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Functional variation of sperm morphology in sticklebacks

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Abstract Some theoretical models of sperm competition make the assumption that in fish species with external fertilisation, sperm length relates positively to swimming speed at the expense of sperm longevity. Few studies have tested this assumption. We used the three-spined stickleback, *Gasterosteus aculeatus* L., to study functional sperm morphology. In this study, the relationship between males' mean sperm length and fertilisation rate was investigated in vitro in a non-competitive situation. Fertilisation at different time points after sperm release was taken into account, and sperm morphology was quantified from scanning electron microscopy images. The time series of artificial fertilisations demonstrated that males which produced sperm with a longer tail fertilised faster, but their sperm had a shorter lifespan (or activity period). It was further suggested that males that produced sperm with a larger midpiece had greater fertilisation chances later on in the fertilisation process. Thus, in sticklebacks, there exists functional variation in sperm morphology, and sperm tail length is traded off against sperm longevity (or activity).

Keywords Sperm morphology · Flagellum length · Midpiece volume · Ratio of head to tail length · Fertilisation rate · *Gasterosteus aculeatus*

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Introduction

There is an incredibly large variation in sperm number, sperm size and sperm morphology among animal species (Jamieson 1991; Matei 1991; Snook 2005; Pitnick et al. 2009a). There are various sources of pre- and post-copulatory selection that influence the evolution of sperm traits like mate choice, mode of fertilisation, cryptic female choice, sexual conflict and sperm competition (Birkhead et al. 2009). Fluctuating or context-dependent selection (e.g. Johnson et al. 2012) and non-adaptive variation due to, for instance, relaxed selection (e.g. Firman and Simmons 2009) or genetic constraints among sperm traits (e.g. Moore et al. 2004; Evans 2011) may further contribute to the variation in sperm traits. Much research has been concentrated on adaptive variation in sperm traits due to sperm competition, which was defined by Parker (1970, 1998) as "the competition of sperm from two or more males for the fertilization of a given set of ova". It is intuitively appealing to assume that any property of sperm that gives it advantages at fertilisation in competition with rival sperm would have a great selective advantage. Thus, adaptive or functional variation in sperm traits would be under strong post-copulatory sexual selection due to sperm competition. Several theoretical models support such an interpretation (e.g. Parker 1993, 1998; Ball and Parker 1996, 1997; Pizzari and Parker 2009).

Some theoretical models predict that when longer sperm swim faster and thereby have an advantage at fertilisation, then under the risk of sperm competition, sperm size will increase if sperm longevity decreases with sperm size (Parker 1993, 1998; Ball and Parker 1996). Sperm size will thus be traded off against sperm longevity. There is some evidence that sperm with longer flagella swim faster (Katz and Drobnis 1990; Gomendio and Roldan 1991; Fitzpatrick et al. 2009; Simpson et al. 2013) and in competition have better chances at fertilisation both at the inter- and intra-specific level, but the evidence here is very ambiguous

(reviewed in Pitnick et al. 2009a). For example, a comparison across fish species suggests a positive relationship between sperm length and the intensity of sperm competition (Balshine et al. 2001; Montgomerie and Fitzpatrick 2009; but see Stockley et al. 1997, who found just the opposite relationship). Ambiguous results may for instance be the consequence of considering non-optimal variables. Recently, Humphries et al. (2008) criticised the assumption that sperm length is directly related to sperm velocity when considering the hydrodynamic sperm environment and instead suggested that the ratio of head (including midpiece) to tail length would be better related to sperm velocity especially in external fertilisers; the lower the ratio of head to tail length the higher sperm velocity. The mode of fertilisation may be another source for ambiguous results (Matei 1991; Pitnick et al. 2009a; Simpson et al. 2013). In fish for instance, sperm length of species with internal fertilisation is greater than that of species with external fertilisation (Stockley et al. 1996). In species with internal fertilisation, there is additional co-evolution taking place between sperm traits and traits of the female reproductive tract (e.g. birds: Briskie and Montgomerie 1992; mammals: Anderson et al. 2006; diving beetles: Higginson et al. 2012), so there is much scope for sperm–female interactions (Pitnick et al. 2009b). Functional sperm morphology is therefore most easily studied in species with external fertilisation because fertilisation rate is here not influenced by characteristics of the female reproductive tract.

In addition to sperm tail length and ratio of head to tail length, the midpiece, which contains mitochondria that produce energy for sperm motility, is a further component of sperm that may evolve under the risk of sperm competition. The relationship between sperm midpiece dimensions and the risk of sperm competition as assessed from interspecific studies can be positive or negative (reviewed in Pitnick et al. 2009a). In many fishes, the midpiece is, however, too small to discern using light microscopy and non-distinguishable from the head component (Gage et al. 2002).

The three-spined stickleback, *Gasterosteus aculeatus* L., is a small territorial, externally fertilising fish species with male only parental care (Wootton 1976, 1984; Bell and Foster 1994). In spring and early summer, males settle a territory in shallow water, develop conspicuous breeding colouration and courtship and build a tunnel-shaped nest of plant material in which they can collect eggs of many females in a short period of time (Kraak et al. 1999; Bakker et al. 2006). Neighbouring males may try to steal fertilisations in the nest in which the female has spawned by ejaculating shortly after (sometimes even before) the nest owner has done so (Largiadèr et al. 2001; Bakker et al. 2006; Candolin and Vlieger 2013). Field data showed that up to 25 % of stickleback nests contained eggs that had been fertilised by sneakers (Largiadèr et al. 2001). There is thus much scope for sperm competition in this species. This is especially true as the mucous around the eggs prolongs sperm longevity (Elofsson et al. 2003). Fertilisation in sticklebacks is slow for a fish with external fertilisation as complete fertilisation takes 10 min or more (Bakker et al. 2006).

The aim of the present study was to investigate functional variation in sperm morphology of three-spined sticklebacks in a non-competitive context. Do males that produce longer sperm have, according to some theories, better fertilisation abilities and do their sperm have, according to some theories, a shorter lifespan? Do males that have sperm with a smaller ratio of head to tail length fertilise, according to expectation, faster, and is it beneficial to males to have sperm with greater midpieces? These questions were approached by linking the rate of the fertilisation process by sperm of single males in vitro to their sperm morphology.

Material and methods

Experimental animals

In the experiment, first-generation fish were used from wild-caught parents of a Dutch anadromous three-spined stickleback population that were caught late March 2001 during spring migration on the island of Texel (53°03′ N, 4°48′ E), the Netherlands. Parents were transported to our institute, reproduced and the offspring were raised in full-sib groups of 10–20 individuals in 50-l tanks filled with tap water. They were daily fed ad libitum with defrosted red and black chironomid larvae and *Artemia*. The tanks were situated in an air-conditioned room of 18±2 °C with a light regime of 16L:8D provided by fluorescent tubes above the tanks. Males with developing breeding colouration were taken from the stock and placed into individual 20-l aquaria ($l \times w \times h$, 40×20×25 cm) containing fine gravel at the bottom and 4-cm pieces of green cotton twine (Patricia), which were boiled off before, to serve as nesting material. The aquaria were separated by opaque grey partitions in order to prevent interactions between the males. Males were regularly stimulated with a gravid female enclosed in a cuvette (12×7×17 cm) in front of their tanks in order to stimulate nest building. Only males with fully developed nuptial colouration that had built a nest were used in the experiments. All males finished their nest within 1 week after the provision of nesting material. Experiments were done 3 days after nest completion. All males used in the experiment were unrelated. Females were held in groups of nine fish in 45-l tanks and fed with defrosted red blood worms. Water in all tanks was cleaned and aerated through an internal filter, and half of it was exchanged once a week with tap water. All tanks were lit by 36-W fluorescent tubes hanging 25 cm above the water surface.

Sperm preparation

In order to collect a male's sperm, the gonads had to be dissected as stripping sperm is not possible in stickleback males (but see de Fraipont et al. 1993). Males' standard body length and body mass (Sartorius LC 221S) were measured before they were quickly killed by decapitation. The testes were carefully dissected, the left and right testis put separately into an Eppendorf tube containing 200 μl isotonic non-activating medium after Fauvel et al. (1999), and homogenised in the Eppendorf tube with a small pestle. The resulting suspension was vortexed and centrifuged (Fisher Scientific MicroV, 3,200 rpm) for about 2 s to remove remaining tissue. The supernatant was used for counting sperm density (after Zbinden et al. 2001) and afterwards for the in vitro fertilisation experiments (see below). For counting, sperm density was reduced 20 times by diluting 10 μl of the supernatant with 190 μl tap water. A Neubauer haemocytometer chamber (Labor Optik, depth 0.1 mm, cell size 0.0025 mm^2) was then filled with a 12-μl sample of this solution. The number of sperm was counted in 64 cells (2.5×10^{-4} μl each) of the chamber. The total number of sperm in the male's left or right testis was calculated as: sperm number $=1/0.00025\times200\times20\times$(mean number of sperm per cell) $=16\times10^6\times$(mean number of sperm per cell) (Mehlis et al. 2012).

Sperm morphometry

Sperm morphometry was performed with scanning electron microscopy (SEM) images, although in *G. aculeatus*, all sperm components can be measured with light microscopy when using appropriate equipment that was not available to us at that time (Mehlis et al. 2013; Rick et al. 2013). Preparation of sperm for SEM was done after Mortimer (1994, pp. 168–173: Preparation of human spermatozoa for electron microscopy) adapted for stickleback sperm (see supporting information in Mehlis et al. 2012). Sperm morphometry was done using 2,500–3,000 magnified sperm SEM images (digitised Leitz AMR 1000). They were digitised using Digital Image Scanning System (Point Electronic, Halle, Germany) and Digital Image Processing System (V 2.5.2.1; Point Electronic, Halle, Germany). Morphometry was performed using the software DISKUS (Carl H. Diskus, Königswinter, Germany).

The sperm of three-spined sticklebacks are morphologically simple. They are typical of derived, externally fertilising teleosts and are termed aquasperm (Jamieson 1991; Mattei 1991). They have a round head without an acrosome, a single, generally unadorned but keeled flagellum and a short midpiece with a prominent cytoplasmic canal (Jamieson 1991, 2009; Lemke et al. 1999; Fig. 1). Sperm tail length (that is, flagellum length without midpiece length using only complete tails recognisable by a pointed end), head width, head length, midpiece width and midpiece length were measured from SEM images (Fig. 1). Comparisons between tail length measurements from SEM and light microscopy images show the reliability of the sperm tail length measurements from SEM images (Online Resource 1). Head and midpiece could be distinguished by a constriction. Width of the head was measured halfway head length and width of the midpiece halfway the midpiece (Fig. 1). From some sperm all traits could be measured, from others only tail length or head and midpiece dimensions. The number of sperm measured per male therefore varied for the different sperm dimensions. The fertilisation experiments (see below) were performed with ten males. Per male, between 10 and 43 sperm (median 19) were used for the measurement of tail length and between 6 and 10 (median 10) for the measurement of head and midpiece dimensions. One sperm (out of 91 measured sperm) was removed from the analyses for the head and midpiece dimensions as it was identified as an outlier (midpiece volume (see below) was more than twice as great as in the other sperm of this male and other males; also the head dimensions were the greatest of this sperm compared to all other measured sperm), although its inclusion did not lead to different conclusions.

Midpiece volume was calculated after (corrected) Anderson and Dixson (2002) assuming the form of a cylinder: $\pi\times$ midpiece $radius^2\times$ midpiece length in cubic micrometer. As in studies of sperm morphology, sperm midpiece and sperm head cannot always be distinguished and thus measured separately (e.g. Gage et al. 2002), we also calculated "head" head inclusive of midpiece volume assuming a spherical shape of the real head: $1/6\times\pi\times((\text{head length}+\text{head width})/2)^3+$(midpiece volume) (see also Mehlis et al. 2012).

Measurement repeatability of sperm dimensions

Sperm from 16 males were independently measured twice in order to determine the repeatability of the measurements and its SE according to Becker (1992). All measurement repeatabilities were highly significant (one-way ANOVA, all $P<0.001$): $r_I\pm SE=0.993\pm0.034$, 0.704 ± 0.130, 0.737 ± 0.118, 0.808 ± 0.090 and 0.663 ± 0.145 for tail length, head length, head width, midpiece length and midpiece width, respectively.

In vitro fertilisation

Artificial fertilisation was done after the protocol of Barber and Arnott (2000) adapted by Bakker et al. (2006). Eggs were obtained by stripping gravid females by gently squeezing the abdomen with thumb and forefinger. The clutch of eggs was put into a watch glass with tap water and carefully divided using two insect forceps into eight more or less equal portions of eggs. Each portion was put into a watch glass with 2 ml of tap water. In half of the experiments, a second gravid female

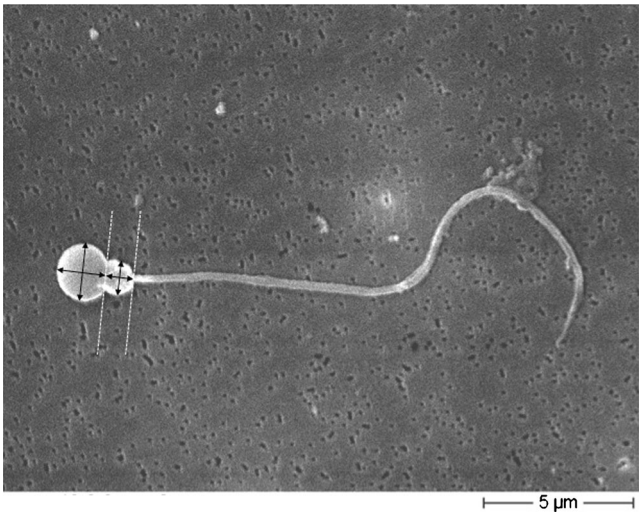

Fig. 1 Scanning electron microscope image of a sperm of a three-spined stickleback consisting of a head, midpiece and tail. Indicated are the separations between head and midpiece and between midpiece and tail by *dotted lines*, the measurements of head width and length and midpiece width and length by *double-headed arrows*

was available, which was also stripped and her eggs also divided into eight equal portions. This means that half of the fertilisation trials involved mixed eggs from two females, while in the other half, eggs from just one female were used. If available, two females were used to increase the reliability of the data as one clutch consisted maximally of about 250 eggs that were divided into eight portions. The eggs of the two females were combined, so that eight about equal portions (Friedman test, $N=10$, $\chi^2=4.599$, degrees of freedom (df)=7, $P=0.71$) were available for artificial fertilisation. Between fertilisation experiments, the portions ranged between 7 and 67 eggs (median 33 eggs). In order to obtain sperm for fertilising the eggs, an unrelated, reproductively active nest-holding male was dissected; his sperm were prepared and sperm concentration was determined (see above). Using a pipette, 10×10^6 sperm were distributed over each of the eight portions of eggs, four portions with sperm from a male's left testis and four with those from his right testis. The number of sperm used corresponded to the average first ejaculate size of males of this population (Zbinden et al. 2001). The time order of fertilisation was systematically changed between males in order to avoid sequence effects. Due to the differences in sperm concentration between males, different volumes of the sperm suspensions were used to fertilise the eggs (median 10.53 µl, range 6.56–24.69 µl).

The fertilisation of the eggs in the eight portions was stopped either after 60, 120, 300 or 600 s. This was done by pipetting 1 ml of fresh commercial sparkling mineral water (Christinen-Mineralwasser, Teutoburger Mineralbrunnen GmbH & Co., Bielefeld, Germany; HCO_3^-, 0.543 g/l; Na^+, 0.371 g/l; Cl^-, 0.304 g/l; SO_4^{2-}, 0.124 g/l; Ca^{2+}, 0.055 g/l; NO_3^-, <0.0003 g/l) into the watch glass. Sparkling mineral water kills spermatozoa without harming the eggs (Bakker et al. 2006). A few minutes after stopping the fertilisation, eggs were transferred to plastic cups filled with aerated tap water and placed in the aquarium rooms. Almost all water in the cups was exchanged twice a day to prevent the development of fungi. After 3–4 days, it was checked under a binocular microscope whether the eggs had been fertilised or not, which in the transparent stickleback eggs can be easily assessed by observing embryo development (Swarup 1958). We repeated the test with ten different males, and each time with new ripe females. The fertilisation rates of portions of eggs fertilised by left and right testes were not significantly different (Wilcoxon signed-rank test, all $P>0.20$) and thus averaged to increase reliability.

Control experiment

As the experimental design may confound sperm age with egg age, a control experiment was done in which freshly gained sperm were added to eggs that had been kept during 9 min in the watch glass. Fertilisation was stopped after 1 min. If the results of the main experiment are due to sperm age rather than egg age, then the results of the control experiment should be comparable to those of the main experiment in which fertilisation was also stopped after 1 min. If egg age played a role, then the results should be more comparable to those of the main experiment in which fertilisation was stopped after 10 min.

In the control experiment, wild-caught, reproductively active three-spined stickleback males and laboratory-bred first-generation females were used. They originated from the 2010 and 2011 Texel population, respectively. They were maintained in the lab under similar conditions as the 2001 sample used for the main experiment.

Artificial fertilisations were done as described above for the main experiment except for some details explained below. In each control trial, the eggs of two females were combined and afterwards carefully divided using two insect forceps into four equal portions of 30 eggs (median 30, range 28–32). Each portion was put into a watch glass with 2 ml of tap water. After the dissection of the males, both testes were pestled in 400 µl non-activating medium (Fauvel et al. 1999) and sperm number was determined as described above. As sperm concentration differed between males, four sperm suspensions per male containing ten million sperm each (median 13.22 µl, range 8.22–24.48 µl) were filled up with non-activating medium in order to obtain the same volume of sperm suspension (100 µl each). Before adding sperm, females' eggs were left in the watch glass for 9 min. Then ten million sperm were distributed over each of the four egg portions using a pipette. Fertilisation was stopped after 60 s, by pipetting 1 ml of fresh commercial sparkling mineral water (Gerolsteiner Sprudel: CO_2, 7 g/l;

HCO_3^-, 1.816 g/l; Na^+, 118 mg/l; Cl^-, 39.7 mg/l; Ca^{2+}, 348 mg/l; NO_3^-, 5.1 mg/l) into the watch glass. After 48 h, fertilisation rate was checked using a binocular microscope (Leica S8AP0). The experiments were conducted with 20 males and 20 females to avoid pseudoreplication. In the control experiment, an improved protocol for artificial fertilisations was used in order to maximise the fertilisation rate. However, the control experiment was only done for checking whether the relationship between tail length and fertilisation rate was positive or negative when using older eggs. Thus, the exact rates of fertilisation of the main experiment and the control experiment needed not to be comparable.

Sperm morphometry was determined using light microscopy (Olympus BX 51, ×100 objective lens) images (see also Mehlis et al. 2013; Rick et al. 2013). Pictures were taken 1–3 h after testis dissection. To that aim, 5 μl sperm suspension was put on a microscope slide. The cover slip was fixed with transparent nail polish. Per male, the tail length of 30 sperm was measured. Measurement repeatability using this method was high (as determined in Mehlis et al. (2013) who used the same method to measure sperm morphometry).

Statistical analyses

Parametric statistics were used, as data and/or residuals in regression analyses did not significantly deviate from normal distributions according to Kolmogorov–Smirnov tests with Lilliefors correction. Associations of fertilisation rate and each of the potential determinants sperm tail length, midpiece volume, "head" volume and ratio of "head" to tail length were analysed for each of the four time stages (fertilisation stopped 60, 120, 300 or 600 s after the adding of sperm) with univariate linear regression analysis using the SPSS 15.0 statistical package (www.spss.com). Changes of the associations over time were analysed with four (one for each potential determinant) linear mixed-effect models (R version 2.9.1 function "lme" in library "nlme"; Pinheiro et al. 2009), with male identity as random factor to control for the multiple use of males in the time series. Although the effect of male identity was not significant in all models (Table 1), male identity was not removed from the models to avoid pseudoreplication. The dependent variable was fertilisation rate, and the explanatory variables were either tail length, ratio of "head" to tail length, midpiece volume or "head" volume, time (60, 120, 300, 600 s) and the trait-by-time interaction. To test the statistical significance of the interaction, these full models were compared with models without the interaction term. The statistical significance of each explanatory variable separately (that is, each sperm trait and time without the trait-by-time interaction) was tested by removing explanatory variables in the order of statistical relevance and these reduced models were subsequently compared with the full models (but without the trait-by-time interaction term). Tests of statistical significance were based on likelihood ratio tests (LRT) that follow a χ^2 distribution. All given P values were based on two-tailed tests.

Results

As expected, fertilisation success increased with time. Mean percentages fertilised eggs ± SD by ten males were 8.29± 6.04, 13.94±13.74, 21.30±10.42 and 25.99±14.75 % after stopping fertilisation 60, 120, 300 and 600 s, respectively, after the addition of sperm. The increase is significant (ordered heterogeneity test after Rice and Gaines, 1994: Friedman test, $\chi^2=12.60$, $df=3$, $P=0.003$, $r_s=1$, $P_c=0.997$, $P<0.001$; see also the significance of time in Table 1). The maximum fertilisation rate attained in the main experiment was 49.15 %.

Sperm dimensions and repeatabilities

The ten males measured 48.80±2.86 mm (mean ± SD, range 46–54 mm) standard length and had a mean sperm store ± SD of $35.0\pm12.1\times10^7$ (range 15.0×10^7–52.6×10^7) sperm cells. The descriptive statistics of their sperm dimensions are listed in Table 2. Consistent differences among males in sperm morphology were expressed as male repeatabilities. These were significant for tail length and head dimensions (head length, head width, "head" volume), but not for midpiece dimensions (midpiece length, midpiece width, midpiece volume) (Table 2).

Fertilisation success related to sperm morphology

When fertilisation was stopped after 60 s, the proportion of eggs that was fertilised correlated significantly positively with the males' mean sperm tail length (Fig. 2a; $y=-48.67+2.72x$, $r^2=0.41$, $N=10$, $F=5.64$, $df=1,8$, $P=0.045$). This was also the case after 120 s (Fig. 2b; $y=-131.50+6.95x$, $r^2=0.52$, $N=10$, $F=8.70$, $df=1,8$, $P=0.018$). But after 300 s, the relationship disappeared (Fig. 2c; $y=-24.99+2.21x$, $r^2=0.09$, $N=10$, $F=0.81$, $df=1,8$, $P=0.395$). And after 600 s, it even tended to become negative (Fig. 2d; $y=153.83-6.11x$, $r^2=0.35$, $N=10$, $F=4.29$, $df=1,8$, $P=0.072$). The regression coefficients changed significantly over time: significant interaction term "tail length×time" according to LRT (Table 1).

Similar but less strong effects were associated with among male differences in ratio of "head" to tail length of their sperm ($P=0.088$, 0.111, 0.397 and 0.088 when stopping fertilisation after 60, 120, 300 and 600 s, respectively). Here, the regression coefficients changed significantly from negative after 60 s to positive after 600 s (interaction term "head" length to tail length×time" in Table 1).

Males that produced sperm with greater midpiece volume achieved higher fertilisation rates in the later fertilisations, that

Table 1 Linear mixed-effect models of associations of fertilisation rate and one of the potential determinants sperm tail length, ratio of "head" to tail length, midpiece volume and "head" volume

Dependent variable	Explanatory variable	χ^2	*P* value	Δdf
Proportion of eggs fertilised [%]	Tail length [μm]×time [s]	18.817	**<0.001**	3
	Tail length [μm]	1.120	0.290	1
	Time [s]	12.785	**0.005**	3
	Male [random factor]	1.729	0.189	1
Proportion of eggs fertilised [%]	ratio of "head" to tail length×time [s]	12.231	**0.007**	3
	ratio of "head" to tail length	0.645	0.422	1
	Time [s]	12.785	**0.005**	3
	Male [random factor]	0.873	0.350	1
Proportion of eggs fertilised [%]	Midpiece volume [μm^3]×time [s]	11.624	**0.009**	3
	Midpiece volume [μm^3]	3.367	0.067	1
	Time [s]	12.785	**0.005**	3
	Male [random factor]	0.190	0.663	1
Proportion of eggs fertilised [%]	"Head" volume [μm^3]×time [s]	9.415	**0.024**	3
	"Head" volume [μm^3]	0.005	0.945	1
	Time [s]	12.785	**0.005**	3
	Male [random factor]	0.980	0.322	1

Time (that is, fertilisation was stopped 60, 120, 300 or 600 s after the addition of sperm to the eggs) was included in each model as well as male identity to account for the use of sperm of each male in four time treatments. N=10 males. Tests of statistical significance were based on likelihood ratio tests that follow a χ^2 distribution. Significant P values (P<0.05) are printed in bold. "Head" refers to head inclusive of midpiece

is, when fertilisation was stopped 600 s after the addition of sperm (y=−52.06+107.08x, r^2=0.71, N=10, F=19.51, df=1,8, P=0.002). At shorter fertilisation times, no significant relationship was detected (60 s: y=16.48−11.24x, r^2=0.05, N=10, F=0.39, df=1,8, P=0.549; 120 s: y=21.63−10.55x, r^2=0.01, N=10, F=0.06, df=1,8, P=0.807; 300 s: y=−2.29+32.36x, r^2=0.13, N=10, F=1.19, df=1,8, P=0.306). The relationship between fertilisation rate and the males' mean sperm midpiece volume changed significantly over time (interaction term "midpiece volume×time" in Table 1).

Males' mean sperm head dimensions (head width, head length) were not significantly correlated to fertilisation rate at any stage (all P>0.13). The associations of "head" volume and fertilisation rate were similar to those of midpiece volume but less pronounced (P=0.213, 0.369, 0.557 and 0.075 when stopping fertilisation after 60, 120, 300 and 600 s, respectively; the association changed significantly over time: Table 1).

As there existed a significant interaction of sperm dimension and time for each tested sperm dimension with respect to fertilisation rate (Table 1), the relationship between the separately tested sperm dimensions and associated fertilisation rates was not significant when time was not taken into account (Table 1; all P>0.067).

Control experiment

The 20 control males had a mean sperm store ± SD of 30.0 ± 9.2×10^7, range 16.3×10^7–48.7×10^7 sperm cells, which is similar to that of males of the main experiment (unpaired t test, t=1.28, df=28, P=0.213). Mean ± SD (range) tail length of the sperm of control males was 24.39±1.83 μm (20.47–27.59 μm), which is, as expected (see Online Resource 1), significantly longer than the mean sperm tail length of the males from the main experiment (unpaired t test, t=5.25, df=

Table 2 Descriptive statistics (mean, SD, range) and male repeatability r_I ± SE (according to Becker 1992) of various morphological sperm traits from ten males

Sperm trait	Mean ± SD (range)	r_I ± SE	n_0	F	df	P
Tail length	20.92±1.43 μm (18.67–23.64)	0.088±0.058	21.15	3.045	9,206	**0.002**
Head length	1.23±0.09 μm (1.14–1.43)	0.158±0.106	8.98	2.684	9,80	**0.009**
Head width	1.26±0.12 μm (1.13–1.51)	0.384±0.139	8.98	6.589	9,80	**<0.001**
"Head" volume	1.77±0.33 μm^3(1.41–2.45)	0.747±0.004	8.98	3.958	9,80	**<0.001**
Midpiece length	0.88±0.05 μm (0.79–0.97)	0.026±0.066	8.98	0.771	9,80	0.644
Midpiece width	1.01±0.06 μm (0.92–1.05)	0.025±0.065	8.98	1.229	9,80	0.289
Midpiece volume	0.73±0.12 μm^3 (0.58–0.93)	0.024±0.065	8.98	1.217	9,80	0.296

The significance of repeatability was tested with a one-way ANOVA. Indicated are the adjusted number of measured sperm per individual (n_0), F statistics, degrees of freedom (df) and P value. Significant P values (P<0.05) are printed in bold. "Head" refers to head inclusive of midpiece

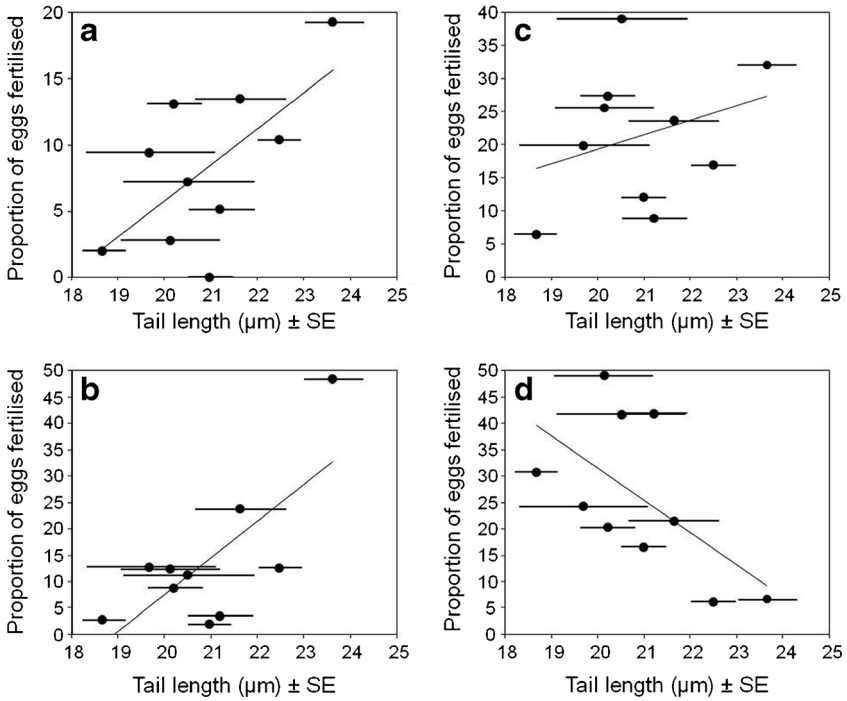

Fig. 2 Proportion of eggs fertilised as a function of mean sperm tail length ± SE (micrometre) per male of ten males when fertilisation was stopped **a** 60 s, **b** 120 s, **c** 300 s and **d** 600 s after the addition of sperm to the eggs. The *lines* are the least square regressions

28, $P<0.001$). Also, the fertilisation success was significantly higher in the control experiment (45.56±14.29 %) than in the main experiment even when fertilisation was stopped there after 600 s (unpaired *t* test, $t=3.50$, $df=28$, $P=0.002$).

In the control experiment, fertilisation success was significantly positively correlated with males' sperm tail length ($y=-44.65+3.70x$, $r^2=0.22$, $N=20$, $F=5.20$, $df=1,18$, $P=0.035$) like in the main experiment when fertilisation was stopped after 60 s. Similarly as in the 60-s main experiment, fertilisation success was negatively correlated, but not significantly, with males' sperm ratio of "head" to tail length (put head between quotation marks)($r=-0.31$, $P=0.182$). Lastly, fertilisation success correlated significantly positively to males' sperm midpiece volume and "head" volume ($r=0.47$, $P=0.037$ and $r=0.45$, $P=0.048$, respectively).

Discussion

By taking fertilisation at different time points into account, in this intra-specific, non-competitive study several predictions of some theoretical models of sperm competition could be confirmed with one data set. Males that produced sperm with longer tails had a higher fertilisation rate in the early stages of the fertilisation process than males with short-tailed sperm, but had lower success in later stages. This is indirect evidence for a trade-off between sperm tail length and sperm longevity (or at least activity). Males that had sperm with large midpieces were more successful in later stages than males that possessed sperm with midpieces of smaller volume. The results make clear that there exists variation in sperm morphology between stickleback males that is obviously functional in a non-competitive context. It is likely that the same functionality is valid in a competitive context (see, e.g. Dean and Nachman 2009). Males with longer sperm (tail length makes up about 90 % of total sperm length in sticklebacks) fertilise faster, but their ejaculated sperm die (or become less active) sooner than those of males with shorter sperm, and males that produce ejaculates with sperm of larger midpieces have advantages at later stages of the fertilisation process. A control experiment showed that sperm age was more important than egg age in generating the time effects involving tail length.

It is generally believed that the advantage of longer sperm is higher swimming speed and thus better fertilisation chances by reaching the site of fertilisation quicker or by having better penetrating ability of the egg (Fitzpatrick et al. 2010). The evidence is mixed and better from inter-specific comparisons (e.g. mammals: Gomendio and Roldan 1991, Tourmente et al. 2011; birds: Lüpold et al. 2009 but see Kleven et al. 2009; fishes: Fitzpatrick et al. 2009) than from intra-specific studies. In the latter, a positive relationship between males' sperm length (or flagellum length) and swimming speed was found in some studies (like in red deer: Malo et al. 2006; rainbow trout: Tuset et al. 2008; cichlid species: Fitzpatrick et al. 2009; sea urchin: Fitzpatrick et al. 2010) but not in others (like in cichlid species: Fitzpatrick et al. 2007, 2009; frog: Dziminski et al. 2009; tree swallow: Laskemoen et al. 2010; house mouse: Firman and Simmons 2010; house sparrow: Helfenstein et al. 2010; mussel: Fitzpatrick et al. 2012).

A recent study suggested that in the physical environment of tiny sperm, the ratio of "head" to tail length is the determinant of swimming speed (Humphries et al. 2008; see Helfenstein et al. 2010 for an empirical example). In our main experiment, the correlations between fertilisation rate and males' sperm ratio of "head" (thus inclusive midpiece) to tail length were less strong than with only males' sperm tail length, but our measurements were not ideal to use this measure as both length measurements were not necessarily done on the same sperm, so that we had to use average values per male. In three-spined sticklebacks from the same population as used in the present study, ratio of "head" to tail length correlated negatively with the linearity of sperm trajectory, which was correlated positively to sperm velocity (Mehlis et al. 2013).

In some theoretical models of sperm competition, the assumption is made that males' sperm length trades off against sperm longevity (Parker 1998). In the present study, we found indirect evidence for this: there was a significant change (from positive to negative) in the relationship between males' mean sperm tail length and fertilisation rate as a function of time. Comparative work consistent with the existence of a trade-off between sperm length and longevity had also been found, for instance, in inter-specific studies of fishes (Stockley et al. 1997) but not in cichlid fishes (Fitzpatrick et al. 2009). In an intra-specific study of house mice, composite sperm velocity was negatively correlated to sperm longevity, although sperm flagellum length did not significantly correlate to sperm velocity but sperm midpiece length did (Firman and Simmons 2010). In the house sparrow, sperm with longer total length (and to a lesser extent longer flagellum length) lived shorter (Helfenstein et al. 2010). A trade-off between average sperm velocity and sperm longevity was measured in the sea urchin (Levitan 2000).

With respect to the function of variation in sperm midpiece size, there exist relatively few direct studies, possibly as a result of difficulties in distinguishing the midpiece with a light microscope (Gage et al. 2002). The few studies in fishes point to a positive effect of midpiece size on sperm velocity, but Evans (2011) found a strong negative genetic correlation between sperm midpiece length and sperm's swimming velocity (average path velocity (VAP)) in guppies, *Poecilia reticulata.* Also in guppies, Skinner and Watt (2007) assessed a positive correlation between sperm velocity (straight line velocity) (VSL) and midpiece area 15 min after activation of the sperm but not 1 min after activation. The changing influence of the midpiece with time is reminiscent of the results of the present study. Interestingly, Elgee et al. (2010) found in an inter-populational study that guppy males of high-predation downstream populations, in which sperm competition is probably stronger due to sneaky matings, had faster swimming sperm with longer midpieces than males in low-predation upstream populations. In swordtails, *Xiphophorus nigrensis*, sneaker males produced sperm with longer midpieces, which was positively correlated with both velocity (VAP) and longevity (Smith and Ryan 2010). As a small ratio of "head" (thus inclusive midpiece) to tail length relates to high sperm swimming velocity (Humphries et al. 2008), too great an increase in midpiece size may rather counteract swimming speed.

A cautionary note has to be added to the conclusions of our study concerning midpiece dimensions as their male repeatabilities were not significantly different from zero. In the analyses, we used the mean dimension scores of a median of ten sperm per male, which should theoretically have a higher repeatability as the phenotypic variance of the means will be lower than that of the individual measurements (Falconer 1989). Additionally, a later, bigger sample size (30 sperm measured from 9 males each) using males from the same Texel population and the same SEM preparation methods yielded male repeatabilities that were all significantly different from zero (Online Resource 2). Finally, other than with tail length, the results of the control experiment could not exclude an effect of egg age on the changing relationship over time between fertilisation rate and midpiece volume. Further experiments are needed here.

Few studies have considered the dynamics of fertilisation in assessing functional variation in sperm morphology, although one should obviously expect time effects, especially in species where fertilisation is not instantaneous and several eggs are simultaneously available as in sticklebacks (Bakker et al. 2006). Exceptions to this are the studies by Levitan (2000), Burness et al. (2004), Skinner and Watt (2007), Pizzari et al. (2008) and Lifjeld et al. (2012). Pizzari et al. (2008) experimentally studied time effects of sperm competition in domestic hens *Gallus gallus domesticus* but at a quite different time scale as in the present study. Their time scale covered egg laying during 12 days, so they studied the outcome of sperm competition in the sperm storage organs. The first eggs were fertilised by large ejaculates of low mobility, but later on, small ejaculates of sperm of high mobility had higher fertilisation chances (Pizzari et al. 2008). Burness et al. (2004) studied sperm competition dynamics of sperm of sneakers and parental males of bluegill, *Lepomis macrochirus.* Sperm of sneakers had faster initial swimming speeds but shorter periods of motility than sperm of parentals. Sneakers were therefore more successful despite having fewer sperm per ejaculate than parentals (Burness et al. 2004). Skinner and Watt (2007) found differences in sperm velocity of sperm of guppies, *P. reticulata*, immediately after activation and 15 min later (i.e. the period of ovarian migration). In pied flycatchers, *Ficedula hypoleuca*, longer sperm were less able to maintain velocity (curvilinear velocity) (VCL) over a time period of 10 min (Lifjeld et al. 2012). In sea urchins, *Lytechinus variegates*, males with faster sperm achieved a higher fertilisation rate when fertilisation was stopped after 10 s (Levitan 2000). As sperm age (measured up to 120 min),

sperm velocity, fertilisation rate (in the first 10 s after the addition of sperm to eggs) and percentage of active sperm decreased (Levitan 2000). Thus, by incorporating time, the understanding of the outcome of sperm competition may be greatly improved.

The reasons for the ambiguous results of some predictions of the tests of sperm competition theory are multifarious (Snook 2005). One reason is variation in fertilisation mode (Snook 2005; Simpson et al. 2013). By studying a fish species with external fertilisation, influences of properties of the female reproductive tract were ruled out. Some influences of the female could not be avoided as the egg slime around the eggs influences sperm longevity in sticklebacks (Elofsson et al. 2003) and also sperm motility in some fishes (e.g. in Artic charr, Urbach et al. 2005). Interacting effects between eggs and sperm also remain (Karr et al. 2009; Gasparini and Pilastro 2011; Butts et al. 2013). Effects of egg age were largely ruled out in a control experiment, at least for tail length and ratio of "head" to tail length. Tests of predictions from sperm competition theory might benefit from taking the dynamics of the fertilisation process into account. In the present study for instance, over a time period of 10 min, the correlation between tail size and fertilisation success changed significantly from positive to negative and thereby supported some predictions of sperm competition theory.

We followed the artificial fertilisation protocol of Barber et al. (2001) adapted by Bakker et al. (2006), but the fertilisation rates achieved in those studies (53 and 83–94 %, respectively) were higher than those obtained in the present study. The reasons for this are unknown, but compared to Bakker et al. (2006) in the present study, on average, three times less sperm per egg were used. However, in the control experiment in which equally low numbers of sperm per egg were used, mean fertilisation rate was as high as 46 %. The higher fertilisation rate was here probably due to a more efficient distribution of sperm cells over the eggs by standardising the amount of sperm-containing fluid to 100 μl, almost ten times as much as in the main experiment. Erroneously, the formula for the calculation of sperm number in Bakker et al. (2006) is incorrect: it should be multiplied by 2, so that the estimations of the sperm numbers in that paper are too low by a factor of 2.

In sticklebacks, females spawn all their ripe eggs at once in a clutch, which may consist of several hundreds of eggs (Wootton 1976). The territory owner (and eventual sneakers) will then ejaculate on the top of the clutch of eggs. The eggs in the clutch vary with respect to their reachability for sperm; some eggs lie on top of the clutch while others are covered by one or more layers of eggs. After ejaculation, the territory owner will, if not disturbed by sneakers, vigorously push on top of the nest in order to flatten the clutch of eggs (Wootton 1976). In this way, further clutches can be collected in the nest. There is thus inter-egg variation in the accessibility for sperm. This variation may be one reason for the relatively high within-male variation and thus low male repeatability of sperm traits in the present study. It may be a strategy to maximise fertilisation success (see also Helfenstein et al. 2010). It will be a challenge to disentangle variation in egg reachability from those of the risk of sperm competition on within-male variation of sperm traits in sticklebacks and animals with comparable reproductive behaviour. In birds, the risk of sperm competition was negatively related to within- and between-male variation in sperm traits both at the inter- (e.g. Lifjeld et al. 2010) and the intra-specific level (Laskemoen et al. 2013). A first comparison of sperm traits between stickleback populations revealed significant differences (Online Resource 1). A comparison of sperm traits of stickleback populations that differ in the risk and intensity of sperm competition may be a first avenue to understand within-male variation in sperm traits.

In summary, in this intra-specific test on functional variation of a male's sperm dimensions in a fish with external fertilisation, we incorporated the dynamics of fertilisation. Initially, males that had sperm with longer tails achieved higher fertilisation rates suggesting that they swim faster or have better penetrating ability. Later on, males that produced sperm with smaller tails tended to have higher fertilisation rates suggesting that long-tailed sperm had a shorter lifespan. Also at a more advanced stage, males that had sperm with a greater midpiece volume seemed to achieve higher fertilisation rates suggesting that energy supply may play a role in the long run. These results not only confirm several predictions of some theoretical models of sperm competition but also show that through the dynamics of fertilisation selection on sperm morphology is not necessarily directional under sperm competition. Different sperm phenotypes may have advantages at different stages of the fertilisation process.

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Ethical standards The experiments comply with the current laws of Germany in which they were performed.

Conflict of interest The authors declare that they have no conflict of interest.

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Online Resource 1

Functional variation of sperm morphology in sticklebacks

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Comparison of sperm dimensions from SEM and light microscopy images

In order to check whether tail length remained undamaged during SEM preparation a comparison was made between tail length measurements from SEM and light microscopy images of sperm from males of the used population as well as of a different population in which the males produce larger sperm lengths (see below). Sperm of 10 males were prepared for light microscopy (according to Balshine et al. 2001). For light microscopy analysis testes were stored in 4 % formalin, slit open, and free milt was distributed on a slide. If free milt was not apparent, testes were squeezed or scraped and the liquid spread finely with formalin (Balshine et al. 2001). The slides were air dried and viewed at 400x magnification. Tail length was measured beginning after the head/midpiece to the end of the tail by tracing a freehand line using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image). For each male the tails of ten sperm of light microscopy images were measured and averaged for the analysis. For the used population, a comparison, albeit indirect, was made between the mean tail length of sperm of 10 males (from the 2001 Texel population and used in the fertilisation experiment) measured from SEM images and the mean tail length of sperm of 10 wild-caught, reproductively active males (from the 2005 Texel population) using light microscopy images. Mean tail length did not significantly differ between the two preparation methods (mean ± SD=20.92 µm ± 1.43 and 20.42 µm ± 2.81 for the SEM and light microscopy method, respectively; unpaired t test, t=0.50, df=18, P=0.62).

In addition, the mean tail lengths of sperm of wild-caught, reproductively active males each from a pond population in Euskirchen (50°38' N, 6°47' E, near Bonn, Germany) prepared according to the same preparation methods (2002 samples using SEM, N=12, and 2005 samples using light microscopy, N=10), were compared, albeit again indirectly. Also here did mean tail length not significantly differ between the two methods (mean ± SD=24.44 µm ± 3.20 and 25.10 µm ± 1.13 for the SEM and light microscopy method, respectively; unpaired t test, t=0.62, df=20, P=0.55). Mean sperm tail length from males of the Euskirchen population was significantly greater than that from males of the Texel population (SEM: t=3.21, df=20, P=0.004; light microscopy: t=4.89, df=18, P<0.001).

In a later study, a direct comparison was made between tail length measurements of sperm of males from the same Texel population using both light microscopy and SEM. The preparation method for SEM was identical to that of the present study (see also Mehlis et al. 2012; Mehlis and Bakker 2013). The preparation method for light microscopy was different (see details given under "Control experiment") in that no air-dried, formalin samples were used but unprepared sperm in solution, so that sperm had not been shrunken in this case and therefore were systematically larger than the SEM samples (Wilcoxon signed ranks test: N=9,

z=2.666, P=0.008). Sperm sizes of the same males as assessed by both preparation methods were significantly correlated (Spearman rank correlation: N=9, r_S=0.733, P=0.025). These comparisons show the reliability of the sperm tail length measurements from SEM images.

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Online Resource 2

Functional variation of sperm morphology in sticklebacks

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Table S1 Male repeatability r_I ± SE of various morphological sperm traits from nine wild-caught males from the 2011 Texel population. Sperm morphometry was performed with SEM images. Per male 30 sperm were measured. The significance of repeatability was tested with a one-way ANOVA. Indicated are the *F* statistics, degrees of freedom (df), and *P*-value. Significant *P*-values ($P<0.05$) are printed in bold. "Head" refers to head inclusive of midpiece

Sperm trait	r_I **± SE**	***F***	**df**	***P***
tail length	0.581 ± 0.001	42.549	8,261	**<0.001**
head length	0.231 ± 0.001	10.019	8,261	**<0.001**
head width	0.279 ± 0.001	12.601	8,261	**<0.001**
"head" volume	0.200 ± 0.001	8.498	8,261	**<0.001**
midpiece length	0.169 ± 0.001	7.084	8,261	**<0.001**
midpiece width	0.061 ± 0.001	2.952	8,261	**0.004**
midpiece volume	0.058 ± 0.001	2.855	8,261	**0.005**
"head" length to tail length ratio	0.404 ± 0.001	21.274	8,261	**<0.001**