), total mass (M_T; ±0.1 g), and somatic mass (M_S; ±0.1 g). Gonads were carefully dissected and removed; the mass of the left testis (Test$_L$; ±0.001 g) and the mass of the right testis (Test$_R$; ±0.001 g) were taken separately. Fulton's condition factor (K), gonadosomatic index (GSI), and testis asymmetry (Test$_A$) were calculated as follows:

$$K = \left(M_S/L_S{}^3 \right) \times 100$$

where M_S represents somatic mass (g) of the fish and L_S represents standard length (cm) of the fish;

$$GSI = (M_G/M_S) \times 100$$

Table 2. General morphological traits of Neumayer's barbs (*Barbus neumayeri*).

Morphological trait	Site						
	Rwembaita Swamp ($N = 16$)	Njuguta Swamp ($N = 10$)	Inlet Stream East ($N = 12$)	Inlet Stream West ($N = 13$)	Mikana Stream ($N = 10$)	Njuguta River ($N = 10$)	Middle of the Dura River ($N = 4$)
L_T (cm)	7.1±0.6	7.5±0.7	7.1±0.9	7.7±0.8	7.5±0.6	7.8±0.9	7.9±0.5
L_S (cm)	5.7±0.6	5.9±0.7	5.7±0.8	6.1±0.7	5.9±0.7	6.2±0.7	6.2±0.4
M_S (g)	3.4±0.9	3.5±1.0	3.9±1.6	4.2±1.3	3.8±1.2	4.0±1.3	4.7±1.0
K	1.8±0.2	1.6±0.1	2.0±0.2	1.7±0.1	1.8±0.1	1.6±0.2	1.9±0.1
GSI (%)	1.3±0.5	0.9±0.6	1.9±0.6	2.7±0.9	3.2±1.7	1.4±0.7	2.1±0.8
$Test_L$ (g)	0.022±0.013	0.018±0.013	0.042±0.023	0.054±0.024	0.075±0.036	0.042±0.022	0.045±0.025
$Test_R$ (g)	0.025±0.017	0.017±0.012	0.035±0.020	0.062±0.025	0.083±0.048	0.042±0.019	0.054±0.021
$Test_A$	0.008±0.014	0.003±0.004	0.009±0.014	0.010±0.009	0.021±0.017	0.005±0.005	0.009±0.007
L_H (μm)	3.18±0.33	2.88±0.19	2.85±0.32	2.90±0.16	3.25±0.32	2.86±0.20	3.18±0.25
W_H (μm)	2.76±0.31	2.61±0.15	2.50±0.32	2.57±0.15	3.08±0.34	2.61±0.15	2.94±0.11
L_F (μm)	34.74±5.74	33.46±4.22	34.86±2.98	31.52±3.78	37.73±10.96	33.61±3.16	38.07±5.39

Note: Values are given as means ± SD and N represents the number of individuals sampled at each site. Fish were collected from seven sites characterized by divergent environments. L_T, total length of the fish; L_S, standard length of the fish; M_S, somatic mass of the fish; K, Fulton condition factor calculated as $(M_S/L_S^3) \times 100$; GSI, gonadal somatic index calculated as $(M_G/M_S) \times 100$, where M_G is total mass of the gonads; $Test_L$, left testis mass; $Test_R$, right testis mass; $Test_A$, testis asymmetry calculated as $Test_R$–$Test_L$; L_H, sperm head length; W_H, sperm head width; L_F, flagellum length.

where M_G represents total mass of the gonads (g) and M_S represents somatic mass (g) of the fish;

$$Test_A = Test_R - Test_L$$

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

Sperm morphometry

Immediately after euthanasia, the abdomen of the fish was patted dry. Using a micropipette, we extracted 1 μL of sperm by gently pressing the abdomen of the fish. Each sperm sample was diluted into 200 μL of 30% formaldehyde solution (Fisher Scientific, Nepan, Ontario, Canada). Immediately after dilution, a subsample was smeared on glass slides, air-dried, and fixed with 30% formaldehyde. The slides were left to dry overnight and then stained the following day by immersion in methanol for 10 min, followed by air-drying, and then immersed in Eosin for 7 min (Bonanno and Schulte-Hostedde 2009). Slides were mounted using Permount (Fischer Scientific, Nepean, Ontario, Canada).

Images of 20 individual sperm per fish were taken using an Infinity 1 camera (Lumenera Scientific, Ottawa, Ontario, Canada) mounted on an Olympus CX41 microscope (Olympus Corporation of the Americas Inc., Center Valley, Pennsylvania, USA). All images were captured at 1000× magnification. All sperm measurements were performed using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Per each individual sperm, three different traits were taken: head length (L_H; μm), head width (W_H; μm), and flagellum length (L_F; μm). Because the midpiece was indistinguishable from the head or tail components using light microscopy, the total L_F included the midpiece; as such, the head and the flagellum were measured separately. Using sperm L_H and W_H, a hydrodynamic ratio (HR) was calculated as L_H/W_H. This ratio provided an estimate of the shape of the sperm head (larger HR values indicate relatively elongated sperm, whereas smaller values indicate relatively round sperm; Malo et al. 2006). Finally, using L_H and L_F, we calculated the ratio of head length to flagellum length (L_H:L_F) as L_H/L_F.

Statistical analyses

We compared the different traits to a normal distribution by plotting histograms for each variable under study. Homogeneity of variance was also confirmed by plotting scatterplots of the residuals. All variables had normal distributions, except for testis asymmetry and sperm traits which were then $\log_{10}(x + 1)$-transformed. Analyses of variance (ANOVAs) or analyses of covariance (ANCOVAs) were performed to assess the degree of variance between each

dependent variable (K, $Test_L$, $Test_R$, M_G, GSI, $Test_A$, HR, and L_H:L_F) and site (environment type). If significant differences were identified, post hoc tests (Tukey's test) were performed to determine where the variation occurred among sites. Principal components (PC) analysis was used to explore which parameters explained the interindividual variation of sperm morphology. We then compared PC1 among sites using a one-way ANOVA and used Tukey's tests to find differences among sites.

Results

Variation across sampling sites

Barbus neumayeri displayed significant differences in general morphological traits (Table 2). We observed variation in L_T among sites ($F_{[6,68]} = 3.78$, $P = 0.003$; Table 3), where the general trend was an increase in size from swamps to streams to rivers. K also significantly differed among sites ($F_{[6,68]} = 10.536$, $P < 0.0001$; Table 3), where fish from the swamps generally displayed lower condition relative to fish from the rivers and streams. As expected, GSI varied significantly among sites ($F_{[6,68]} = 9.25$, $P < 0.0001$; Table 3), where fish from Mikana Stream and Inlet Stream West displayed the greatest GSI relative to fish from the swamps. Furthermore, $Test_A$ was significantly different among sites ($F_{[6,68]} = 2.54$, $P = 0.028$; Table 3), principally due to a difference between fish from Mikana Stream and Njuguta Swamp. In this study, we also observed variation in $Test_L$ ($F_{[1,68]} = 7.98$, $P < 0.0001$) and $Test_R$ ($F_{[6,68]} = 8.94$, $P < 0.0001$) among sites (Table 3).

Principal components analysis explained a high proportion of the individual variation in sperm morphology (Table 4). About 75% of the total variance was explained by the first component. There was significant variation in PC1 among sites ($F_{[6,68]} = 0.763$, $P < 0.0001$; Fig. 1). Specifically, Mikana Stream differed from all other sites as follows: Inlet Stream East ($P = 0.0043$), Inlet Stream West ($P = 0.003$), Njuguta Swamp ($P = 0.021$), and Njuguta River ($P = 0.02$). Interestingly, there was a significant difference in L_H:L_F ($F_{[6,68]} = 4.384$, $P = 0.001$; Fig. 2a), where Inlet Stream East significantly differed from Mikana Stream ($P = 0.011$) and Rwembaita Swamp ($P = 0.004$), and Rwembaita Swamp significantly differed from Njuguta River ($P = 0.043$). Furthermore, HR also showed differences among sampling sites ($F_{[6,68]} = 6.884$, $P < 0.0001$; Fig. 2b). In general, Mikana Stream differed from Rwembaita Swamp ($P < 0.0001$), Inlet Stream East ($P = 0.001$), and Inlet Stream West ($P < 0.0001$).

Variation across DO environments

To examine variation of the traits measured based on oxygen levels, sites were categorized as hypoxic or normoxic based on the

Table 3. Specific statistical differences on the measured morphological traits of Neumayer's barbs (*Barbus neumayeri*) among sampling sites.

Morphological trait and site	Site						
	Rwembaita Swamp (RWS)	Njuguta Swamp (NJS)	Inlet Stream East (ISE)	Inlet Stream West (ISW)	Mikana Stream (Mik)	Njuguta River (NJR)	Middle of the Dura River (Dmid)
L_T (cm)							
ISE	NS	NS	—	NS	NS	$P = 0.010$	NS
K							
NJS	$P = 0.001$	—	NS	NS	NS	NS	NS
ISE	NS	$P < 0.0001$	—	$P = 0.006$	$P = 0.008$	$P < 0.0001$	NS
Dmid	$P < 0.0001$	$P = 0.043$	NS	NS	NS	NS	—
GSI (%)							
ISW	$P = 0.001$	$P < 0.0001$	NS	—	NS	NS	NS
Mik	$P < 0.0001$	$P < 0.0001$	NS	NS	—	NS	NS
$Test_L$ (g)							
ISW	$P = 0.008$	$P = 0.007$	NS	—	NS	NS	NS
Mik	$P < 0.0001$	$P < 0.0001$	$P = 0.018$	NS	—	$P = 0.025$	NS
$Test_R$ (g)							
ISW	$P = 0.003$	$P = 0.001$	NS	—	NS	NS	NS
Mik	$P < 0.0001$	$P < 0.0001$	$P = 0.001$	NS	—	$P = 0.009$	NS
$Test_A$							
Mik	NS	$P = 0.008$	NS	NS	—	NS	NS

Note: L_T, total length of the fish; K, Fulton condition factor calculated as $(M_S/L_S^3) \times 100$, where M_S is somatic mass of the fish and L_S is standard length of the fish; GSI, gonadal somatic index calculated as $(M_G/M_S) \times 100$, where M_G is total mass of the gonads; $Test_L$, left testis mass; $Test_R$, right testis mass; $Test_A$, testis asymmetry calculated as $Test_R–Test_L$; NS, not significant.

Table 4. Results of principal components (PC) analysis for sperm size of Neumayer's barbs (*Barbus neumayeri*) and sampling sites.

Sperm trait	PC1
Head length (μm)	0.942
Head width (μm)	0.935
Flagellum length (μm)	0.698
Eigenvalue	2.251
Proportion of total variance	0.750

Note: Factor loadings are shown for the retained component (PC1).

Fig. 1. Principal component scores by population of Neumayer's barbs (*Barbus neumayeri*). RSW, Rwembaita Swamp; NJS, Njuguta Swamp; ISE, Inlet Stream East; ISW, Inlet Stream West; Mik, Mikana Stream; NJR, Njuguta River; Dmid, middle of the Dura River. Values are means ± SE.
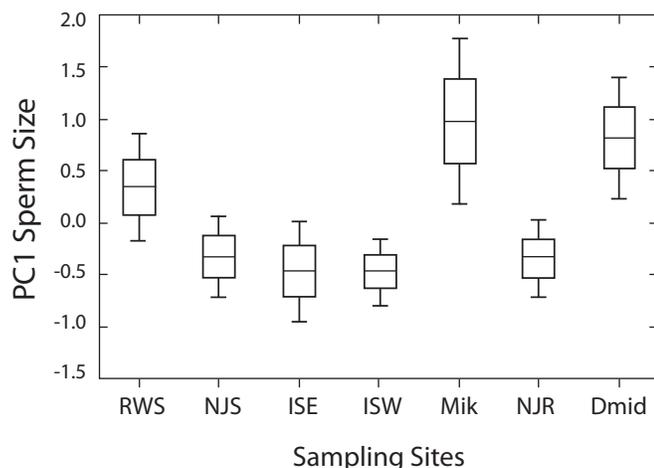
long-term DO levels (Table 1). We found significant variation in L_T ($F_{[1,73]} = 6.504$, $P = 0.013$) and M_T ($F_{[1,73]} = 8.193$, $P = 0.005$), where fish from hypoxic sites were smaller in length than individuals from normoxic sites. As expected, GSI varied significantly between DO levels ($F_{[1,73]} = 27.02$, $P < 0.0001$), where fish from hypoxic sites displayed lower GSI than fish from normoxic sites. Overall, testes

mass of fish from normoxic sites was approximately twice the value of the testes mass of fish from hypoxic sites, where $Test_L$ ($F_{[1,73]} = 28.49$, $P < 0.001$) and $Test_R$ ($F_{[1,73]} = 23.21$, $P < 0.001$) were significantly different between DO levels. Finally, we only observed a significant difference in HR between individuals from hypoxic sites and from normoxic sites ($F_{[1,73]} = 4.79$, $P = 0.0032$), where sperm from individuals living in hypoxic waters were more elongated (had larger HR values) than sperm from fish in normoxic waters.

Discussion

Various studies have shown that at these sampling sites, pH, conductivity, water temperature, water transparency, and DO are lower in the swamp sites than in the nearby river and stream sites (Schaack and Chapman 2004; Langerhans et al. 2007). Variation in these environmental factors has the potential of changing different aspects of fish physiology, and in particular, traits related to fitness and reproduction as the ones studied here. Past studies show a great deal of variation in *B. neumayeri* at the level of gill morphometry, where swamp fish possess larger gill filaments than fish in normoxic environments (Chapman and Liem 1995), which is related to their ability to withstand low DO levels. A significant difference in dietary regime has also been observed in *B. neumayeri*, where fish from the swamps seem to feed more on seed pods and hard insect exoskeletons compared with fish in the streams and rivers that seem feed more on plant matter and soft-body material (Schaack and Chapman 2004). Recently, it was found that forest stream fish consume a diet rich in mayflies and caddisflies, which are rich in energy content, whereas stream fish consume a diet rich in chironomids and oligochaetes, which are poor in energy content (V. Fugère, personal communication). Because of this difference in the dietary regime among the sampling sites, it is not surprising to find that key foraging muscles near the branchial region are smaller in swamp fish with large gills, which could cause trade-offs in efficiency of food uptake (Schaack and Chapman 2003, 2004). These studies led us to suggest that differences in nutritional intake ultimately could influence fitness-related traits, such as condition, and gonad development as observed in other fish.

Overall, fish seem to display similar levels of variation in the morphological traits measured based on site type (swamp, stream, or river) and on DO levels. Specifically, fish living in hypoxic

Fig. 2. Variation in sperm morphometry of Neumayer's barbs (*Barbus neumayeri*) across sampling sites: (*a*) head length to flagellum length ratio (L_H:L_F) and (*b*) hydrodynamic ratio (HR). RSW, Rwembaita Swamp; NJS, Njuguta Swamp; ISE, Inlet Stream East; ISW, Inlet Stream West; Mik, Mikana Stream; NJR, Njuguta River; Dmid, middle of the Dura River. Values are means ± SE.
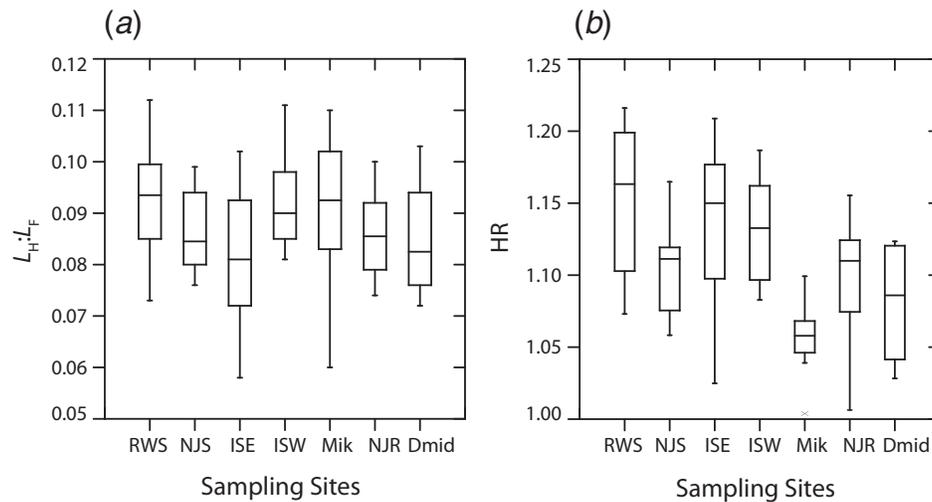
swamps were shorter in length and lighter in mass compared with fish living in normoxic streams and rivers. Variations in general morphometry such as fish length, fish mass, K, M_G, and GSI are likely due to changes in energy allocation under varying conditions (Wendelaar Bonga 1997; Iwama 1998; Schreck et al. 2001; Wingfield and Sapolsky 2003). If hypoxic swamps are perceived to be of less quality or stressful by *B. neumayeri*, then our findings are in agreement with those found in other studies. Chabot and Dutil (1999) demonstrated that reduced growth in length and mass, as well as decreased physical condition, occurred in Atlantic cod (*Gadus morhua* L., 1758) exposed to nonlethal hypoxic conditions. In a reciprocal transplant acclimatization study using *B. neumayeri*, we found that independently of the site of origin (swamp or stream), exposure to hypoxia induced a reduction in growth of *B. neumayeri* (Martínez et al. 2011), supporting the idea that hypoxia is a stressor and with great potential to cause detrimental effects on the biology of this species.

There are two proposed patterns of energy allocation under environmental stress that could result in condition and reproductive trade-offs: (1) maintenance of body mass and adjustment in reproductive tissue production or (2) maintenance of productive tissue at the expense of somatic tissues (Schreck et al. 2001; Wu et al. 2003). Hypoxia is an endocrine disruptor, where chronic exposure leads to reduced growth, decreased overall physical condition, reduced gonad size and mass, decreased GSI, and the appearance of testis asymmetry (Chabot and Dutil 1999; Schreck et al. 2001; Landry et al. 2007; Fitzpatrick et al. 2009). As expected, we found that GSI was lower in individuals from hypoxic swamps than from normoxic streams and rivers, likely, because of reduced energy investment into reproduction under less favourable conditions (Stockley et al. 1997; Landry et al. 2007; Burness et al. 2008). Even though the overall trend was an increase in traits from swamps to streams to rivers, K and GSI were determined to be highest in stream environments, contrary to our predictions. There are a few possible explanations for this observation. The first is that stream systems undergo a larger variation in DO compared with swamps and rivers (Chapman and Frank 2000; Martínez et al. 2011). During the rainy season, water levels become so high that swamp water overflows into the streams (Chapman and Liem 1995), which potentially allow the flow of other food sources into the streams. Despite the increase water levels during the rainy seasons, the rate of migration between sampling sites is very low (Harniman et al. 2013; C. Baltazar, personal communication). Another possible explanation is that river fish may face

greater predation risk than swamp or stream fish. Many natural predators are hypoxia sensitive and will not venture past some streams; hence, one can suggest that river fish are forced to hide more often, reducing their foraging time, which in turn may decrease their physiological fitness (Reardon and Chapman 2009).

If trade-offs exist between somatic growth and reproduction for this species, a reduction in the physiological condition of swamp fish should be accompanied by reduced development of reproductive tissue (gonad mass), as found in other fish (Wu et al. 2003; Landry et al. 2007; Reardon and Chapman 2009). In the dwarf Victoria mouthbrooder (*Pseudocrenilabrus multicolor victoriae* Seegers, 1990), an African cichlid that inhabits environments with divergent oxygen regimes as does *B. neumayeri*, hypoxia has significant effects on female reproductive potential (Friesen et al. 2012). During the reproductive season, the swamp environment has a significantly lower number of brooding female *P. m. victoriae* than normoxic environments; this reduction may be due to an increase in testosterone levels and testosterone to estradiol ratio, which will affect gonad maturation (Friesen et al. 2012). Similarly, we found that male GSI was reduced in fish from hypoxic sites compared with fish from normoxic sites. This finding can also be supported by the fact that hypoxia can have direct effects on the endocrine system and, subsequently, indirect effects on reproductive capacity through the hypothalamic–pituitary–gonadal axis. Gonad development and gametogenesis are under the control of the brain via the hypothalamic–pituitary–gonadal axis (Wendelaar and Bonga 1997; Iwama 1998; Thomas et al. 2007; Reardon and Chapman 2009). This axis stimulates the secretion of two forms of gonadotropin-releasing hormones (GTH-I and GTH-II) from the pituitary gland. GTH-I controls gonad development and the early stages of gametogenesis, while GTH-II controls the final gamete maturation stages (Wendelaar Bonga 1997; Iwama 1998; Wu et al. 2003). All these studies provide arguments to support the presence of trade-offs in the reproductive biology of *B. neumayeri* across the sampling sites in these regions.

Sperm competition

Sperm competition is widespread in fish and occurs when ejaculates produced by two or more males compete to fertilize the ova produced by a female (Fuller 1998; Anderson et al. 2004; Bonanno and Schulte-Hostedde 2009). *Barbus neumayeri* tends to display a batch-spawning behaviour, producing clouds of sperm with little or no involvement of mate courtship (Chapman and Frank 2000; Greenwood 2010). Sperm competition has a powerful influence on

selection and is responsible for variations in male behaviour, anatomy, and physiology (Wedell et al. 2002; Anderson et al. 2004; Rubolini et al. 2007). In the presence of sperm competition, theoretical models predict that individual males should increase investment into reproduction through ejaculates (Kime et al. 2001; Rurangwa 2004; Burness et al. 2008; Richards et al. 2009). Ejaculate quality ultimately determines reproductive success and can be measured using a number of traits including testis size, GSI, sperm density, flagellum length, sperm motility, sperm velocity, and adenosine triphosphate stores (Olsson et al. 1997; Stockley et al. 1997; Kime et al. 2001; Bonanno and Schulte-Hostedde 2009; Gasparini et al. 2010). Therefore, a male's reproductive success greatly depends on the energy invested into its reproductive structures, as well as the capacity of its sperm to reach and fertilize an ovum first (Stockley et al. 1997; Fuller 1998; Bonanno and Schulte-Hostedde 2009). As a result, spermatozoa can be subjected to strong selective pressures (Agrawal 2001; Gasparini et al. 2010). This is particularly true of natural populations, where there are significant differences in fertility among males (Malo et al. 2006).

The use of GSI, testis size, and testis asymmetry to measure the degree of sperm competition in a species, as well as overall ejaculate quality, is now ubiquitous (Anderson et al. 2004; Burness et al. 2008; Bonanno and Schulte-Hostedde 2009). Therefore, reproductive capacity and potential sperm competition can be explored by examining relative testis size (Burness et al. 2008). In this study, ejaculate investment was dependent on body condition, where individuals with greater K displayed increased M_G. The increased M_G and GSI in males from normoxic stream and river environments suggests more intense sperm competition than in hypoxic or swamp environments (Stockley et al. 1997; Kleven et al. 2008). Burness et al. (2008) found that *C. clupeaformis* displayed testis asymmetry, a phenomenon not widely reported in fish, yet widespread in other taxa (Anderson et al. 2004; Møller 1994; Kleven et al. 2008; Blukacz et al. 2010).

Sperm morphometry

Hypoxia may specifically impact sperm performance and sperm velocity because sperm have been found to be particularly susceptible to low DO levels (Urbach et al. 2007; Fitzpatrick et al. 2009; Richards et al. 2009). Spermatozoa of fish consist of a head, which contains the male haploid genome, and a flagellum, which is responsible for sperm motility (Morrow and Gage 2001; Gage et al. 2004; Malo et al. 2006). The flagellum consists of two components: (1) the midpiece, where the mitochondria are stored, and (2) the rest of the flagellum, which uses beating movements to propel the spermatozoan forward (Malo et al. 2006). Flagella of sperm are powered by energy in the form of adenosine triphosphate, which is generated in the sperm midpiece through oxidative phosphorylation (Kime et al. 2001). This process relies heavily on the presence of oxygen for cellular pathways (Landry et al. 2007). Because fertilization success is related to sperm performance, hypoxia may induce problems in sperm function, resulting in decreased reproductive capacity (Landry et al. 2007; Fitzpatrick et al. 2009).

Large variation in sperm morphometry has been observed in different taxa, both between and within species (Gage et al. 1998, 2002, 2004; Malo et al. 2006; Gasparini et al. 2010). In fish in particular, there is large variation in sperm size (Gasparini et al. 2010). Sperm sizes are documented to range from 13 µm in the flathead grey mullet (*Mugil cephalus* L., 1758) to nearly 100 µm in the channel catfish (*Ictalurus punctatus* (Rafinesque, 1818)) (Thünken et al. 2007). In some taxa, it has also been demonstrated that longer sperm are produced in the presence of sperm competition (Stoltz and Neff 2006). Increased L_F has been associated with increased testis size (Burness et al. 2008). Males with relatively larger testes are expected to produce spermatozoa with longer flagella (Stockley et al. 1997; Bonanno and Schulte-Hostedde 2009). We did not find any significant variation of sperm L_F among sampling sites. Nev-

ertheless, in other species, the shape of the sperm head is also a relatively important trait in regards to sperm hydrodynamics and thus velocity (Morrow and Gage 2001; Urbach et al. 2007; Humphries et al. 2008; Simpson et al. 2014). In *B. neumayeri*, we did observe variation in $L_H:L_F$ and $L_H:W_H$ across sites. Evidence in various species suggest that sperm with elongated heads (i.e., greater $L_H:W_H$ values) swim faster due to their increased hydrodynamic efficiency and streamlined shape (Humphries et al. 2008; Simpson et al. 2014). On the other hand, Malo et al. (2006) found that spermatozoa with elongated heads swam faster, as well as those that possessed longer relative lengths of the flagellum. This is because a linear relationship has been identified between L_F and sperm velocity (Kime et al. 2001; Kleven et al. 2008; Gasparini et al. 2010).

The variation in sperm traits of *B. neumayeri*, such as the hydrodynamic shape of the head and the propulsion generated by the relative size of the flagellum, suggest that there is a link between the degree of variation of these traits and sperm velocity. It is not immediately clear what the apparent advantage of an increased HR is in the hypoxic Rwembaita Swamp and Njuguta Swamp sites, where sperm W_H decreased to provide an increased streamlined shape. We can suggest that this change in shape is advantageous in environments where water flow is very low, as in the swamp sites (Langerhans et al. 2007), and perhaps where water viscosity is higher due to the presence of dense vegetation found at this type of sites (Kobashi and Mazda 2005). In some organisms, the viscosity of the medium where they need to swim has a great impact on their swimming performance (Kupriyanova and Havenhand 2005; Kirkman-Brown and Smith 2011; Sohn et al. 2013). If hypoxic environments are more viscous than normoxic sites, sperm would need to generate more power to swim through this medium. The speed that a sperm can generate will hence depend on the proper balance between the thrust created by the longer flagellum producing higher beat frequencies and the drag force generated by the head shape as the sperm moves through the medium (Humphries et al. 2008; Simpson et al. 2014). Future studies in this species should include analysis of sperm swimming performance to elucidate whether variation in sperm morphometry across sampling sites can be related to variation in swimming performance. Results from this study contribute to the increase evidence suggesting that hypoxia exposure significantly impairs male reproduction (Wu et al. 2003; Thomas et al. 2007; Landry et al. 2007; Fitzpatrick et al. 2009; Thomas and Rahman 2009, 2010). With the rapid increase in the frequency and extension of hypoxia across freshwater and marine environments (Diaz and Rosenberg 1995; Keeling et al. 2010; Pörtner 2010), more studies involving species that do reproduce in hypoxic water are required to establish trends that can be extrapolated to other species in aquatic systems susceptible to becoming hypoxic.

Acknowledgements

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