



Sperm size is negatively related to relative testis size in West African riverine cichlid fishes

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Abstract

Fishes show a great diversity of mating systems and fertilization mechanisms. This diversity creates an enormous potential for sperm competition. Typically, monogamous species face a low risk of sperm competition and invest less into sperm, and thus show smaller relative testis mass compared to polygamous species with high sperm competition. In cichlids, sperm competition risk is very variable. In lacustrine East African cichlids, large sperm are interpreted as an adaptation to sperm competition, as in those species sperm length correlates with sperm swimming speed. The aim of the present study was to examine variation in sperm and testis traits of substrate breeding cichlids from West African river systems and its relationship to sperm competition. Therefore, sperm traits (total sperm size, flagellum-, midpiece-, and head size) and sperm number were related to the gonadosomatic index (GSI), an indicator of sperm competition, in eight species of two large informal tribes, the chromidotilapiines and the haplotilapiines. We found significant differences between species in all examined sperm traits, sperm number, and GSI with pronounced differences between chromidotilapiines and haplotilapiines. We used a generalized least-squares approach to control for non-independence of data. GSI was positively correlated with sperm number but negatively correlated with total sperm size (also negatively with the flagellum and head size but not significantly with midpiece size). Sperm number and sperm size were negatively correlated suggesting a trade-off between sperm size and quality. Our results suggest that large sperm can evolve in species with relatively low sperm expenditure and probably in absence of high sperm competition between males.

Keywords *Pelvicachromis taeniatus* · West African cichlids · Sperm competition · Mating system · Sexual selection · Gonadosomatic index

Introduction

Sperm competition is defined as the competition between sperm of different males in fertilizing a female's eggs (Parker 1970). Anisogamy creates an enormous potential,

e.g., for the evolution of sperm variation through different selection pressures on males and females. Thus, distinct reproductive strategies can evolve (Parker et al. 1972). There is usually a trade-off between gamete number and gamete size: smaller gametes can be produced in larger numbers and larger zygotes can be produced with more resources, and thus have a higher chance of survival (Parker et al. 1972; reviewed in Kokko et al. 2006). Therefore, males usually produce small and many sperm to compete for large but few female eggs. This leads to competition between males to fertilize a female's eggs, e.g., multiple mating in internal fertilizers (Jennions and Petrie 2000), simultaneous sperm release in external fertilizers (Byrne et al. 2003), and evolution of male or sperm traits that increase a male's fertilization success (Simmons 2001; Kokko et al. 2006).

Sperm competition theory predicts an increase in sperm production, i.e., sperm number, with increasing sperm competition. Accordingly, males of polygamous species should have larger testes relative to their body mass, as they are exposed to

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a higher sperm competition risk than males of monogamous species (Parker 1982, 1990a, b, 1993; Parker and Begon 1993). These theoretical predictions were supported by numerous empirical studies across a range of taxa (reviewed in Gomendio and Roldan 2008). Relative testis size should be positively correlated with sperm demand according to theoretical models, and in many taxa, relative testis size increases with the level of sperm competition (Parker 2016). Accordingly, relative testis mass, e.g., gonadosomatic index (GSI) is ubiquitously used as proxy for sperm competition risk (e.g., Møller and Briskie 1995; Balshine et al. 2001; Anderson et al. 2004; Burness et al. 2008; Bonanno and Schulte-Hostedde 2009; Martinez et al. 2015).

While the relationship between sperm competition and sperm number is well understood, the relationship between sperm competition and sperm size (equals length) remains controversial. Sperm size and morphology vary extremely between taxa (Pitnick et al. 1995; Pitnick et al. 2009) as well as within species (e.g., Fitzpatrick et al. 2010). Two theoretical models try to explain this variation in sperm size, the instantaneous fertilization model for internal fertilizing species (sperm size is not affected by sperm competition (Parker 1993)), and the continuous fertilization model for external fertilizing species according to which sperm size increases with sperm competition intensity (Ball and Parker 1996). Furthermore, sperm swimming speed increases with sperm size at a cost to sperm longevity (Ball and Parker 1996). In external fertilizers, sperm competition is higher than in internal fertilizers as males release sperm simultaneously to fertilize a limited number of eggs (Ball and Parker 1996). Thus, males should increase their sperm number and sperm swimming speed to enhance their fertilization success. Empirical data are often in accordance with the theoretical predictions but not always. Contrary to the predictions of the instantaneous fertilization model, in mammals, sperm size is longer in polygamous species than in monogamous species with longer sperm swimming faster than shorter sperm (Gomendio and Roldan 1991; Gomendio and Roldan 2008). In passerine birds, in one family (Fringillidae) (Immler and Birkhead 2007), sperm midpiece length and flagellum length were positively related to relative testis mass, and thus sperm competition risk, while in another family (Sylviidae) sperm traits were negatively related to relative testis mass. In fishes, Stockley et al. (1997) found that sperm length was negatively related to sperm competition intensity as opposed to predictions of the continuous fertilization model (but see Montgomerie and Fitzpatrick 2009).

Moreover, to maximize the number of sperm, males often produce smaller sperm, and thus have more sperm available to compete successfully for fertilizations (Parker 1982). However, in some species, males produce giant sperm although at a cost to sperm number (Pitnick et al. 1995; Pitnick 1996). This trade-off between sperm number and

sperm size was shown, e.g., in chipmunks (Schulte-Hostedde and Millar 2004) and in passerines (Immler et al. 2011).

Especially in fishes, sperm competition is widespread due to the high number of external fertilizing species and the high diversity of mating systems and spawning patterns (Petersen and Warner 1998; Taborsky 2008). In external fertilizers, it is more difficult for males to monopolize access to partners and, thus, sperm of different males often compete over fertilizing the eggs of a given female (Taborsky 2008). Accordingly, different male mating tactics (e.g., sneaking) can evolve that can influence sperm traits (e.g., motility, morphology), testis, and ejaculate size (e.g., Parker 1998; LaMunyon and Ward 1999; Gage and Morrow 2003; Gage et al. 2004; Snook 2005). In fishes, sperm size ranges from 13 µm in the flathead mullet *Mugil cephalus* to 97 µm in the channel catfish *Ictalurus punctatus* (Stockley et al. 1997). However, not only sperm competition risk can have an impact on sperm and testis traits in external fertilizers but also environmental factors such as different habitats, spawning behaviors, or spawning site characteristics.

Testis and sperm traits of East African cichlids have been investigated in several studies. Balshine et al. (2001) examined closely related Lake Tanganyika cichlids and found that sperm length is significantly longer in polygamous species than in monogamous species and is related to relative testis mass. Furthermore, sperm length varied between the place of fertilization, with substrate fertilizing cichlids having longer sperm than buccal fertilizing cichlids (Balshine et al. 2001). Fitzpatrick et al. (2009) found phylogenetically controlled evidence that polygamous species have a higher sperm swimming speed due to higher sperm competition than closely related monogamous species. Within Lake Tanganyika cichlids, fast and large sperm evolved from slow and small sperm in response to increasing sperm competition (Fitzpatrick et al. 2009). Furthermore, Fitzpatrick et al. (2009) showed that sperm length was related to sperm swimming speed. As these studies show, sperm competition influences the enormous variation of sperm size in cichlids that ranges from 15.5 µm in the monogamous *Asprotilapia leptura* to 33.3 µm in the polygamous *Telmatochromis vittatus* (Balshine et al. 2001). However, in the monogamous West African cichlid *Pelvicachromis taeniatus*, sperm size is more than twice as long reaching a length of up to 69 µm (Thünken et al. 2007; Langen et al. 2017) suggesting a much higher variation of sperm size in cichlids. The function of these long sperm still remains unclear and is in contrast to the continuous fertilization model that predicts longer sperm with increasing sperm competition.

While the East African lacustrine cichlids are well studied, there is less knowledge on testis and sperm traits of West African cichlids and especially of riverine cichlids. Although in East African cichlids, sperm size is positively related to

sperm competition, it is of special interest to study this relationship in West African cichlids as well: is there more than one species with larger-sized sperm or is there overall higher variation in sperm size of West African cichlids? Sperm size variation across fishes has been shown to be negatively correlated with sperm competition (Stockley et al. 1997; but see Balshine et al. 2001; Montgomerie and Fitzpatrick 2009).

The aim of the study was to compare testis and sperm traits of West African riverine cichlids in the context of sperm competition. As detailed information about the mating systems of many of these species is missing, we used relative testis size, i.e., GSI as indicator for the strength of sperm competition. Several studies in cichlids show, that GSI is a good proxy of mating patterns and sperm competition level, not only across species but also within species (Taborsky 1994; Sato et al. 2004; Desjardins et al. 2008; Ota et al. 2010; Schütz et al. 2010). We examined total sperm length as well as flagellum-, head-, and midpiece length of sperm of eight cave-breeding species of chromidotilapiines and haplotilapiines. Within the chromidotilapiines, four species were examined: the socially and genetically monogamous *P. taeniatus* (Langen et al. 2017), the sister species *Pelvicachromis pulcher* with different mating tactics (monogamy, harem, and satellite males) (Martin and Taborsky 1997), *Pelvicachromis humilis* without information on mating type, and the socially monogamous *Nanochromis parilus*, although harems have been observed as well in this species (Lamboj 2004). The riverine haplotilapiine species *Lamprologus werneri*, *Steatocranus casuarius*, and *Steatocranus tinanti* are socially monogamous, although there are some reports of polygyny in *S. tinanti* (according to Lamboj 2004) and polygamy in *S. casuarius* as more than two individuals per breeding cave were observed showing parental care in natural habitats (J. Schwarzer, personal observation). Additionally, the facultative polygynous lacustrine haplotilapiine cichlid *Lamprologus ocellatus* that breeds in snail shells was examined (Walter and Trillmich 1994). The results were compared against those of a socially monogamous

substrate-breeding hemichromine species that served as outgroup as it is a very basal species (see Fig. 1).

Methods

Experimental fish and maintenance

A simplified phylogeny of the nine examined species is given in Fig. 1 that is based on a phylogeny of chromidotilapiines (Schwarzer et al. 2015), giving known information about sperm morphology, breeding system, mating system, and flow velocity of the habitat. Sperm and GSI values of four species of chromidotilapiines were analyzed: *P. taeniatus* (lab-reared F2 generation of wild-caught fish from the Moliwe River in Cameroon, $N=14$); *P. pulcher* (F1 generation and wild-caught fish from Nigeria, $N=13$); *P. humilis* (wild-caught fish from Liberia, $N=9$, Aquarium Glaser, Frankfurt); and *N. parilus* (wild-caught fish from Pool Malebo, Lower Congo River, $N=10$, Aquarium Glaser, Frankfurt). Sperm and GSI values of three riverine haplotilapiines were analyzed: *S. casuarius* (wild-caught fish from Pool Malebo, Lower Congo River, $N=10$, Mimbon Aquarium, Cologne); *S. tinanti* (bred fish, $N=8$, Knauber, Bonn); and *L. werneri* (wild-caught fish from Pool Malebo, $N=11$, Aquarium Glaser, Frankfurt). Additionally, to compare lacustrine and riverine species, sperm and GSI values of *L. ocellatus*, a lacustrine snailshell-breeding haplotilapiine from Lake Tanganyika, were analyzed (bred fish, $N=7$, Aquarium Glaser, Frankfurt). To rule out any influence of the reproduction mode, only cave-breeding cichlids were chosen. A basal hemichromine substrate breeder, *Hemichromis guttatus* (bred fish, $N=10$, Aquarium Glaser Frankfurt) was analyzed as outgroup. Furthermore, by choosing species out of one family, confounding variables can be minimized (Immler and Birkhead 2007; Fitzpatrick et al. 2009). The different species were chosen due to their relatedness, although logistic reasons also played a role, e.g., limited availability and capabilities of purchase.

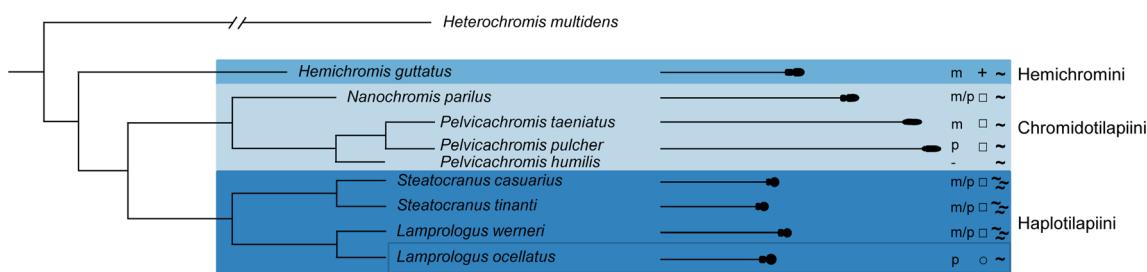


Fig. 1 Simplified phylogeny (Schwarzer et al. 2015) of the nine cichlid species examined in this study (blue = *Hemichromis*; light blue = chromidotilapiini; dark blue = haplotilapiini, blue box = lacustrine haplotilapiini). A scaled illustration of sperm morphology of each species is given. Monogamous species are indicated with *m*, polygamous with *p*, and species with both observations with *m/p*; no information about the

mating system is indicated with a minus sign. Substrate breeders are indicated with plus sign, cave breeders with a white square, and snail shell breeders with a white circle. Flow velocity of habitats is presented by a single tilde for low flow velocity and three tildes for strong flow velocity

The analyzed species occur in different habitats (see Fig. 1 for an overview). *N. parilus* inhabits low-flow littoral zones of the lower Congo River and its branches, and *Pelvicachromis* and *H. guttatus* littoral zones of small rivers and streams in woodland with low flow velocity (Lamboj 2004). In contrast, *Steatocranus* and *L. werneri* are rheophilic in the Lower Congo River, preferring rapids and areas with strong flow velocity.

To ensure reproductive activity, males were confined with a female until they displayed courtship behavior and pair bonding. Tank size measured 50 cm × 30 cm × 30 cm (L × B × H), water level was 24 cm, and water temperature was 25 ± 1 °C. Tanks were equipped with a gully filter, sand, java moss, and a spawning site (stone caves for cave breeders, vineyard snail shells for *L. ocellatus*, and flat stones for the substrate breeder *H. guttatus*). *L. ocellatus* had more sand at its disposal (2 cm layer) because this species digs the shells into the sand.

Following observations of courtship behavior by males, the standard length and body mass of each were measured. The nine species differed significantly in body mass and standard length (body mass: Kruskal-Wallis test, $\chi^2 = 61.481$, df = 8, $p < 0.001$; standard length: Kruskal-Wallis test, $\chi^2 = 41.692$, df = 8, $p < 0.001$).

Testis and sperm traits

Afterward, males were dissected and testes surgically extracted. Each testis was weighed three times with a fine scale (XS205 Dual Range, Mettler Toledo, error ± 0.1 mg). A mean value per testis was calculated to obtain testis mass and to calculate the GSI according to de Vlaming et al. (1982) using the following formula: GSI = (gonad mass [g]/body mass [g]) × 100. Afterward, the left testis was ground in 440 µl distilled water and 12 µl of the resulted sperm suspension was placed in a Neubauer counting chamber (Labor Optik, chamber depth: 0.1 mm, smallest square: 0.0025 mm²) and enumerated. Sperm in 64 small squares of the chamber were counted (≥ 0) under a microscope (Universal Zeiss, × 40 object lens, × 10 eyepiece lens, × 1.25 Optovar) with × 500 magnification. The total estimated number of sperm (= sperm number) per testis (S_T) was calculated using the following equation: $S_T = S_A \times 4000 \times 440$ (S_A is the mean number of sperm counted in the 64 smallest squares, 440 is the dilution factor, 4000 is 1/0.00025 µl (1/volume of one smallest square)). The right testis was stored in 4% formalin until preparation for scanning electron microscopy (SEM). Prior to SEM preparation, the sperm number of this testis was counted as well. Next, sperm were fixed for at least 6 h at 4 °C in 200 mmol⁻¹ cacodylate buffer containing 2.5% glutaraldehyde and 4% sucrose. Afterward, the glutaraldehyde fixation was washed out and samples were dehydrogenated along an ascending ethanol series (40–98%), dried, and evaporated with gold. With the SEM (digitized Leitz-AMR 1000, DISS:

Digital Image Scanning System, Point Electronic, Halle, Germany) microphotographs of up to 20 sperm per male (mean ± SD = 18 ± 4) were taken using the software DIPS (Digital Image Processing System, Version 2.5.2.1, Point Electronic GmbH, Halle, Germany). The total sperm length, the flagellum-, head- and midpiece length, and the head and midpiece width were measured using ImageJ v 1.45. Additionally, the head-to-tail ratio ((head length + midpiece length)/flagellum length) (Humphries et al. 2008) was calculated, and the coefficient of variation of sperm length within males (CV_{wm}) and between males (CV_{bm}) (see Laskemoen et al. 2007; Laskemoen et al. 2013) was calculated and adjusted for sample size (Sokal and Rohlf 1995). Furthermore, the maximum sperm length per individual was noted and a mean was calculated for each species.

Repeatability

Total sperm length of about 10% randomly chosen measured sperm per individual (10% of 20 sperm per individual = two sperm per individual) were measured a second time to check the reliability of the used method. Repeatability, r , was calculated according to Lessells and Boag (1987) with $n_0 = 2$ (because two measurements were compared). The standard error of r was calculated according to Becker (1984) and revealed that the measurements of total sperm length were significantly repeatable ($r = 0.994 \pm \text{SE } 0.0001$, $p < 0.001^{***}$).

Statistics

The statistical software package R v. 2.9.1 was used for statistical analyses (R Development Core Team 2009). Parametric tests were performed when data were normally distributed according to the Shapiro-Wilk test. When not normally distributed, data were transformed or non-parametric tests were applied. P values are two-tailed throughout. Differences among species were analyzed using a Kruskal-Wallis test. Afterward, each species was compared to the other species using a t test for independent samples or a Wilcoxon signed-rank test.

We used a generalized least-squares approach (GLS) (Grafen 1989; Martins and Hansen 1997; Pagel 1999; Freckleton et al. 2002) in a phylogenetic context to control for dependence of species data points in comparative tests that arise from shared ancestry (Harvey and Purvis 1991) using the “caper” package in R (Orme et al. 2013). This approach investigates whether a dependent trait is predicted by one or more independent traits (Nunn 2011). As there is little or lacking knowledge about the mating systems of the analyzed species, and thus a strict classification of species into monogamous or polygamous mating system is not possible, we used relative testis mass, i.e., the GSI as proxy for sperm competition (e.g., Stockley et al. 1997; Neff et al. 2003; Hellmann

et al. 2015). The application of the GSI can be problematic when there is a positive or negative allometric relationship between testis mass and body mass (Tomkins and Simmons 2002); this, however, was not the case in our data set. Across species, the relationship between testis mass and body mass did not significantly differ from isometry (slope = 1.214, SE = 0.430, $t = 2.306$, $P = 0.629$). Therefore, we used sperm length and sperm number as dependent traits and GSI as the explanatory variable. Data were log-transformed for analyses and mean values for each trait for each species were calculated. In order to only analyze and compare West African cichlids, the East African cichlid *L. ocellatus* was excluded for the GLS analysis. A phylogeny of chromidotilapiines based on mitochondrial and nuclear sequences (see Schwarzer et al. 2015) were included in the analysis. *L. werneri* was not in the phylogeny, but the sister species *Variabilichromis moorii* was. Lamprologines are a monophyletic group so we could use *L. werneri* in the tree instead of *V. moorii*. The scaling parameter λ was calculated using likelihood ratio tests (LRTs) to assess the degree of phylogenetic independence of our data (Pagel 1997; Freckleton et al. 2002). Normally, λ varies between 0 and 1; values of $\lambda = 0$ indicate phylogenetic independence of traits while values of $\lambda = 1$ indicate that traits evolved according to Brownian motion (Pagel 1997; Freckleton et al. 2002).

We found no *P. humilis* sperm for measurements with the SEM, probably because of a problem during SEM preparation, so we have no data on sperm morphology for this species.

Results

Variation in testis and sperm morphology

Testis morphology varied between species (Table 1). Compared to haplotilapiines, GSI and testis mass were very low in chromidotilapiines with the highest values in *N. parilus* and the lowest values in *P. humilis*. The riverine haplotilapiines had bigger testes than the chromidotilapiines. *S. casuarinus* had the highest testis mass and GSI while the lacustrine *L. ocellatus* had the lowest testis mass and GSI. Testis mass and GSI of the substrate breeder *H. guttatus* are similar to those of the chromidotilapiines (especially to *N. parilus*).

Sperm morphology varied between the two tribes (Fig. 1, 2, Table 1). The chromidotilapiines had very long sperm with long heads and small midpieces (Fig. 2a–c). *P. pulcher* had with, on average, 59.76 μm the longest sperm followed by *P. taeniatus* and *N. parilus*. The *Pelvicachromis* species had rod-shaped heads (Fig. 2a, b) and were very similar while *N. parilus* had oval heads (Fig. 2c). *H. guttatus* had globular to oval heads and a midpiece of nearly the size of the

head (Fig. 2d). All haplotilapiines had short sperm ranging from 20.12 μm in *L. ocellatus* to 27.15 μm in *L. werneri* with globular heads and midpieces of 0.33–0.52 μm in length (Fig. 2e–h). With regard to sperm, flagellum and head length, sperm morphology of the basal *H. guttatus* can be classified in between the chromidotilapiines and haplotilapiines.

Testis mass and GSI both were significantly different between species (testis mass: Kruskal-Wallis test, $\chi^2 = 44.169$, df = 8, $P < 0.001$; GSI: Kruskal-Wallis test, $\chi^2 = 41.249$, df = 8, $P < 0.001$) with haplotilapiines having heavier testes and higher GSI than chromidotilapiines and *Hemichromis* (Fig. 3a). Sperm number differed significantly between species (Kruskal-Wallis test, $\chi^2 = 68.469$, df = 8, $P < 0.001$) with haplotilapiines having more sperm than chromidotilapiines and *Hemichromis* (Fig. 3b). Species differed significantly in sperm length and flagellum length that were both longer in chromidotilapiines (sperm length: Kruskal-Wallis test, $\chi^2 = 48.968$, df = 7, $P < 0.001$, Fig. 3c; flagellum length: Kruskal-Wallis test, $\chi^2 = 48.652$, df = 7, $P < 0.001$). While sperm heads were longer in chromidotilapiines than in haplotilapiines (Kruskal-Wallis test, $\chi^2 = 47.768$, df = 7, $P < 0.001$), head width was smaller in chromidotilapiines than in haplotilapiines (Kruskal-Wallis test, $\chi^2 = 50.733$, df = 7, $P < 0.001$). Midpiece length (Kruskal-Wallis test, $\chi^2 = 46.787$, df = 7, $P < 0.001$, Table 1) and midpiece width (Kruskal-Wallis test, $\chi^2 = 34.455$, df = 7, $P < 0.001$, Table 1) differed significantly as well with the lowest values in *P. taeniatus* and *P. pulcher*. The head-to-tail ratio was significantly different between species (Kruskal-Wallis test, $\chi^2 = 43.767$, df = 7, $P < 0.001$, Fig. 3, Table 1) with a lower head-to-tail ratio in chromidotilapiines (between 0.056 and 0.065) than in haplotilapiines (between 0.062 and 0.101) and highest ratios in *H. guttatus* and *L. ocellatus*. The adjusted coefficient of variation within males (CV_{wmadj}) differed significantly between species (Kruskal-Wallis test, $\chi^2 = 27.851$, df = 7, $P < 0.001$, Table 1) with variation in *L. ocellatus* being twice as large than in *L. werneri*.

Sperm traits in relation to GSI

The GLS analyses revealed phylogenetic independency of data, except for total sperm length correlated to sperm number and flagellum length correlated to head length (see Table 2). Sperm number significantly increased with higher GSI (Table 2), but sperm length significantly decreased with GSI (Fig. 4a, Table 2) as well as flagellum length and head length with GSI, while midpiece length did not significantly decrease with GSI (Table 2). Flagellum length and head length were significantly correlated, as well as head length and width, but there existed no significant relationship between midpiece length and width. Furthermore, sperm length significantly decreased with sperm number (Fig. 4b, Table 2).

Table 1 Descriptive statistics of measured traits. Means \pm standard deviations are given for each of the nine species examined

Trait/species	<i>P. taenius</i>	<i>P. pulcher</i>	<i>P. humilis</i>	<i>N. parilus</i>	<i>S. casuarinus</i>	<i>S. tinanti</i>	<i>L. werneri</i>	<i>L. ocellatus</i>	<i>H. guttatus</i>	hemichromines
chromidotilapiines										
Standard length (cm)	5.5 \pm 0.59	6.02 \pm 0.40	5.01 \pm 0.52	6.01 \pm 0.37	5.75 \pm 0.36	5.65 \pm 0.42	5.83 \pm 0.50	3.44 \pm 1.60	6.09 \pm 0.41	
Body mass (g)	4.04 \pm 0.92	4.63 \pm 0.74	2.24 \pm 0.50	3.91 \pm 0.93	4.79 \pm 0.98	2.98 \pm 0.89	3.43 \pm 0.78	1.01 \pm 2.83	6.87 \pm 1.93	
Testis mass (mg)	3.2 \pm 1.51	2.94 \pm 1.39	0.60 \pm 0.37	4.95 \pm 2.69	28.13 \pm 13.03	8.54 \pm 4.95	28.66 \pm 21.38	1.97 \pm 7.18	10.55 \pm 7.00	
GSI	0.08 \pm 0.03	0.06 \pm 0.03	0.02 \pm 0.01	0.13 \pm 0.07	0.59 \pm 0.28	0.31 \pm 0.24	0.54 \pm 0.41	0.23 \pm 0.11	0.11 \pm 0.57	
Sperm number ($\times 10^6$)	2.13 \pm 1.41	2.01 \pm 1.80	2.46 \pm 0.90	4.99 \pm 3.09	179.39 \pm 156.28	65.96 \pm 49.17	66.29 \pm 102.27	3.15 \pm 2.29	6.01 \pm 1.72	
Total sperm length (μm)	54.14 \pm 6.73	59.76 \pm 9.83	—	40.75 \pm 8.14	23.91 \pm 3.91	21.83 \pm 4.51	27.15 \pm 3.68	20.12 \pm 4.85	31.54 \pm 4.85	
Max. sperm length (μm)	64.38 \pm 4.57	71.97 \pm 3.34	—	56.44 \pm 3.91	31.19 \pm 4.83	31.13 \pm 5.30	32.86 \pm 1.98	30.14 \pm 6.19	37.50 \pm 1.84	
Flagellum length (μm)	51.14 \pm 6.71	56.32 \pm 9.88	—	38.15 \pm 8.15	22.34 \pm 3.88	20.25 \pm 4.49	25.48 \pm 3.68	18.3 \pm 4.68	28.89 \pm 4.80	
Head length (μm)	2.62 \pm 0.20	2.86 \pm 0.37	—	1.89 \pm 0.20	1.17 \pm 0.12	1.17 \pm 0.12	1.14 \pm 0.11	1.31 \pm 0.36	1.52 \pm 0.20	
Head width (μm)	0.63 \pm 0.05	0.58 \pm 0.08	—	0.95 \pm 0.09	1.27 \pm 0.12	1.24 \pm 0.15	1.13 \pm 0.10	1.26 \pm 0.28	0.98 \pm 0.14	
Midpiece length (μm)	0.28 \pm 0.10	0.28 \pm 0.10	—	0.57 \pm 0.11	0.33 \pm 0.12	0.33 \pm 0.13	0.44 \pm 0.15	0.523 \pm 0.27	1.2 \pm 0.35	
Midpiece width (μm)	0.44 \pm 0.10	0.39 \pm 0.13	—	0.73 \pm 0.10	0.69 \pm 0.20	0.76 \pm 0.21	0.76 \pm 0.16	0.74 \pm 0.19	0.72 \pm 0.14	
Head-to-tail ratio	0.057 \pm 0.004	0.056 \pm 0.005	—	0.065 \pm 0.005	0.067 \pm 0.003	0.074 \pm 0.007	0.062 \pm 0.003	0.101 \pm 0.025	0.094 \pm 0.006	
Adjusted CV _{Wm}	10.54	14.34	—	20.11	14.27	18.70	12.51	26.39	14.69	
Adjusted CV _{Bm}	12.82	15.46	—	20.97	16.94	21.32	13.46	24.53	16.03	

Discussion

The present study revealed significant differences in testis and sperm traits among West African cichlid species. Across species, relative testis mass, i.e., GSI was negatively correlated with sperm length and species with a lower GSI had longer sperm. GSI is often used as indicator of risk of sperm competition. Thus, our results are contrary to findings in Lake Tanganyika cichlids (Balshine et al. 2001; Fitzpatrick et al. 2009) and other studies where sperm length increases with sperm competition risk (Gomendio and Roldan 1991; Gage 1994; Briskie et al. 1997; but see Stockley et al. 1997). We found a negative correlation between sperm length and sperm number across species, suggesting a trade-off between the two traits, which were also not present in Lake Tanganyika cichlids.

Lake Tanganyika cichlids differ in various aspects from West African riverine cichlids. First, environmental conditions like water velocity or other characteristics at the spawning site, all of which are assumed to affect GSI and sperm traits and their interrelationships, are highly divergent. Here, the different habitats can affect GSI and sperm characteristics, as the East African cichlids inhabit lakes and the analyzed West African cichlids inhabit rivers. In rivers, the flow velocity of the water is much stronger than in lakes, and differs between the different rivers and streams which the analyzed West African cichlids inhabit. In rivers with rapids and high flow velocity, fertilization must be harder. Although fishes will occupy territories or nest caves in areas with lower current, in habitats with high current, the average current at the nest or the water turbulence will be higher than in habitats with low current. A male's spawning behavior, and thus, testis and sperm traits, have to be adapted to higher flow velocities to reach the female's eggs compared to small rivers or streams (or lakes) with low flow velocity. Maybe different ways of sperm release have evolved like releasing the sperm upstream in the presence of a strong current so that sperm will not be washed away before fertilizing the eggs. Second, sperm of East African cichlids are easier to activate than those of West African ones (see supplementary information for information on sperm activation in *P. taenius*). This might point to fundamental differences in gonadal traits between the two groups. Especially, West African chromidotilapiines differ from the East African lake species with respect to sperm traits with chromidotilapiines having extraordinarily long sperm and very small GSI. Because males of those species probably experience only weak or no sperm competition, our results indicate that large sperm can evolve in absence of strong sperm competition suggesting that the evolution of sperm size is influenced by various factors. To assess the function of large

Fig. 2 Scanning electron micrographs with size standards showing sperm morphology of **a** *P. taenius*, **b** *P. pulcher*, **c** *N. parilus*, **d** *H. guttatus*, **e** *S. casuarinus*, **f** *S. tinanti*, **g** *L. werneri*, **h** *L. ocellatus*

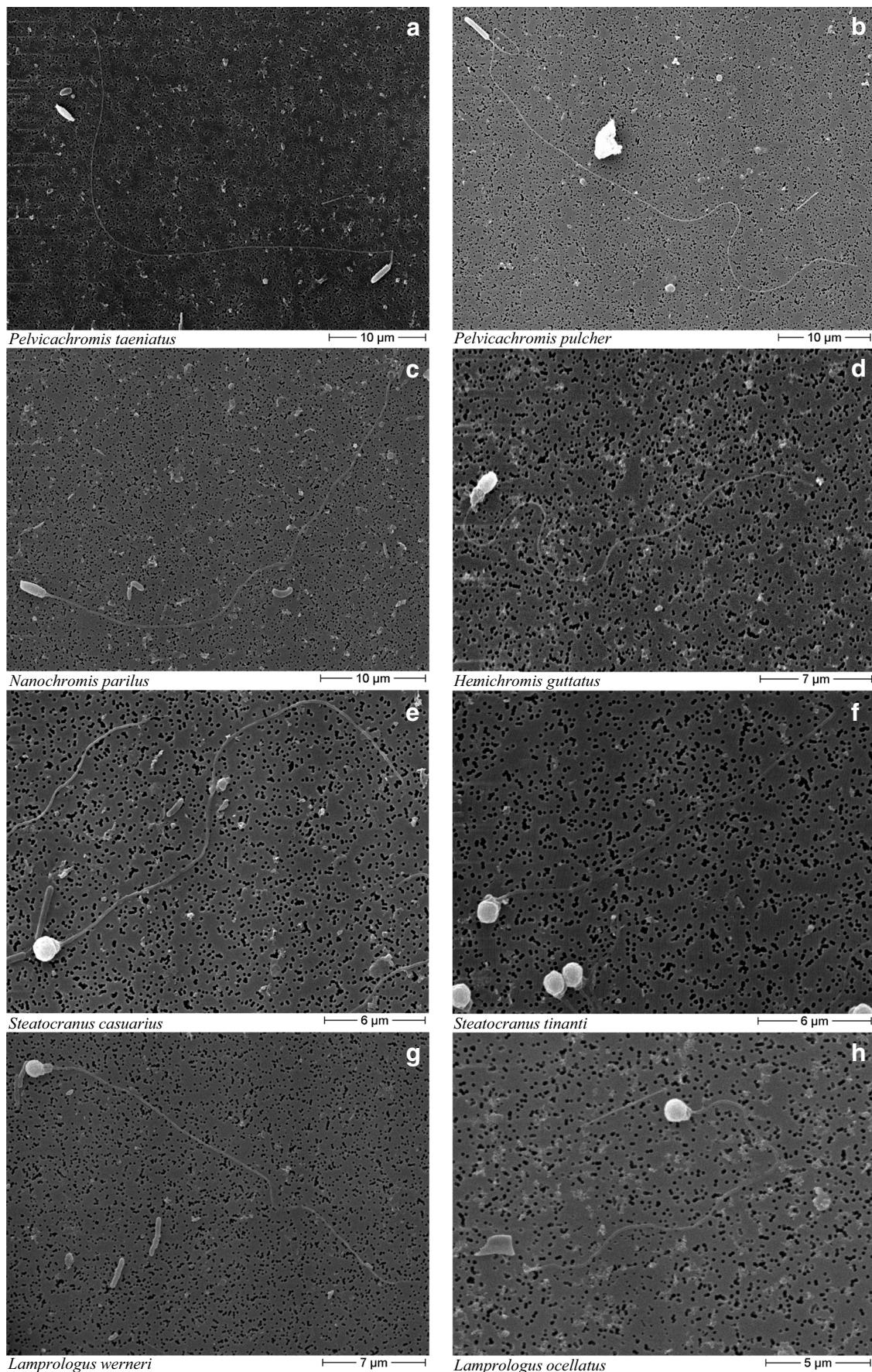
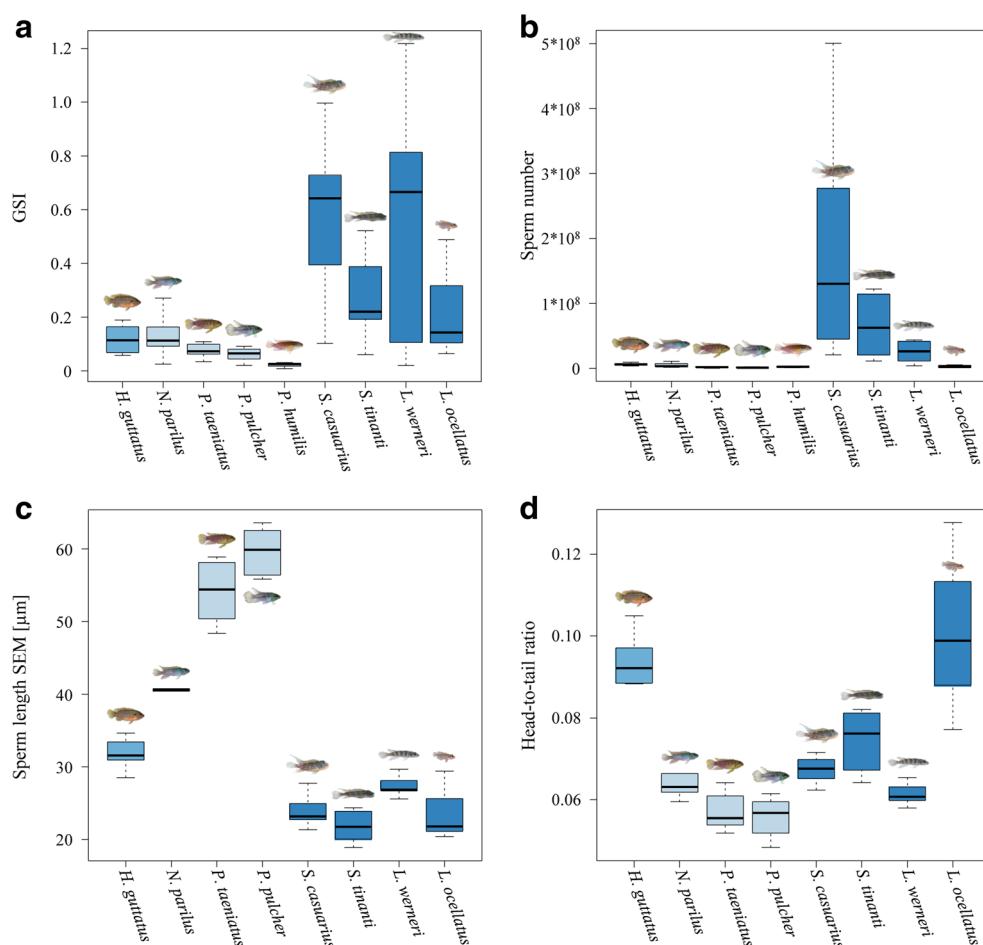


Fig. 3 Boxplots of **a** GSI, **b** sperm number, **c** sperm length, and **d** sperm head-to-tail ratio of the analyzed cichlids (blue = *Hemichromis*; light blue = chromidotilapiini; dark blue = haplotilapiini). Given are median, interquartile range (IQR) and whiskers. The whiskers show the lowest/highest value within the range between the 1st quartile $- 1.5 \times \text{IQR}$ and the 3rd quartile $+ 1.5 \times \text{IQR}$



sperm, it would be relevant to know whether it is associated with swimming speed. Unfortunately, despite considerable effort, we have to date no data on sperm swimming speed and longevity of the various species, except for *S. casuarinus* (see supplementary information and Table S1 for further information on *P. taeniatus*). Thus, we can only speculate about the relationship between sperm size and other sperm traits and velocity. In some species, longer sperm are positively associated with sperm speed (Malo et al. 2006; Gomendio and Roldan 2008); however, other studies find no significant relationship (reviewed in Humphries et al. 2008). If sperm swimming speed is higher for longer sperm, the chances to reach the micropyle are enhanced and this should lead to a production of faster sperm (Gage et al. 2004; Fitzpatrick et al. 2009). The midpiece generates ATP and thus the energy for sperm motility. For the bluegill (*Lepomis macrochirus*), it was shown that the ATP level in the sperm's midpiece is positively related to sperm swimming speed (Burness et al. 2005) and in the Atlantic salmon (*Salmo salar*) sperm length was positively related to ATP level (Vladić et al. 2002). Males using alternative reproductive tactics often have a higher sperm ATP level than conventional males (Vladić and Jarvi 2001; Vladić et al. 2002; Burness et al. 2004; Burness et al. 2005). More ATP

correlates to larger midpieces but not necessarily to longer sperm (Fitzpatrick et al. 2009). However, in our study, GSI was not significantly correlated with midpiece length. This could suggest that there is no selection on midpiece size, and thus no selection for higher sperm swimming speed in long sperm. But in fishes, the relationship between midpiece size and sperm swimming speed is mixed (for references see, e.g., Bakker et al. 2014).

If we look at the two tribes, sperm morphology differed considerably between chromidotilapiines and haplotilapiines. Spherical sperm heads, such as those found in haplotilapiines, have lower drag compared to long (prolate spheroid) sperm heads, such as those found in the chromidotilapiines (Humphries et al. 2008). Long sperm heads convey a sperm motility advantage that is important for species exposed to sperm competition risk. Those West African haplotilapiines analyzed here live in the Lower Congo River in rapids and fast flowing water where spherical-shaped sperm heads can be advantageous as they have a lower drag in the water, while chromidotilapiines included in this study prefer habitats of low water velocity along shallow banks of small streams and in the Lower Congo River. In the lacustrine haplotilapiine *L. ocellatus*, sperm number is significantly lower than in the

Table 2 Result of the generalized least-squares approach (GLS), relationship between sperm traits (sperm length, sperm number, flagellum-, midpiece-, head traits), testis traits (testis mass, GSI), and body mass

controlled for phylogenetic independence using GLS of the seven West African riverine cichlid fishes examined

Trait	λ	Predictor	Estimate	t	P	Multiple r^2	Adjusted r^2	df	CI	F-statistics		
										F	df	p
Testis mass	1.000 ^{ns,ns}	Body mass	1.995	2.483	0.068	0.606	0.508	4	NA,NA	6.16	4	0.068
Total sperm length	0.000 ^{ns,ns}	GSI	-0.423	-3.867	0.018	0.789	0.736	4	NA,NA	14.95	4	0.018
Sperm number	0.000 ^{ns,ns}	GSI	2.175	15.920	<0.001	0.9845	0.981	4	NA,NA	253.4	4	<0.001
Flagellum length	0.000 ^{ns,ns}	GSI	-0.426	-3.692	0.021	0.771	0.716	4	NA,NA	13.6	4	0.021
Head length	0.000 ^{ns,ns}	GSI	-0.394	-3.939	0.017	0.795	0.744	4	NA,NA	15.51	4	0.017
Midpiece length	1.000 ^{ns,ns}	GSI	-0.047	-0.160	0.881	0.006	-0.242	4	NA,NA	0.03	4	0.881
Flagellum length	0.000 ^{ns,*}	Head length	1.091	20.772	<0.001	0.991	0.989	4	NA, 0.693	431.5	4	<0.001
Head length	1.000 ^{ns,ns}	Head width	-1.100	-9.479	<0.001	0.957	0.947	4	NA,NA	89.85	4	<0.001
Midpiece length	1.000 ^{ns,ns}	Midpiece width	1.049	1.270	0.270	0.287	0.109	4	NA,NA	1.612	4	0.273
Total sperm length	1.000 ^{ns,*}	Sperm number	-0.198	-0.448	0.011	0.832	0.790	4	NA,0.992	19.78	4	0.011

The scaling parameter λ was used to assess phylogenetic dependence. Superscripts on λ represent significance levels of likelihood ratio tests with λ compared with 0 (first position) and 1 (second position). Significance levels are denoted as ns, not significant, and a single asterisk, significant with $P < 0.05$. A t test was used to compare the observed slope against a slope of 0. Degrees of freedom (df), 95% confidence intervals and F -statistics are presented

other three investigated haplotilapiines, maybe suggesting that riverine haplotilapiines may produce more sperm to enhance their fertilization success in fast flowing water as many sperm will be washed away before reaching the eggs.

There are not only differences in sperm and testis traits between the two groups but also within the tribes that should not be neglected. In chromidotilapiines, we found the longest sperm known in cichlids so far. Within this tribe, *N. parilus* had more than twice as much sperm, a higher GSI, but smaller sperm as the other analyzed chromidotilapiines. From this species, we know that besides a monogamous mating system, also mating in harems has been observed (Lamboj 2004). A higher GSI concomitant with a higher sperm number on cost of sperm lengths underline these observations. Furthermore, chromidotilapiines possess a low head-to-tail ratio that has been suggested to be advantageous in sperm competition situations in species with much smaller sperm size, because in those species, sperm with a low head-to-tail ratio swim faster (Lüpold et al. 2009; Hilke 2011). In contrast, the low GSI in chromidotilapiines (0.02–0.13) compared to haplotilapiines (0.23–0.59), rather points to a very low sperm competition risk.

Within the haplotilapiines, *S. tinanti* and *L. ocellatus*, which originated from lab-bred individuals, showed lower GSI and shorter sperm than the other two examined haplotilapiines. Long-lasting lab breeding could have affected GSI and sperm length. However, especially in *L. ocellatus*, the most plausible explanation is the breeding system and the environmental factors that affect fertilization success in a lake. Overall, variation in sperm size of African cichlids does not only seem to be determined by between-male sperm

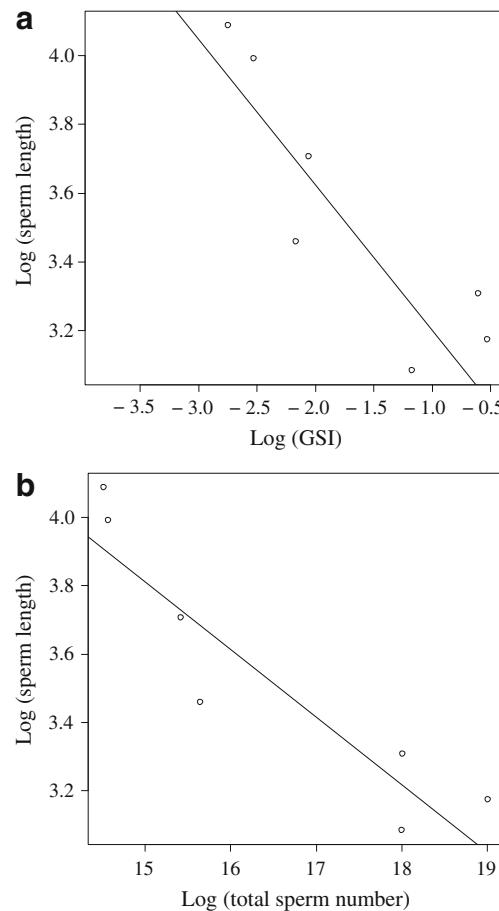


Fig. 4 Relationship between **a** sperm length and GSI and **b** sperm length and sperm number between the seven West African riverine cichlid fishes examined. Data in the figure are not controlled for phylogeny; results of GLS are given in Table 2

competition but by various other factors including phylogeny or habitat characteristics.

In conclusion, we found a negative correlation between GSI and total sperm size and between sperm size and sperm number in West African riverine cichlids. The latter might suggest that males facing competition produce more sperm to enhance their fertilization chances but at the cost of sperm length. The results of the West African chromidotilapiines suggest that long sperm can evolve or be maintained in the absence of sperm competition or when the risk is low. In monogamous species, it could be optimal for males to produce only a smaller number of longer, high-quality sperm as a male's sperm do not have to compete with other males' sperm for fertilization. Alternatively, Parker and Begon (1993) suggest in the absence of between-male sperm competition the evolution of large sperm under certain conditions (when sperm of an ejaculate of a single male compete among each other and sperm size is under haploid control; for further references, see Thünken et al. 2007). Detailed knowledge about the causal relationships between sperm size, velocity, and longevity is necessary to clarify these questions.

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