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Sperm design and function in the redside dace *Clinostomus elongatus*

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A study was undertaken to examine sperm morphometry in relation to sperm velocity and sperm longevity in the redside dace *Clinostomus elongatus*. There was significant between-male variance in sperm size and shape metrics (total sperm length, sperm head length, flagellum length and sperm head length to width ratio) and positive relationships were found between these morphometrics and sperm velocity. There were no significant relationships found between sperm morphometry and sperm longevity, nor was there a trade-off between sperm velocity and sperm longevity.

Key words: fertility; fertilization success; flagellum; sperm competition; sperm quality.

Considerable interest in the evolution of externally fertilizing fish species' sperm shape, size and the resulting hydrodynamics exists because male reproductive success relies ultimately on the ability of sperm to reach and fertilize eggs in an aqueous environment. Sperm swimming velocity is a major determinant of male fertilization success in both non-competitive (Casselman *et al*., 2006; Tuset *et al*., 2008) and competitive (Gage *et al*., 2004; Linhart *et al*., 2005; Liljedal *et al*., 2008) contexts. Few studies, however, have attempted to identify which aspects of sperm size and shape are associated with sperm velocity (Fitzpatrick *et al*., 2009). There are three key hypotheses that attempt to explain the relationship between sperm morphometry and sperm velocity. First, it is hypothesized that longer sperm (whose length is primarily determined by flagellum length) enable faster swimming speeds (Gomendio & Roldan, 1991, 2008) although they have not always been found (Gage & Freckleton, 2003). Higher sperm velocities may be achieved because the longer flagella may have higher beat frequencies and higher wave propagation velocity compared to short flagella (Turner, 2003). Concordantly, sperm velocity may be traded off with sperm longevity because energy stores are quickly depleted in order to achieve high rates of velocity (Ball & Parker, 1996; Vladic *et al*., 2002). Second, it is hypothesized that the size of the midpiece positively correlates with sperm velocity if a larger midpiece is presumed to possess more energy stores or mitochondria. For example, Vladic *et al*. (2002) found that in Atlantic salmon *Salmo salar* L., midpiece length correlated positively with adenosine triphosphate (ATP)

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content, a source of energy for sperm motility (Burness *et al*., 2005). Third, it is hypothesized that the shape of the sperm head will affect sperm velocity via resistance and drag through differential sperm hydrodynamics. Sperm with elongated heads are predicted to exhibit less hydrodynamic resistance and as a result swim faster than sperm with rounder heads (see Malo *et al*., 2006).

This study examined sperm of redside dace *Clinostomus elongatus* (Kirtland) from a wild population to test hypotheses related to whether or not sperm morphometry is associated with sperm swimming velocity. *Clinostomus elongatus* are a small cyprinid with external fertilization and a promiscuous non-resource-based mating system (*i.e.* males provide no parental care or any other material benefit to their young). During the spawning season, the fish congregate in deeper parts of clear, cool streams with woody debris whose bottoms are composed of gravel, sand or bedrock (Koster, 1939; B. Zimmerman, J. Miner & T. E. Pitcher, unpublished data). *Clinostomus elongatus* are apparently obligate nest parasites and spawn in riffles and shallow, flowing pools, parasitizing the nests of, *e.g.* creek chub *Semotilus atromaculatus* (Mitchill) or common shiner *Luxilus cornutus* (Mitchill), depending on the location within their range. Females often separate from a shoal of conspecifics and approach a spawning bed with between 2 and 10 males in pursuit. Females move forward into the depression of the host species' nest where the males stay in close proximity to the female until both sexes release their respective gametes. Spawning acts can be repeated four to six times within the span of 1 min and once a spawning sequence is complete, males and females return to the shoal from which they originated. This behaviour apparently continues to occur until spawning is complete (Koster, 1939; T.E. Pitcher, unpublished data).

Between 28 May 2008 and 1 June 2008, 45 reproductively active *C. elongatus* males were caught from the Rathburn Run tributary (40°49′ N; 82°01′ W) in Wayne County, Ohio, U.S.A., using standard seining techniques. Milt was collected from each individual by applying gentle pressure to the abdomen of the fish. Care was taken to ensure that the milt was not exposed to any water to prevent activation of the sperm. Sperm velocity (after activation with 10 µl of water, maintained at 13° C (the temperature of the river where spawning was occurring), from the river where fish were collected) was video-recorded through a microscope and analysed with sperm tracking software. Video recording was performed using a CCD B/W video camera module at 50 Hz vertical frequency (www.sony.com), mounted on a negative phase-contrast microscope (C×41 Olympus; www.olympus.com) with a ×10 objective. Video-recordings were analysed using the HTM-CEROS sperm tracking package (CEROS version 12, Hamilton Thorne Research; www.hamiltonthorne.com), an objective tool for studying sperm motility in fishes (Kime *et al*., 2001; Pitcher *et al*., 2009). Sperm velocity was assessed as the curvilinear velocity (V_{CL}; average velocity on the actual point-to-point track followed by the cell; Tuset *et al*., 2008) at 5 s post-activation (see Table I). Five seconds post-activation was used as the standard because fertilization of eggs probably occurs within the first few seconds following ejaculation in external fertilizing species (Hoysak & Liley, 2001; Liley *et al*., 2002; Yeates *et al*., 2007). These velocity estimates are the mean velocity of all motile cells analysed (mean ± S.D. number of sperm examined per male = 47 ± 27, range = 16–118); *e.g.*, for each male, the velocity of each individual sperm cell was measured but the estimate used in the final analyses corresponds to a mean over all individual sperm cells. For the sake of brevity, only V_{CS} data are presented

TABLE I. Mean ± S.E. and range for sperm traits of *Clinostomus elongatus* ($n = 45$)

Trait	Mean	S.E.	Range (minimum—maximum)
Sperm head length (μm)	3·03	0·01	2·8–3·2
Sperm head width (μm)	2·62	0·01	2·5–2·7
Flagellum length (μm)	41·20	0·22	38·4–44·1
Total sperm length (μm)	44·20	0·22	41·2–47·2
Head length to head width ratio	1·16	0·00	1·05–1·23
Sperm velocity at 5 s post activation (μm s^{-1})	165·20	2·60	129·7–196·2
Ejaculate longevity (s)	34·00	0·70	26·5–50·5

here, but V_{CS} was correlated with path velocity (V_{AP}) and progressive velocity (V_{SL}) and they gave qualitatively similar results in all analyses. Sperm longevity was also estimated as the time from activation until 95% of the spermatozoa within the field of view were no longer motile (*i.e.* showing no forward movement; Gage *et al.*, 2002). Two measures of sperm longevity were estimated for each individual and because the two scores were highly correlated ($r = 0{\cdot}90, n = 45, P < 0{\cdot}001$), the mean sperm longevity was used in all analyses to reduce the likelihood of measurement error (Yezerinac *et al.*, 1992).

A sub-sample of milt (5 μl) was used to assess sperm morphometry using techniques similar to those described by Leach & Montgomerie (2000). Sperm in saline solution (with 2·5% gluteraldehyde) were dispensed onto a glass slide to present two-dimensional images for measurement. Sperm were observed at ×1000 magnification under oil immersion and digital images were taken. The head and flagellum were measured separately (the midpiece was too small to discern using light microscopy and was indistinguishable from the head component; Gage *et al.*, 2002) using ImageJ software (www.rsb.info.nih.gov/ij) and care was taken to only measure (at the same zoom level for each component; 25% for flagellum and 50% for head size metrics) intact sperm without flagellar damage. Head length (L_H, which included the midpiece) was the measurement from the insertion of the flagellum across the mid-line of the sperm head to its forward apex; flagellum length was measured from its insertion to the end of the terminal filament. Total sperm length was determined by combining L_H and the flagellum length. Sperm head width (W_H) was measured at the midpoint of the sperm head's length. The L_H: W_H ratio was also calculated to provide an estimate of the shape of the sperm head (larger values of the L_H: W_H ratio indicate relatively elongated sperm and smaller values of the L_H: W_H ratio indicate relatively round sperm; Malo *et al.*, 2006). Twenty sperm per male were measured and the mean of the measures taken on total sperm length, L_H, W_H, flagellum length and L_H: W_H ratio were used in all analyses.

There was significant variation in total sperm length among the males (ANOVA, $F_{44,854}$, $P < 0{\cdot}001$; Fig. 1 and Table I). Separate analyses showed that this among-male variance in total sperm length was attributable to differences in both L_H ($F_{44,854}$, $P < 0{\cdot}001$; Table I) and flagellum length ($F_{44,854}$, $P < 0{\cdot}001$; Table I). There was also significant variation in W_H ($F_{44,854}$, $P < 0{\cdot}001$; Table I) and L_H: W_H ratios ($F_{44,854}$, $P < 0{\cdot}001$; Table I) among males. No associations were found between L_H, W_H and flagellum length (Pearson correlation, all $P > 0{\cdot}05$).

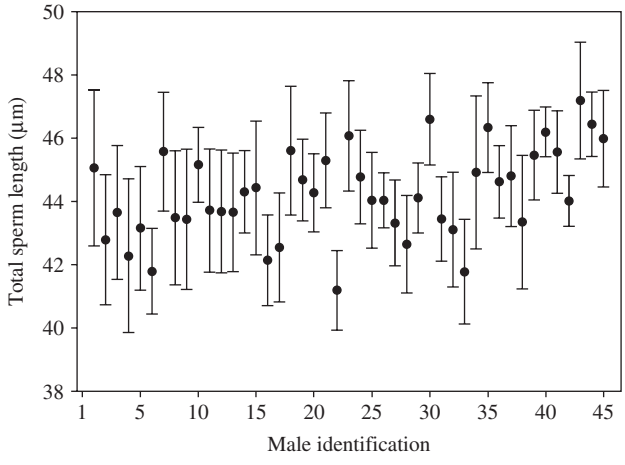

FIG. 1. Variation within and among *Clinostomus elongatus* ($n = 45$) for total sperm length. Mean ± 95% CI values per individual are represented. Male identification reflects arbitrary assignments.

There was a significant relationship between sperm velocity and total sperm length [$r = 0{\cdot}40$, $n = 45$, $P < 0{\cdot}01$; Fig. 2(a)]. Separate analyses showed that there was a significant relationship between sperm velocity and L_H [$r = 0{\cdot}41$, $n = 45$, $P < 0{\cdot}01$; Fig. 2(b)] and flagellum length [($r = 0{\cdot}38$, $n = 45$, $P < 0{\cdot}01$; Fig. 2(c))]. There was no significant relationship between W_H and V_{CL} ($r = -0{\cdot}01$, $n = 45$, $P > 0{\cdot}05$), however, there was a significant relationship between V_{CL} and L_H: W_H ratio [$r = 0{\cdot}41$, $n = 45$, $P < 0{\cdot}01$; Fig. 2(d)], even after removing one outlier [$r = 0{\cdot}32$, $n = 44$, $P < 0{\cdot}05$; Fig. 2(d)]. From a mechanistic perspective, a multiple linear regression was used to assess the relative contributions of each aspect of sperm morphology on sperm velocity. There was a significant relationship between sperm velocity and the length of the sperm head and flagellum, but not with the width of the sperm head (Table II). There were no significant relationships found between sperm longevity and any of the sperm size metrics (Pearson correlation, all $P > 0{\cdot}05$). Furthermore, there was no evidence of a trade-off between sperm velocity and sperm longevity ($r = 0{\cdot}24$, $n = 45$, $P > 0{\cdot}05$).

Consistent with other studies examining intraspecific variation in sperm traits in fishes (Gage *et al*., 2002; Casselman *et al*., 2006; Burness *et al*., 2008), this study found significant inter-individual variation in sperm traits among male *C. elongatus*. Because sperm competition is probably intense in *C. elongatus*, the existence of intraspecific variation is perplexing as sperm design in theory should be subject to intense stabilizing selection pressure, which would result in low inter-individual variation (Birkhead *et al*., 2005; Calhim *et al*., 2007). Potential explanations for the inter-male variation in sperm traits include differences in ontogeny (Evans *et al*., 2002), genetic diversity (Gage *et al*., 2006) and body condition (Burness *et al*., 2008) among males.

The results of this *in-vitro* study did support the hypothesis that the effect of sperm length metrics and shape upon sperm velocity is considerable. Sperm L_H (which included the midpiece) was significantly associated with V_{CL}. Adenosine triphosphate (ATP) hydrolysis is required to maintain flagellar motility and ATP is

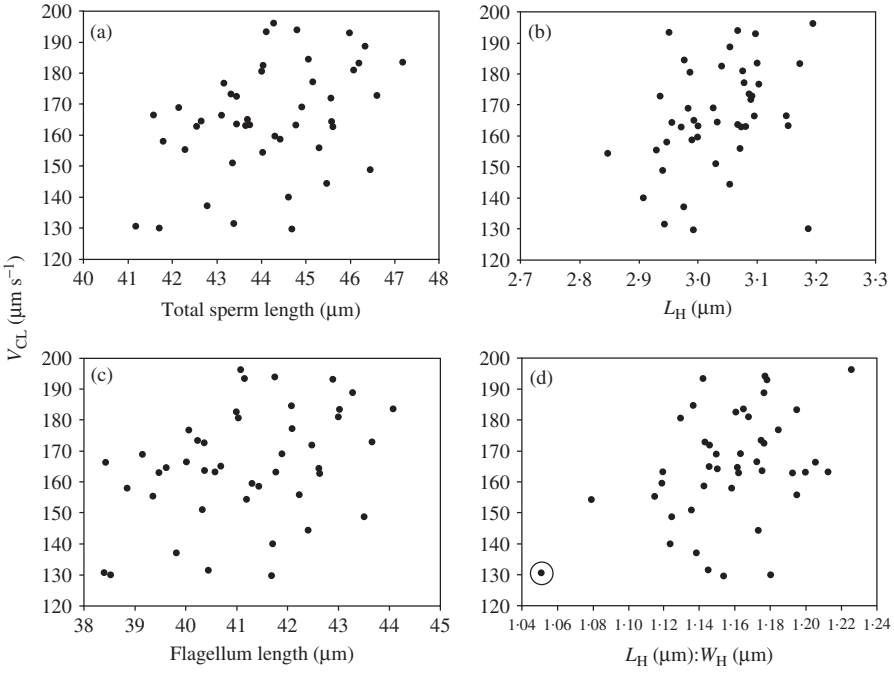

FIG. 2. Relationship between sperm velocity (V_{CL}) (at 5 s post activation) and (a) total sperm length, (b) sperm head length (L_H), (c) flagellum length and (d) L_H to head width (W_H) ratio in *Clinostomus elongatus* ($n = 45$). The relationship between V_{CL} and L_H:W_H remained significant when one outlier (⊙) was removed.

mainly stored in the midpiece (Christen *et al*., 1987; Zilli *et al*., 2004). Thus, the size of the midpiece may be indicative of ATP content because this section contains the densely packed array of mitochondria that provides energy for sperm motility (Vladic *et al*., 2002). Unfortunately, the midpiece could not be distinguished from head size using light microscopy (Gage *et al*., 2002). Future studies should assess midpiece size using transmission electron microscopy of longitudinal sections of *C. elongatus* sperm. This study also found that the length of the flagellum appears to influence sperm swimming speed and this may be because longer sperm are able to produce more propulsion. Elongated sperm head shape was also associated with higher sperm

TABLE II. Results from a linear multiple regression with sperm velocity as the dependent variable. Independent variables tested were sperm head length, sperm head width and flagellum length. ß is the standardized regression coefficient and reflects the direction and magnitude of the effect and *P* refers to corresponding significance

Source*	ß	*P*
Head length	0·42	<0·01
Head width	−0·08	>0·05
Flagellum length	0·36	<0·01

*Overall model: $r^2 = 0{\cdot}26$, $F_{3,41} = 6{\cdot}06$, $P < 0{\cdot}01$.

velocity. This finding supports the hypothesis that sperm hydrodynamics may play a role in determining sperm velocity. Factors that determine fishes' sperm head size (which houses the male's haploid contribution) are poorly understood. Because the nucleus comprises a large proportion of the head size in fishes (Jamieson, 1991), genetic-related variables such as genome mass or number of chromosomes may explain the variation. Contrary to theory (Ball & Parker, 1996), there was no apparent trade-off between sperm longevity and sperm velocity. Instead, a nearly significant positive relationship was found between these two sperm quality metrics, which is consistent with two other studies of external fertilizing fish species (Kortet *et al*., 2004; Pitcher *et al*., 2009).

In summary, the results of this study suggest that the main determinants of sperm velocity in *C. elongatus* are the shape and length of the sperm. Thus, swimming velocity may be the result of the combined design of different sperm components. The large inter-male variation in sperm design found in this natural population of *C. elongatus* underlies differences in sperm swimming speed, which in turn, may determine differences in sperm competition success in this highly promiscuous species.

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