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# Comparing sperm swimming speed

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## ABSTRACT

**Question:** Do current methods correctly compare sperm swimming speed (SSS) between males, species or species groups?

**Data incorporated:** Thirty-four studies published since 2009.

**Method of analysis:** Logical and statistical examination of several assumptions behind comparisons of SSS measurements.

**Conclusions:** If sperm swimming speed is measured with a time lag from sperm activation, false-positive correlations can arise between sperm swimming speed and sperm longevity, and between initial and later sperm swimming speed. Comparing the mean, rather than the maximum, sperm swimming speed per ejaculate across subjects may also mask trade-off effects. Environmental and genotype × environment interaction effects can complicate, or invalidate, comparisons of sperm swimming speed. Unlike a previous study, we found that sperm swimming speed and sperm longevity in Tanganyikan cichlids were related to mouthbrooding, not to sperm competition.

*Keywords*: automated sperm analysis, mouthbrooding, sperm age, sperm competition, sperm senescence, sperm velocity, trade-off.

## INTRODUCTION

Sperm swimming speed is considered a key male fitness trait in several areas of ecological and evolutionary research. In sexual selection, sperm swimming speed is suggested to mediate the competition between ejaculates of different males (Snook, 2005). In speciation research, Levitan *et al.* (2004) showed that sperm ageing (among other factors) caused reproductive isolation between synchronously spawning coral species. Sperm swimming speed has also been the focus of animal breeding research (Billard and Cosson, 1992; Froman *et al.*, 2006). Finally, in ecotoxicology, the rate of decline in the sperm of swine has been used as a toxicity bioassay (e.g. Hoornstra *et al.*, 2004). Studies have compared sperm swimming speed between males, populations, species or functional groups (e.g. Burness *et al.*, 2004; Fitzpatrick *et al.*, 2007, 2009; Kleven *et al.*, 2009). Here we discuss potential pitfalls of this approach, especially in

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light of recent advances in automated measurements of sperm swimming speed by computer-assisted sperm analysis [CASA (World Health Organization, 2010)] or optical tweezers (König *et al.*, 1996; Nascimento *et al.*, 2008), which allow researchers to generate large data sets on sperm swimming speed. For example, the number of studies measuring sperm velocity or sperm swimming speed in non-human animals has increased significantly (e.g. 4 in 2000, 15 in 2005, and 22 in 2010 in Web of Science). Specifically, we focus on the rapid, non-linear and environment-dependent decline of sperm swimming speed and illustrate the challenges faced when comparing sperm swimming speed within and between ejaculates, as well as between males, species, and functional or taxonomic groups. Our considerations apply to comparisons of sperm swimming speed at all these hierarchical levels, for external and internal fertilizers (see Table 1) and for pre- and post-storage sperm movement (Fig. 1). Of the episodes between ejaculation and fertilization that influence sperm function and paternity (Fig. 1), sperm swimming speed likely represents a more immediate male fitness measure in external than in internal fertilizers because the sperm storage of internal fertilizers may balance out the differences in pre-storage sperm swimming speed or other characters of sperm metabolism (Ribou and Reinhardt, 2012).

## CHARACTERIZING THE TEMPORAL DECLINE IN SPERM SWIMMING SPEED

Sperm swimming speed, and its more immediate fitness measure, the fertilization ability of sperm, has been shown to decline rapidly over time in a variety of organisms representing different ecologies, contexts and taxa, such as marine and freshwater animals, external and internal fertilizers, vertebrates and invertebrates (Gray, 1955; Billard and Cosson, 1992; Levitan, 2000; Hoysak and Liley, 2001; Hoornstra *et al.*, 2004; Levitan *et al.*, 2004; Casselman *et al.*, 2006; Fitzpatrick *et al.*, 2007, 2009; Kleven *et al.*, 2009). The rapid drop in sperm swimming speed and other aspects of sperm function (Hoysak and Liley, 2001) led Kime *et al.* (2001) to recommend the first measurement of sperm swimming speed in fish to be made within 10 s of sperm activation.

The reason for this decline in sperm swimming speed may be related to cellular ageing (for an overview, see Reinhardt, 2007), including a depletion of resources in the sperm cell or the accumulation of reactive oxygen species in the cell arising from the oxidative phosphorylation that fuels the sperm cell (Aitken and Baker, 2004). The decline may also be related to a life-history-like model of sperm function (Levitan, 1995; Taborsky, 1998), where selection favours

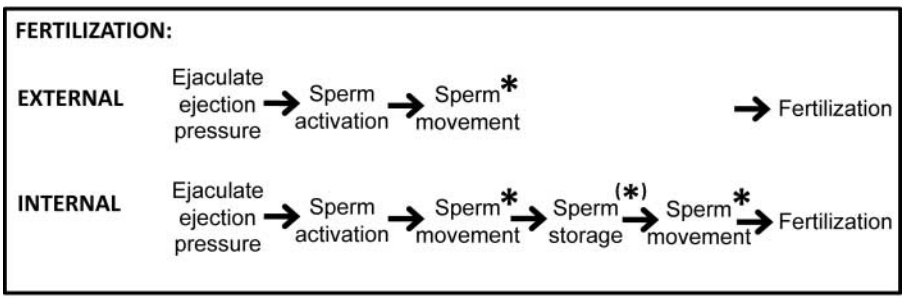


**Fig. 1.** The main factors that affect sperm function from ejaculation to fertilization in external and internal fertilizers. Processes where sperm swimming speed is important are marked with an asterisk. Due to the lack of sperm storage, sperm swimming speed likely represents a more immediate fitness measure in external than internal fertilizers.

high early and low late sperm swimming speed, or to evolutionary models of ageing, which suggest that ageing results from a lack of selection, i.e. high sperm swimming speed is not required late in a sperm cell's life.

Regardless of whether ageing or life-history-like effects are responsible for its decline, sperm swimming speed can be described as the sigmoidal decline of functionality that is predicted by general physiological ageing models (e.g. Novoseltsev *et al.*, 2003) and by some empirical studies that kept the time between sperm activation and the measurement of sperm swimming speed to a minimum (Billard and Cosson, 1992; Burness *et al.*, 2004; Casselman *et al.*, 2006). Sperm swimming speed may be separated into the three fundamental components identified by Novoseltsev *et al.* (2003): an initial plateau in sperm swimming speed (corresponding to Novoseltsev's reproductive capacity, RC), here called $SSS_0$, which lasts until sperm swimming speed declines at the critical time $T$ (corresponding to critical age $T$), usually at an exponential rate $\tau$ (Fig. 2a). The rapid and sigmoidal decline in sperm swimming speed has two consequences: (i) when sperm swimming speed is not measured before $T$, there may be a false-positive correlation between sperm swimming speed and sperm longevity, and (ii) differences in the decline curve between subjects can complicate the comparison of sperm swimming speed between subjects when based on cross-sectional measurements.

## THE CORRELATION BETWEEN SPERM SWIMMING SPEED AND SPERM LONGEVITY

Consider sperm swimming speed (SSS) is measured on three occasions after sperm activation: before $T$ ($t_0$), some time after $T$ ($t_1$), and when sperm swimming speed has declined by 95% ($t_x$, a measure of sperm longevity that corresponds to the time when 95% of the sperm in an ejaculate have stopped moving). Compare two species, or males, H and L, with high ($SSS_0^H$) and low initial sperm swimming speed ($SSS_0^L$), respectively. Under the sperm ageing and the sperm life-history model, sperm reach the critical time earlier in male H than male L ($T^H < T^L$), or show a faster decline in $\tau$ ($\tau^H > \tau^L$) and do not maintain sperm swimming speed as long (Fig. 2b). The resulting negative correlation between $SSS_0$ and $SSS_1$, or between $SSS_0$ and $t_x$, is a fundamental pattern that has been confirmed empirically in the form of the trade-off between sperm velocity and sperm longevity (Levitan, 2000) and early vs. late sperm function (e.g. Billard and Cosson, 1992; Burness *et al.*, 2004; Casselman *et al.*, 2006). These studies measured sperm swimming speed within a few seconds of sperm activation, and thus the first measurement of sperm swimming speed likely represented $SSS_0$. Summarizing 34 recent studies that measured sperm swimming speed (Table 1), we found that the time course of sperm swimming speed from the instant of sperm activation was not known for any of the 100 or so study species. In particular, information was lacking on whether sperm swimming speed was measured before the critical time $T$ and whether the decline in sperm swimming speed reported in most studies corresponds to the decline in $SSS_0$ or $SSS_1$ (see Fig. 2b). Five species of fish or external fertilizers were analysed within the recommended 10 s, and more than 70 species within 30 s. Most studies did not examine, or did not state, the relationship between an early and a late measurement of sperm swimming speed or between sperm swimming speed and sperm longevity (Table 1). Two recent studies of 30 freshwater fish species that reported a positive correlation between sperm swimming speed and sperm longevity recorded sperm swimming speed for the first time at 30 and 10 s after sperm activation, respectively (Fitzpatrick *et al.*, 2009; Janhunen *et al.*, 2009). While this remains a valid possibility, there is an alternative explanation. Assuming that sperm swimming speed declines very rapidly also in these fish species, the first measurement of sperm swimming

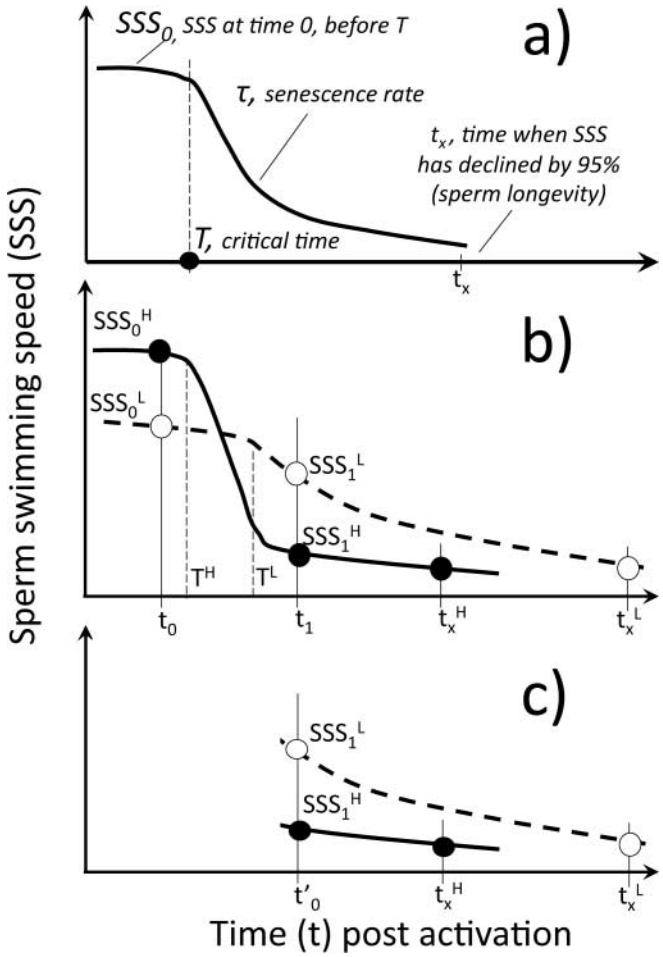


**Fig. 2.** (a) Theoretical time course and parameters proposed to analyse and compare sperm swimming speed (SSS): the initial sperm swimming speed, $SSS_0$; the onset of decline, critical time $T$; and the rate of senescence, $\tau$. (b) Time course of sperm swimming speed in two males showing high (H) and low (L) initial $SSS_0$. Sperm swimming speed is measured at $t = 0$ ($t_0$), $t = 1$ ($t_1$) (after $T$), and when 95% of the sperm have stopped moving ($t_x$), corresponding to sperm longevity. Note the negative correlation between $SSS_0$ and $SSS_1$, and between $SSS_0$ and $t_x$. (c) The same time course as in (b) showing the effect when sperm swimming speed is not measured immediately after activation ($t_0$) but later at a point $t_0'$, which is assumed to be $t_0$. In this example, $SSS_1$ is assumed to be $SSS_0$. In this comparative example, one would (i) falsely attribute a high sperm swimming speed to the male with low initial $SSS_0$, (ii) reach the false conclusion of a positive correlation between sperm swimming speed and sperm longevity, and (iii) be unable to calculate the true $SSS_0$.

speed may have been taken after $T$, i.e. at $t_0'$ instead of $t_0$, despite all efforts. Sperm swimming speed may then represent $SSS_1$ and not $SSS_0$, which would lead to a false-positive correlation between sperm swimming speed and $t_x$ across males (Fig. 2c). Unfortunately, because the time course of sperm swimming speed, and hence $T$, is unknown in these and most other studies, it is currently impossible to predict the true $SSS_0$ for most studies

**Table 1.** Studies in areas of ecology and evolution published since 2009 that examine sperm swimming speed (SSS) and sperm velocity

| Study species | Time lag between sperm activation and first measurement | Time lag smaller than critical time $T$? | Relationship between early SSS and sperm longevity | Relationship between early and later SSS | Reference |
|---|---|---|---|---|---|
| **Birds** | | | | | |
| 40 passerine species | 'Immediately after dilution', unknown time after collection | ? | Not measured | SSS measured only once | Lüpold *et al.* (2009) |
| 42 passerine species | <30 s | ? | Not measured | Not stated | Kleven *et al.* (2009) |
| *Taeniopygia guttata* | Not stated | ? | Not measured | SSS measured only once | Mossman *et al.* (2009a) |
| *Taeniopygia guttata* | Within 27–587 s | ? | Not measured | No difference 10 and 30 min after dissection | Mossman *et al.* (2009b) |
| *Gallus gallus* | 3 min | ? | Not measured | SSS measured only once? | Cornwallis and O'Connor (2011) |
| *Parus major* | 'Immediately after mixing and transfer to microscope', unknown time after collection | ? | Not measured | Not stated | Helfenstein *et al.* (2010b) |
| *Passer domesticus* | 25 s | ? | Not stated (negative correlation between number of motile sperm and longevity) | Not stated | Helfenstein *et al.* (2010a) |
| *Tachycineta bicolor* | <30 s | ? | Not measured | Not stated | Laskemoen *et al.* (2010) |
| **Freshwater fish** | | | | | |
| 29 species | 30 s | ? | Positive | Positive | Fitzpatrick *et al.* (2012) |
| *Astatotilapia burtoni* | 15 s | ? | Not stated | Not stated | Kustan *et al.* (2012) |

(*continued*)

**Table 1.**—(*continued*)

| Study species | Time lag between sperm activation and first measurement | Time lag smaller than critical time $T$? | Relationship between early SSS and sperm longevity | Relationship between early and later SSS | Reference |
|---|---|---|---|---|---|
| *Clarias gariepinus* | Not stated | ? | Not measured | Not stated | Noor Azlina *et al.* (2011) |
| *Clinostomus elongatus* | 5 s | ? | ? | Not stated | Beausoleil *et al.* (2012) |
| *Clinostomus elongatus* | 5 s | ? | No significant relationship | SSS measured only once | Pitcher *et al.* (2009) |
| *Neolamprologus pulcher* | 1 min | ? | Not measured | SSS measured only once? | Desjardins *et al.* (2011) |
| *Oncorhynchus tshawytscha* | 5 s | ? | Not measured | Not across three populations over 10 s | Lehnert *et al.* (2012) |
| *Poecilia reticulata* | <30 s | ? | Not measured | SSS measured only once? | Boschetto *et al.* (2011) |
| *Poecilia reticulata* | 5 s | ? | Not measured | SSS measured only once? | Elgee *et al.* (2012) |
| *Poecilia reticulata* | 'Immediately', unknown time after collection | ? | ? | Positive for two successive measurements (significant repeatability) | Evans (2009) |
| *Poecilia reticulata* | Not stated | ? | Not measured | Not stated | Gasparini *et al.* (2009a) |
| *Poecilia reticulata* | Not stated | ? | Not measured | Not stated | Gasparini *et al.* (2009b) |
| *Poecilia reticulata* | Not stated | ? | Not measured | Not stated | Gasparini and Pilastro (2011) |
| *Poecilia reticulata* | Not stated | ? | Not measured | Not stated | Gasparini *et al.* (2012) |
| *Salvelinus alpinus* | 10 s | ? | Positive | Not stated | Janhunen *et al.* (2009) |

| | | | | | |
|---|---|---|---|---|---|
| *Salvelinus alpinus* | 10 s | ? | Not measured | Not stated | Kekäläinen *et al.* (2010) |
| *Salvelinus alpinus* | 10 s | ? | Not measured | Negative across two male morphs | Haugland *et al.* (2009) |
| *Xiphophorus nigrensis* | 1 min | ? | Not measured | SSS measured only once | Smith and Ryan (2009) |
| *Xiphophorus nigrensis* | 1 min | ? | Not measured | SSS measured only once | Smith (2012) |
| **Amphibians** | | | | | |
| *Crinia georgiana* | unknown | ? | Not measured | SSS measured only once? | Dziminski *et al.* (2009) |
| **Mammals** | | | | | |
| *Mus domesticus* | 90 min | ? | ? | Not stated | Firman and Simmons (2010) |
| *Myodes glareolus* | 15–30 min after dissection or incubation | ? | Not measured | SSS measured only once | Lemaitre *et al.* (2012) |
| *Sus scrofa* | Not stated | | Not measured | Not stated | Holt *et al.* (2010) |
| **Bivalves** | | | | | |
| *Crassostrea gigas* | <10 s | ? | Not measured | Not stated | Havenhand and Schlegel (2009) |
| *Mytilus galloprovincialis* | 7.4 ± 0.4 (S.E.) min (range −5 to 22 min) | ? | ? | Positive (significant repeatability) | Fitzpatrick *et al.* (2012) |
| **Echinoderms** | | | | | |
| *Heliocidaris erythrogramma* | 10 min | Unknown for study species. Yes for related ones | Not measured | SSS measured only once | Fitzpatrick *et al.* (2010) |

*Note*: The selection of journals is restricted to those our institution had a subscription for. The time between sperm activation and the first measurement of sperm swimming speed, its relation to the time of critical decline in sperm swimming speed, and the relationship between successive measurements of sperm swimming speed and sperm longevity were extracted.

(Figs. 2b, c). This example illustrates that measuring sperm swimming speed before $T$ is of utmost importance (see also discussion in Kime *et al.*, 2001; Rosengrave *et al.*, 2009). Below we illustrate that the comparison of sperm swimming speeds experiences an additional complication when males or species differ in $T$.

## FACTORS INFLUENCING BETWEEN-SUBJECT COMPARISONS OF SPERM SWIMMING SPEED

Most recent studies (Vladic and Järvi, 2001; Gage *et al.*, 2004; Fitzpatrick *et al.*, 2007, 2009; Kleven *et al.*, 2009; Rosengrave *et al.*, 2009) (see Table 1) measured sperm swimming speed at the same, discrete, time point after sperm activation for all males or all species to be compared. This can be difficult if subjects differ in $T$: the first measurement of sperm swimming speed may be taken before $T$ in one male or species but after $T$ in another. Consider again the H and L males with high and low initial sperm swimming speed – $SSS_0^H$ and $SSS_0^L$, respectively. If the first measurement occurs after the crossover of $SSS^H$ and $SSS^L$, male H with in reality higher $SSS_0^H$ would erroneously be classified as having a lower sperm swimming speed than male L (compare Figs. 2b and c) (this can also occur if sperm swimming speed declines linearly, not sigmoidally). In the following, we discuss four common scenarios that influence the comparison of sperm swimming speed via their effect on the time course of sperm swimming speed or via the within-sample variation. We do not address the variation between males in the time lag of sperm activation and sperm motility caused by ejaculate dilution, as this problem is discussed extensively elsewhere (Billard and Cosson, 1992).

### Comparison of sperm swimming speed between different methods

Sperm swimming speed can be measured along the actual swimming path (curvilinear track velocity, VCL), an integrated (smoothed) path (average-path velocity, VAP), a straight-line path (straight-line velocity, VSL), or in six other established ways (World Health Organization, 2010). In one species, *Telmatochromis vittatus*, the decline in sperm swimming speed was independently sampled at the same geographical location and with almost identical sample sizes (Fitzpatrick *et al.*, 2007, 2009) (Fig. 3). This species shows a markedly different time course of sperm swimming speed at the same location when measured by different methods (Fig. 3). This difference between methods suggests that only measurements of sperm swimming speed obtained by the same method should be compared in meta-analyses.

### Environmental influences on sperm swimming speed

The decline in sperm swimming speed varies across physical and chemical environments, such as temperature, pH and other ion concentrations (e.g. Lillie, 1919; Lindroth, 1947; Billard and Cosson, 1992; Kime *et al.*, 2001; World Health Organization, 2010). Standardizing or quantifying such environments during the measurement of sperm swimming speed is vital for between-study comparisons. We note that such comparisons may be severely hampered because a substantial number of studies have not even provided basic environmental conditions, such as temperature or pH values at which sperm swimming speed or other sperm measurements were taken, including one of our own (Ribou and Reinhardt, 2012).

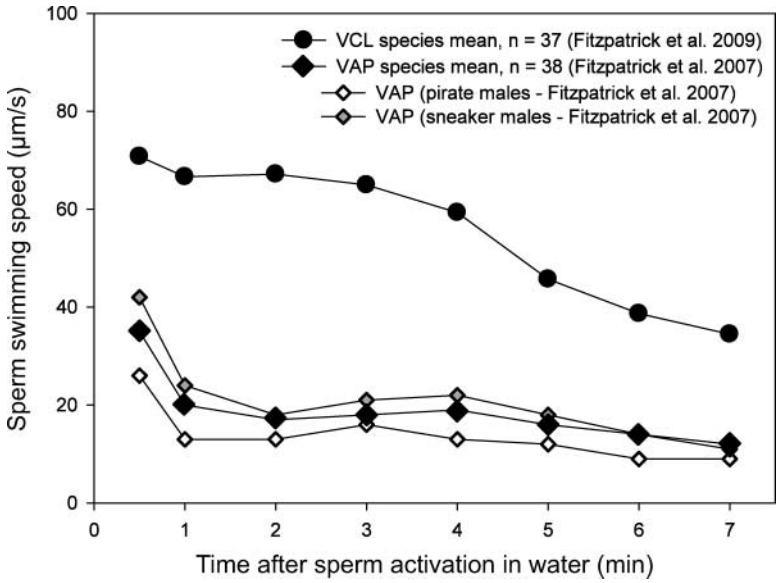


**Fig. 3.** Effect of reproductive strategy and method of measurement on the temporal change in sperm swimming speed of a cichlid species, *Telmatochromis vittatus*. Black dots are curvilinear track velocity (VCL) [data courtesy of Fitzpatrick *et al.* (2009), $n = 37$, Kasakalawe Bay, Lake Tanganyika], black diamonds are from the smoothed path velocity (VAP) of a different sample of the same species from the same location [Fitzpatrick *et al.* (2007), $n = 38$]. Black diamonds are means of several morphs that differ in sperm swimming speed, of which the two most extreme are shown [grey diamonds: sneaker males; white diamonds: pirate males; redrawn from Fitzpatrick *et al.* (2007)]. The two measurement methods result in a different temporal pattern, and hence different values for SSS, $T$, and $\tau$ (as illustrated in Fig. 2). Note that $SSS_0$ is unknown for both measurement methods (cf. Fig. 2c).

## Genotype × environment interaction effects on sperm swimming speed

Environmental effects on sperm swimming speed have another consequence. Under the current view that sperm swimming speed evolves (e.g. Snook, 2005; Fitzpatrick *et al.*, 2009), its components $T$, $\tau$ (and $t_x$) can also be expected to evolve – or be phenotypically plastic (cf. Crean and Marshall, 2008). We note, however, that the mechanism of selection on the male for mitochondria-dependent and hence maternally dependent traits such as oxidative phosphorylation is currently not clear. As environmental factors affect sperm swimming speed, models of SSS evolution should also predict that natural, rather than sexual, selection acts on sperm swimming speed. As a consequence, populations or species from different habitats can be expected to differ in $SSS_0$, $T$, $\tau$, and $t_x$. We realize how difficult it is to account for such genotype × environment interactions on sperm swimming speed, and only a few studies have ever examined such effects on any aspect of sperm performance. For example, the statistical table in Clark and colleagues' (1999) study on *Drosophila melanogaster* allowed us to calculate that up to 19% of the variation in paternity was due to a genotype × laboratory setting interaction. It is likely that at least some of this variation is explained by sperm traits [see also Axelsson *et al.* (2010) for sperm nuclear genotype × environment interactions]. Lindroth (1947) found species × temperature interactions on sperm motility duration in freshwater fish. Here we illustrate the strong effect that

genotype × environment interactions in sperm swimming speed have on the interpretation of results. Fitzpatrick *et al.* (2009) took their first measurement of sperm swimming speed at 30 s after activation because, citing Hoysak and Liley (2001), '30 s post activation is the likely period during which most fertilization occurs'. If there were species × environment interactions on sperm performance, it is likely that fertilization would occur much earlier because Hoysak and Liley (2001) obtained their data for the Pacific salmon (*Oncorhynchus nerka*), a species that spawns, and whose sperm swimming speed has presumably evolved in the cold rivers around the North Pacific, not the warm water of Lake Tanganyika. Kime *et al.* (2001) recommended measuring the sperm swimming speed of fish at lower temperatures to delay the rapid drop in speed. However, Hoysak and Liley (2001) showed in their study that the fertilization rate of salmon sperm had already dropped to 2–20% after 20 or 40 s.

Genotype × environment interaction effects on sperm swimming speed appear especially important when the common garden laboratory environment is more similar to the natural environment (in which sperm swimming speed has presumably evolved) of one than another species or population. For example, some species of fish have evolved to transfer sperm within a mucus layer, but others have not. The effect is dramatic. Scaggiante *et al.* (1999) showed that, in seawater, sperm of the grass goby *Zosterisessor ophiocephalus* die within 83 min. However, in the mucus that males of this species ejaculate alongside sperm cells into seawater, sperm mortality was delayed by 15 times and sperm died after an average of 20 h (Scaggiante *et al.*, 1999). Comparing the sperm swimming speed of mucus- and non-mucus-using species is complicated if sperm are activated in water rather than mucous because it represents the natural environment for only the non-mucus-using species. Mucus coats of ejaculated sperm are also known from cichlid species in which females take up sperm and eggs, and fertilize them in their mouth cavity (Wickler, 1965, 1997; Grier and Fishelsen, 1995; Fishelsen, 2003) – so-called mouthbrooders. Because male-derived mucus (and the female mouth cavity) represents a different environment than the lake water used to activate sperm, genotype × environment interactions on sperm function would predict that different time courses have evolved in substrate breeders and mouthbrooders. We found support for this prediction in the data of Fitzpatrick *et al.* (2009): the decline in sperm swimming speed differed between substrate breeders and mouthbrooders, to the extent that they did not overlap for a considerable part of the range (Fig. 4). More specifically, sperm swimming speed dropped by a mean of 4.33 $\mu m \cdot s^{-1}$ between 30 s and 60 s after activation in substrate breeders and by 9.93 $\mu m \cdot s^{-1}$ in mouthbrooders. This decline was affected by whether the species was a mouthbrooder or substrate breeder (black + green lines vs. blue + pink lines in Fig. 4; linear regression, slope estimate $-6.305 \pm 2.21$, $t = 2.849$, $P = 0.008$). It was not affected, as previously suggested, by whether the species was monogamous or polygamous (solid vs. broken lines in Fig. 4; slope estimate $0.619 \pm 0.705$, $t = 0.878$, $P = 0.388$) (overall model: $F_{2,26} = 4.076$, $P = 0.0289$; interaction term excluded: $P = 0.738$, adj. $R^2 = 0.18$).

In the same data set, it is also possible that genotype × environment interactions affect the trade-off between early and late sperm swimming speed predicted by sperm ageing or physiological models (see above). As seen in Fig. 4, the stronger decline in early sperm swimming speed in mouthbrooders than substrate breeders was reversed during later decline. For example, arbitrarily using the decline from minute 3 to minute 4 post activation as a measure of late sperm swimming speed, substrate breeder species show a mean decline of 10.57 $\mu m \cdot s^{-1}$ and mouthbrooder species one of 3.56 $\mu m \cdot s^{-1}$. Specifically, sperm swimming speed differed with respect to mouthbrooders vs. substrate breeders (slope estimate $6.961 \pm 1.761$, $t = 3.954$, $P < 0.001$) but not sexual selection (monogamy or

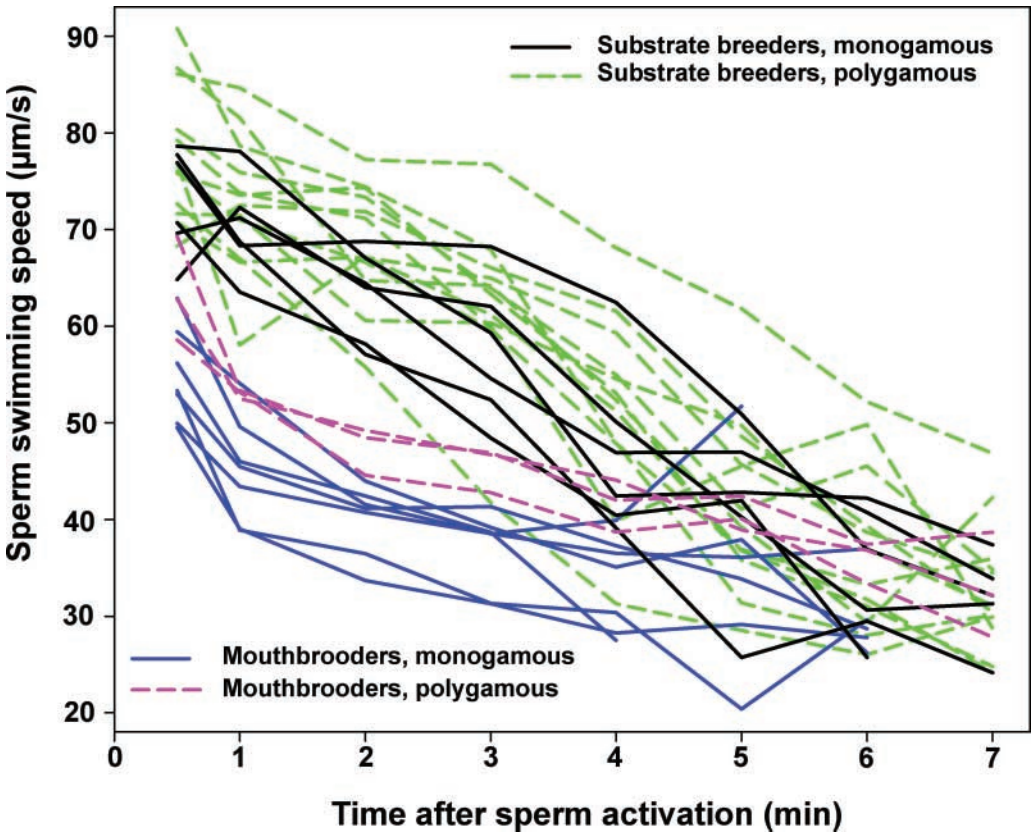


**Fig. 4.** The effect of mouthbrooding or substrate breeding on sperm swimming speed (VCL) in cichlid fish compared with the influence of the mating system. Lines represent species means [data courtesy of Fitzpatrick *et al.* (2009)]. In all species, sperm slow down as they age. The decline in sperm swimming speed differs between mouthbrooding and substrate breeding species but mating system (monogamy = solid lines; polygamy = broken lines) does not play a role. Sperm swimming speed in mouthbrooding species (blue and pink lines) initially declines more rapidly than in substrate breeding species (black and green lines). Later, the decline is smaller in mouthbrooding than substrate breeding species. The different decline curves prevent the prediction of sperm swimming speed at the time of sperm activation (cf. Fig. 2c; also see text for details). Genotype × environment interaction effects may also be significant because the freshwater of the laboratory environment, in which sperm swimming speed was measured, is probably more similar to lake water to which the sperm of substrate fertilizers are exposed than to the seminal mucus and female mouth environment to which sperm of mouthbrooders are exposed.

polygamy) (slope $0.0466 \pm 0.561$, $t = 0.083$, $P = 0.934$) (overall model: $F_{2,26} = 4.192$, $P < 0.001$; interaction term excluded: $P = 0.921$, adj. $R^2 = 0.37$). Similarly, within species, the late decline was greater than the early decline in one of 10 mouthbrooder species but in 17 of 19 substrate breeder species ($\chi^2_2 = 17.576$, $P < 0.0001$). Changes in sperm swimming speed within individuals were not available to us. If the measurements of sperm swimming speed were comparable between groups, our analysis suggests that it is a plausible hypothesis that genotype × environment effects related to mouthbrooding selected for

sperm swimming speed, rather than sperm competition *per se* (Fitzpatrick *et al.*, 2009). Alternatively, mouthbrooders and substrate breeders differ so fundamentally in *T* to prevent any judgement to be made as to whether the first measurement of sperm swimming speed was before or after *T* (cf. Figs. 2b, c). In other words, if the storage of sperm in the female's mouth (cf. internal fertilizers in Fig. 1) selects for different sperm traits than the lake water used by substrate brooders (cf. Fig. 2a), then sperm swimming speed at 30 s after activation may not represent the same fitness measure in the two groups.

### Sperm density effects on the decline in sperm swimming speed

At high densities, sperm cells of both external and internal fertilizers have reduced metabolic rate, prolonged lifespan or function (see references in Reinhardt, 2007; Holman, 2009), and increased fertilization rates (e.g. Lillie, 1919; Levitan, 1995; Hoysak and Liley, 2001). This is known as the 'respiratory-dilution effect' (Chia and Bickell, 1983). How sperm density is related to sperm swimming speed is unknown for most species; however, for cuttlefish *Sepia apama* (Naud and Havenhand, 2006) and the zebra finch (Mossman, 2008), positive correlations have been reported. Given these results and the respiratory-dilution effect, we suggest that values of sperm swimming speed should be experimentally or statistically controlled for sperm density (e.g. Kleven *et al.*, 2009; Mossman *et al.*, 2009a, 2009b), and presented alongside the uncorrected values. Note that sperm density is likely correlated with the concentration of seminal fluid, which is 'a key issue' in measuring sperm movement (Billard and Cosson, 1992). We suggest statistical corrections should use the actual sperm density per sample (Kleven *et al.*, 2009; Mossman *et al.*, 2009a, 2009b) as a covariate. Using testis size relative to body size (e.g. Fitzpatrick *et al.*, 2009; Desjardins *et al.*, 2011; Kustan *et al.*, 2012) is inferior because the proportion of body mass allocated to testes is not an indicator of how many or how densely sperm are transferred. Relative testis size, as a linear ratio (the gonadosomatic index, GSI), should not be used in comparative analyses (e.g. Freckleton, 2009).

## COMPARING SPERM SWIMMING SPEED BETWEEN SUBJECTS WHEN THERE IS WITHIN-SUBJECT VARIATION

The final point we consider is that $SSS_0$, or any other parameter associated with sperm swimming speed, is usually analysed as an average obtained from different hierarchical levels. For example, a taxon's mean sperm swimming speed consists of the mean of the species' sperm swimming speeds, which consists of the means of several males, which themselves consist of the means of several sperm cells. Each time a mean is used in this hierarchy (taxon–species–male–sperm cell), the question arises as to the valid representation of the mean sperm swimming speed in the next higher hierarchical level. Given that in most species most of the sperm will not reach an egg, but that high sperm swimming speed is important in achieving fertilization, we propose that maximum sperm swimming speed per ejaculate – but also per male, species or species group – may be more informative than mean sperm swimming speed (for a similar argument, see Haugland *et al.*, 2009). Thus, under selection for high sperm swimming speed, the swimming speeds of, say, the 70% or 90% slowest sperm in an ejaculate may not be biologically important but are incorporated into the statistical mean [for correlations between mean and maximum sperm swimming speed per ejaculate in zebra finch, see Mossmann *et al.* (2009a)]. While excluding most sperm from the data set may seem an inappropriate inflation of sample means, we argue that the incorporation of data that are

likely irrelevant to the fertilization outcome will also strongly affect the interpretation of the biological relevance. For example, Haugland *et al.* (2009) did not find a difference in sperm velocity between dominant and subordinate males of a fish species when analysing total ejaculate means. However, there was a difference when looking only at the 10%, 5%, and 1% fastest sperm. Similarly, Fitzpatrick *et al.* (2009) did not find a trade-off between the species' mean sperm swimming speed at 30 s after contact with water and sperm longevity. However, restricting the analysis of their data to the upper first standard deviation of sperm swimming speed, we found that species with higher sperm swimming speed at 30 s also showed a faster rate of decline over the following minutes (Pearson's correlation, $r = 0.420$, $P = 0.023$). We thus advocate the use of maximum or some upper proportion of sperm swimming speed per ejaculate (for examples, see Haugland *et al.*, 2009; Mossmann *et al.*, 2009a). Such a procedure requires similar variance in sperm swimming speed, and many studies show large differences between males in the number of sperm measured. We suggest that consideration be made of maximum values and variance in sperm swimming speed when addressing questions related to sperm competition because males of polygamous species usually produce more sperm than males of monogamous species (e.g. Simmons, 2001). Therefore, more sperm per male may be available for the measurement of sperm swimming speed from polygamous than monogamous species and so may increase the sample mean merely by increased variance in the former.

A second example of within-subject representation at a higher hierarchical level is in comparative analyses of a species when there are distinct differences in sperm swimming speed between males. For example, one fish species, *Telmatochromis vittatus*, occurs as four reproductive morphs that show differences in sperm swimming speed (Fitzpatrick *et al.*, 2007) (see Fig. 3 for the two morphs that differ the most in sperm swimming speed). These morph differences in sperm swimming speed approach, and sometimes surpass, differences between species (Fitzpatrick *et al.*, 2009) (cf. Fig. 4). Therefore, the representation of the different reproductive morphs in the sample will influence the species mean sperm swimming speed.

Similarly, in a comparative analysis, the clade mean, or maximum, sperm swimming speed may be distorted when the sperm swimming speeds of more abundant species are over-represented. How to deal with such large within-species variation depends on the biological question being asked. If the evolution of sperm swimming speed is to be reconstructed in a comparative analysis, it may be possible to allocate quasi-species status to the morphs. If the total density of sperm of all species – or morphs – in the water column is important in the ecology of reproductive isolation [for an example in broadcast spawners, see Levitan *et al.* (2004)], it may be more important to compare sperm swimming speed according to the species' – or morphs' – relative abundance or the abundance of their sperm.

## CONCLUSION AND FUTURE DIRECTIONS

The measurement of sperm swimming speed can be methodologically challenging, especially under field conditions. We suggest that some of the potential pitfalls discussed above can be circumvented by taking some precautions: (1) Measuring and comparing sperm swimming speed among males or species will be most successful when obtaining $SSS_0$, i.e. measuring sperm swimming speed from the moment of sperm activation (Levitan, 2000; Burgess *et al.*, 2004; Casselmann *et al.*, 2006). (2) Often, it may be biologically more meaningful to compare $SSS_0$, the critical time $T$, and the decline rate $\tau$, rather than comparing fixed time

points. This may also allow one to compare changes in sperm swimming speed across species with their respective time window of fertilization (Rosengrave *et al.*, 2009). Comparing $SSS_0$, $T$, and $\tau$ regardless of the fertilization window may be more useful for evolutionary comparisons of cellular ageing. (3) By contrast, comparing sperm swimming speed cross-sections at fixed time points is useful when sperm swimming speed is ecologically important in the reproductive isolation of spawning species (Levitan *et al.*, 2004) or competing reproductive morphs (Burness *et al.*, 2004). (4) Sperm density per frame should be used as a covariate. (5) We advocate the use of some maximum or higher percentile value of sperm swimming speed (Haugland *et al.*, 2009; Mossman *et al.*, 2009a) coupled with variance considerations.

We also suggest that at least two areas warrant future research. First, no data exist on how well *in vitro* studies represent *in vivo* circumstances. The differences are potentially large given that: (i) even crude sperm function measures such as survival differ markedly between the buffers used (Holman, 2009); (ii) there is large and transient variation when sperm swimming speed is examined in different ovarian fluids (Rosengrave *et al.*, 2009); and (iii) mitochondria-driven metabolism strongly depends on the environment (e.g. Murphy, 2009). Second, no studies exist for internal fertilizers that examine the relationship between sperm swimming speed before, during, and after sperm storage. This relationship could be important in accounting for currently unexplained fitness variation. Although in some studies pre-storage sperm traits are positively correlated with post-storage traits and hence predict paternity well, in others they are not (Ribou and Reinhardt, 2012) and have low predictive power of paternity or conception (Clark *et al.*, 1999; for humans, see discussion in Aitken, 2006).

Finally, given the very rapid decline of sperm function documented for freshwater fish, we think it a worthwhile challenge to document the decrease in sperm swimming speed from the very moment of sperm collection and/or sperm activation in other well-studied species in sperm biology, such as the guppy, the Arctic charr, the zebra finch, and domestic fowl under well-defined environmental conditions.

In conclusion, we have illustrated potential pitfalls in measuring and comparing sperm swimming speed in relation to sperm longevity, genotype × environment interactions, sperm density, and the within-subject distribution of sperm swimming speed. Rather than discouraging researchers owing to the substantial complexity involved, we hope we have contributed to improving the comparability of sperm swimming speed between studies and achieving further necessary standardization of sperm swimming speed (and other sperm traits) in studies in ecology and evolution (Pacey, 2009).

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