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ORIGINAL PAPER

The influence of ambient water temperature on sperm performance and fertilization success in three-spined sticklebacks (*Gasterosteus aculeatus*)

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Abstract Acute and long-term temperature changes caused by global warming could lead to severe ecological and physiological consequences for aquatic organisms. This might be reflected in a higher mortality rate or a reduced hatching success but elevated temperatures might also lead to accelerated growth and egg development due to higher metabolic rates. Thermal conditions could also act on male gamete function that is known to be under strong sexual selection especially in mating systems where sperm competition frequently occurs. The three-spined stickleback (*Gasterosteus aculeatus*) is an externally fertilizing fish species. Males are sperm limited over the course of one breeding season and the risk of sperm competition is known to be high. To study the impact of ecologically relevant water temperatures in relation to different sperm numbers on reproductive performance standardized in vitro fertilization experiments were conducted, using two different immediate temperature changes (15 and 25 °C) as all test individuals were held under the same conditions prior to the experiments. The results revealed that fertilization success differed significantly when using different sperm numbers in different water temperatures. At higher temperatures a lower number of sperm was sufficient to fertilize 50 % of the eggs. However, with increasing sperm number fertilization success saturated at 75 % in the 25 °C treatment but at about 90 % in the 15 °C treatment. A further experiment dealt with sperm swimming ability at different temperatures (5, 15 and 25 °C), showing that both sperm velocity and linearity significantly increased whereas the percentage of motile sperm decreased at higher temperatures, suggesting that under these conditions sperm might reach an unfertilized egg more rapidly but live shorter. The observed results stress the role of current and future levels of naturally occurring ambient water temperatures as an important environmental factor influencing fitness related traits.

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Introduction

During the past decades a net increase in water temperatures for large parts of aquatic ecosystems has been observed (Levitus et al. 2000; IPCC 2007, 2013), resulting in negative consequences for organisms especially in fluctuating environments both in freshwater (Xenopoulos et al. 2005) and marine (Knowlton 2001) habitats. Recent studies stress the harmful effects of increased temperatures also on human life or health. For example, the rise in sea surface temperatures is associated with higher frequency of *Vibrio* spp., which is a pathogen bacterium that is responsible for severe infections in humans and other animals (Vezzulli et al. 2013). Moreover, during the past four decades global warming had negative consequences on commercial fishing catch rates (Cheung et al. 2013) and it is expected to cause complex changes in fish community structure through direct and indirect effects on fish metabolism (see Jeppesen et al. 2010 for an overview).

Acute and long-term temperature changes caused by global warming are known to have far-reaching biological consequences (Hughes 2000) also for the development and maintenance of reproductive traits (see Grazer and Martin 2012 and citations therein). For example, in the whitefish (*Coregonus lavaretus*) elevated temperatures (given are test ranges: 4–14 °C) led to a higher proportion of unfertilized eggs (Cingi et al. 2010) and in an Indo-Pacific coral species (*Acropora tenuis*) a negative impact of water temperature on fertilization success was found (27–30 °C; Albright and Mason 2013) underlining the adverse consequences on reproductive performance. The effect of higher temperatures on sperm velocity yielded ambiguous results: either a negative (*Solea senegalensis*: Beirão et al. 2011; *Salmo trutta*: Lahnsteiner 2012), no relationship (*Sparus aurata*: Lahnsteiner and Caberlotto 2012), or a temperature optimum (*Gadus morhua*: Purchase et al. 2010; *Lota lota*: Lahnsteiner and Mansour 2012) have been found. These differing results may be species specific or related to varying temperature ranges between studies. Other studies have shown that individuals which were raised or held under different environmental conditions developed sperm that differ in their swimming abilities. For example, in the poeciliid fish *Gambusia holbrooki* sperm of warm-acclimated males swam faster (18–30 °C; Adriaenssens et al. 2012). In addition, a recent study showed that guppies (*Poecilia reticulata*), which were reared under higher temperatures had slower sperm (23–30 °C; Breckels and Neff 2013). However, general conclusions cannot be drawn due to large variation in experimental conditions and differences in fertilization mode of the used study animals. Additionally, sperm of different species simply have been adapted to different conditions and may show different associations.

The three-spined stickleback (*Gasterosteus aculeatus*) is an externally fertilizing fish species with male only parental care, which is distributed throughout the Northern Hemisphere, where it inhabits both coastal regions and freshwater habitats (lakes and streams) (Wootton 1976). During the second half of the twentieth century the average Northern Hemisphere temperature was higher than during any other 50-year period in the last 500 years (e.g. IPCC 2007). Due to their wide geographical distribution temperature preferences of sticklebacks vary between 4 and 20 °C (Guderley 1994). However, survival rate decreased at higher temperature (Blahm and Snyder 1975) whereas growth rate and

egg development increased due to higher metabolic rates (Allen and Wootton 1982). In three-spined sticklebacks reproductive behavior is stimulated by long photoperiods in combination with increasing temperatures (Borg et al. 2004) and thus occurs during spring and summer (April–August). During this time reproductively active males build a tunnel-shaped nest in shallow freshwater habitats, which are characterized by rapid changes in temperature, but it is known that nesting males can tolerate water temperatures as high as 30 °C (Guderley 1994). Hopkins et al. (2011) showed that an increase in water temperature had detrimental effects on stickleback males' parental-care behavior. For example, males suffered from an increased likelihood of mortality when caring for their eggs under higher temperatures, which resulted in a lowered reproductive success (Hopkins et al. 2011). This behavior was highly consistent across individuals, indicating consequences at the population level (Hopkins et al. 2011). Sticklebacks, that were reared under low temperatures early in life showed growth compensation at later life stages. However, these individuals suffered from a reduced reproductive investment over the following two breeding seasons (Lee et al. 2012). Moreover, under higher temperatures parasite growth and transmission (*Schistocephalus solidus*) was favored, which underlines the extensive effects of thermal variation on the life history of three-spined sticklebacks (Macnab and Barber 2012).

Although the influences of enhanced water temperatures on survival and growth are rather well examined in sticklebacks, studies dealing with the direct impact of acute temperature changes on sperm function are lacking thus far. Thus, we conducted standardized in vitro fertilization experiments to assess the influence of ecologically relevant water temperatures on fertilization success in relation to different sperm numbers. In addition, we quantified sperm swimming performance (sperm velocity, sperm linearity, the percentage of motile sperm and sperm longevity) under different water temperatures as especially in externally fertilizing species it is expected that males with faster and/or more motile sperm will have a higher fertilization ability (reviewed in Pizzari and Parker 2009).

Materials and methods

In vitro fertilization

Sticklebacks originated from an anadromous population (Texel, the Netherlands). Fish were caught during their spring migration in April 2010. During this time they leave the cold North Sea to reproduce in shallow near coastal freshwater habitats (breeding season: April–August). Meteorological data collected at the weather station Den Helder over the last 10 years (2002–2012) show that between April and August the minimum air temperature is about 2 °C and the maximum averaged around 30 °C so that fish from our study population, are exposed to high and fluctuating temperatures during spring and summer. In addition, water temperatures measured in comparable freshwater habitats in the Netherlands peak in the range 15–25 °C during summer (June/July 2009; see Schrier-Uijl et al. 2011 for details) revealing that the applied test temperatures in our study fall within the range of naturally occurring water temperatures at the sticklebacks' breeding sites (see also Wootton 1984; Guderley 1994; Frommen 2001).

Approximately 500 individuals were kept in a large outdoor tank (750 l) with a constant supply of tap water (3 l min^{-1}). Between June 22th and July 20th, 40 randomly chosen nuptially colored males were isolated in single tanks measuring 30 cm × 20 cm × 20 cm (length × width × height), each equipped with a sand-filled petridish (Ø 9 cm) and 2 g of java moss (*Vesicularia dubyana*) for nest-building under standardized laboratory

conditions (day length: 16 h light, 8 h dark; water temperature 15 ± 1 °C). Nest-building was stimulated by daily presenting a gravid female originating from the outdoor tank for 15 min in a transparent container in front of each tank (see Mehlis et al. 2010). On average males needed 1.23 ± 0.10 (mean ± SE) days after introduction to finish their nest.

About 2 days (1.95 ± 0.13; mean ± SE) after a male has finished its nest it was stimulated again using a receptive female (see above). This was done to ensure that the used test males were in a comparable reproductive state. Directly thereafter, male's standard length (SL), body mass (M) and body condition (BC = 100 × M/SL^3, following Bolger and Connolly (1989)) was determined and it was quickly sacrificed by decapitation as sperm stripping is not possible in our study species (the only exception in de Fraipont et al. 1993). Both testes were removed and weighed to the nearest milligram (variable: testis mass TM; OHAUS, Explorer, E11140, Göttingen, Germany) in order to calculate the gonadosomatic index ((GSI = TM/M) × 100; de Vlaming et al. 1982). In three-spined sticklebacks sperm supply is not renewed during the breeding season (see Borg 1982 for details). Thus, testes of reproductively active males contain solely mature sperm.

Male's sperm number was quantified using a Neubauer improved counting chamber (Labor Optik, 0.0025 mm^2, depth 0.1 mm, Lancing, United Kingdom). Therefore, both testes were pestled in one Eppendorf tube containing 400 µl of a non-activating medium (NAM: NaCl: 3.5 g/l; KCl: 0.11 g/l; $MgCl_2$: 1.23 g/l; $CaCl_2$: 0.39 g/l; NaH_2CO_3: 1.68 g/l; glucose: 0.08 g/l, ph = 7.7; Fauvel et al. 1999) and the extrapolation of the average sperm count in 64 cells of the Neubauer chamber yielded the total number of sperm (see Mehlis et al. 2012). Using the same male's sperm and the same female's eggs (see below) ten in vitro fertilization experiments were conducted using different sperm numbers for each fertilization experiment (5,000, 50,000, 100,000, 150,000, 300,000, 500,000, 1,000,000, 1,500,000, 3,000,000, and 5,000,000 sperm per egg, respectively). The total number of sperm in the testes differed between males (range 3.25×10^8–7.82×10^8). Thus, each sperm suspensions per male (ranging from 100,000 to 100,000,000 sperm for the fertilization of 20 eggs) was filled up with the non-activating medium (see above) in order reach a constant volume of 500 µl for the fertilization experiments.

Immediately after sperm counting, the same female, which was used for male's stimulation (see above), was gently stripped and served as egg donor. Female's standard length, body mass and body condition was determined before and after egg stripping as described above. In vitro fertilizations were done using ten sub-clutches (20 eggs each, 200 eggs in total) of the same female, which were separately put into glass petri dishes that already contained 1,000 µl of tap water, which was either tempered at 15 ± 1 °C (N = 20) or at 25 ± 1 °C (N = 20). The remaining eggs were counted to quantify female's clutch size.

A pipette was used for the fertilization process to ensure that the each sperm suspension (constant volume 500 µl, only differing in sperm number) was equally distributed over the portion of 20 eggs. This was repeated for each ten portions in random order to control for sequence effects. In sticklebacks, complete clutch fertilization takes up to 10 min which is atypically long for an externally fertilizing species (see Bakker et al. 2006 for details). To ensure comparable fertilization rates the fertilization process was stopped standardizedly after 60 min using 1,000 µl of fresh commercial sparkling mineral water, which did not harm the eggs (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; Bakker et al. 2006). Thereafter, each portion of eggs was separately stored at the respective treatment temperature (15 or 25 °C) in a 1 l box, which was aerated by an airstone. The proportion of fertilized eggs was checked 24 h later using a binocular (Leica S8AP0, Wetzlar, Germany) (see Swarup 1958). In addition,

ten randomly selected fertilized eggs per female were weighed to the nearest milligram. To avoid pseudoreplication all test individuals (males and females) were used only once.

Sperm swimming performance

Males used in the second experiment were captured 1 year later (April 2011) but originated from the same population as described above. Before testing, individuals were kept together in a large outdoor tank (see above). Between August 7th and August 9th sperm swimming speed was quantified under three different ambient water temperatures for 36 randomly chosen nuptially colored males ($N_{5\ °C} = 12$, $N_{15\ °C} = 12$; $N_{25\ °C} = 12$). Males' body measurements were determined as described above.

For sperm motility measurements, males were killed quickly by decapitation, both testes were removed, weighed to the nearest milligram (see above) and put in an Eppendorf tube containing 500 µl of artificial "ovarian fluid" (NaCL: 3.0 g/l; KCL: 0.1 g/l; $CaCl_2$: 0.07 g/l; after Elofsson et al. 2006), which was already stored at a constant temperature (5, 15 or 25 °C) for at least 15 min (Thriller, PEQLAB_V0410E, Erlangen, Germany). Testes were pestled, and exactly 2 min (1st sperm motility measurement) and 30 min (2nd sperm motility measurement) later sperm movement was videotaped (see also Rick et al. 2014). At each time point a Leja counting chamber (depth 12 µm, Nieuw-Vennep, the Netherlands) was loaded with 3 µl of the mixed sperm suspension. Subsequent sperm motility determination was based on six randomly chosen one-second-lasting sequences, which were analyzed via CASA (ImageJ) resulting in the following variables: (1) percentage of motile sperm (PM), (2) velocity curvilinear (VCL [µm s^{-1}]), (3) velocity average path (VAP [µm s^{-1}]), (4) velocity straight line (VSL [µm s^{-1}]), (5) straightness (STR = VSL/VAP × 100 [%]), (6) linearity (LIN = VSL/VCL × 100 [%]) and (7) wobble (WOB = VAP/VCL × 100 [%]). Averaged values of about 308.43 ± 43.46 (1st motility measurement) and 278.43 ± 36.06 (2nd motility measurement) (mean ± SE) single motile sperm per male were used for further analyses. Overall, the threshold values for excluding immotile sperm were specified as 10 µm s^{-1} for VCL, 5 µm s^{-1} for VAP and 2 µm s^{-1} for VSL (see also Mehlis et al. 2013; Rick et al. 2014).

Statistical analyses

In vitro fertilization

To examine the influence of temperature and sperm number on fertilization success, we fitted a linear mixed effect model ("lme") using the 'lme' function in the "nlme" library of the R 3.0.2 statistical package. The proportion of fertilized eggs significantly deviated from normal distribution according to Kolmogorov–Smirnov tests and could not be transformed. Thus, a corrected α-level was determined using a permutation test. After 100,000 simulations, the corrected α-level for the "lme" was set as 0.036. In the "lme" temperature as well as sperm number and the interaction between these two variables were included as explanatory variable. Trial number was set as random factor and never removed to control for repeated measures. The statistical significance of the interaction term was tested by comparing the full model with a model without the interaction term. To test the statistical significance of each explanatory variable separately (temperature and sperm number) explanatory variables were removed in the order of statistical relevance and these reduced models were subsequently compared with the full model (but without the interaction term).

Tests of statistical significance were based on likelihood ratio tests. All given p values were based on two-tailed tests.

Sperm swimming performance

One male (25 °C) had to be excluded from the analyses as no sperm movement could be detected neither in the 1st nor in the 2nd measurement, leading to the following final sample size ($N_{5\ °C} = 12$, $N_{15\ °C} = 12$; $N_{25\ °C} = 11$). As VCL, VAP and VSL were highly significantly positively correlated with each other (Pearson correlations: $N = 70$ (1st and 2nd sperm motility measurement), all $r_P > 0.669$, all $p < 0.001$) a principal component analyses (PC) was conducted in R 3.0.2 statistical package, resulting in a single significant factor for sperm velocity (eigenvalue: 1.614, proportion of variance: 86.79 %). In addition, the trajectory of sperm movement is best described by the three velocity ratios (LIN, STR and WOB) and a principal component analysis yielded a single significant factor for the trajectory of sperm movement (sperm linearity; eigenvalue: 1.615, proportion of variance: 86.89 %). The PC1 of sperm velocity was significantly positively correlated with the single velocity variables (VCL, VAP and VSL), which was also true for PC1 of sperm linearity and the three single velocity ratios (LIN, STR (exponentially transformed, to achieve a normal distribution according to Kolmogorov–Smirnov tests) and WOB; Pearson correlations: $N = 70$, all $r_P > 0.856$, all $p < 0.001$). Furthermore, as a measure of sperm longevity the absolute difference in the percentage of motile sperm between the 1st and the 2nd sperm motility measurement was calculated for each male (see also Rick et al. 2014).

To analyze sperm swimming performance, linear mixed-effect models were fitted in R 3.0.2 including male identity as random factor, which was never removed to control for repeated sperm motility measurements per male. Sperm velocity, sperm linearity and the percentage of motile sperm were used as dependent variable and the temperature treatment (5, 15 and 25 °C) was set as explanatory variable. Tests of significance were based on likelihood-ratio tests and the residuals of the best explanatory model did not differ significantly from normal distribution (Kolmogorov–Smirnov). All given p values were based on two-tailed tests.

Results

In vitro fertilization

Males used in the two temperature treatments did not differ significantly among the temperature treatments in standard length (unpaired t test: $N_{15\ °C} = 20$, $N_{25\ °C} = 20$, $df = 38$, $t = 0.656$, $p = 0.516$), body condition (unpaired t test: $N_{15\ °C} = 20$, $N_{25\ °C} = 20$, $df = 38$, $t = -0.607$, $p = 0.547$), gonadosomatic index (unpaired t test: $N_{15\ °C} = 20$, $N_{25\ °C} = 20$, $df = 38$, $t = 1.131$, $p = 0.265$) or sperm density (unpaired t test: $N_{15\ °C} = 20$, $N_{25\ °C} = 20$, $df = 38$, $t = 0.408$, $p = 0.685$). In addition, also females did not differ significantly with respect to their standard length (unpaired t test: $N_{15\ °C} = 20$, $N_{25\ °C} = 20$, $df = 38$, $t = -0.392$, $p = 0.697$), body condition (before and after spawning; unpaired t test: $N_{15\ °C} = 20$, $N_{25\ °C} = 20$, $df = 38$, $t = 0.815$ and 0.787, $p = 0.420$ and 0.436, respectively), egg mass or clutch size (unpaired t test: $N_{15\ °C} = 20$, $N_{25\ °C} = 20$, $df = 38$, $t = 1.529$ and 1.040, $p = 0.135$ and 0.305, respectively). For descriptive data see Supplementary Material Table S1a.

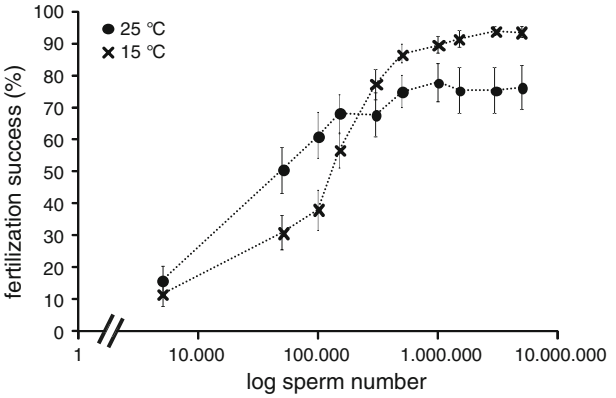

Fig. 1 Ambient water temperature significantly influenced fertilization success in relation to different sperm numbers. In the 25 °C treatment (*circles*) a lower number of sperm per egg was sufficient to fertilize 50 % of the eggs than in the 15 °C treatment (*crosses*). Furthermore, when the number of sperm was increased the fertilization success also increased in the 15 °C treatment close to 100 % whereas fertilization success in the 25 °C treatment saturated at about 75 %. Note the logarithmic scale on the x-axis. Shown are mean ± SE

The results of the "lme" revealed a significant interaction between temperature and sperm number ("lme": $N_{15\ °C} = 20$, $N_{25\ °C} = 20$, $\Delta df = 9$, $\chi^2 = 58.862$, $p < 0.001$; Fig. 1), indicating that the fertilization success (proportion of fertilized eggs) was influenced by the ambient water temperature in relation to the different sperm numbers used in the in vitro fertilization experiment. In detail, in the 25 °C treatment a lower number of sperm per egg was sufficient to fertilize 50 % of the eggs than in the 15 °C treatment (see Fig. 1). However, when the number of sperm per egg increased the fertilization rate also increased in the 15 °C treatment to well above 90 % whereas the proportion of fertilized eggs in the 25 °C treatment saturated at about 75 % (see Fig. 1). Due to the significant interaction term between temperature and sperm number in relation to fertilization success, the effect of temperature and associated fertilization rates was not significant when sperm number was not taken into account ("lme": $N_{15\ °C} = 20$, $N_{25\ °C} = 20$, $\Delta df = 1$, $\chi^2 = 0.323$, $p = 0.570$; Fig. 1). However, as expected sperm number alone significantly influenced the proportion of fertilized eggs ("lme": $N_{15\ °C} = 20$, $N_{25\ °C} = 20$, $\Delta df = 9$, $\chi^2 = 311.177$, $p < 0.001$; Fig. 1).

Sperm swimming performance

Males used in this experiment did not differ significantly in standard length (ANOVA: $N_{5\ °C} = 12$, $N_{15\ °C} = 12$, $N_{25\ °C} = 11$, $df = 2$, $F = 1.223$, $p = 0.308$), body condition (ANOVA: $N_{5\ °C} = 12$, $N_{15\ °C} = 12$, $N_{25\ °C} = 11$, $df = 2$, $F = 0.215$, $p = 0.808$) or gonadosomatic index (ANOVA: $N_{5\ °C} = 12$, $N_{15\ °C} = 12$, $N_{25\ °C} = 11$, $df = 2$, $F = 0.805$, $p = 0.456$). As sperm concentration was not standardized during motility measurements, we additionally recorded the total number of sperm cells that were detected by CASA (ImageJ) for each male, which did not differ significantly between the temperature treatments (ANOVA: $N_{5\ °C} = 12$, $N_{15\ °C} = 12$, $N_{25\ °C} = 11$, $df = 2$, $F = 1.033$, $p = 0.368$, using averaged values for the 1st and 2nd sperm motility measurement).

Our results showed that sperm velocity was significantly influenced by the different test temperatures ("lme": $N_{5\ °C} = 12$, $N_{15\ °C} = 12$, $N_{25\ °C} = 11$, $\Delta df = 2$, $\chi^2 = 8.497$,

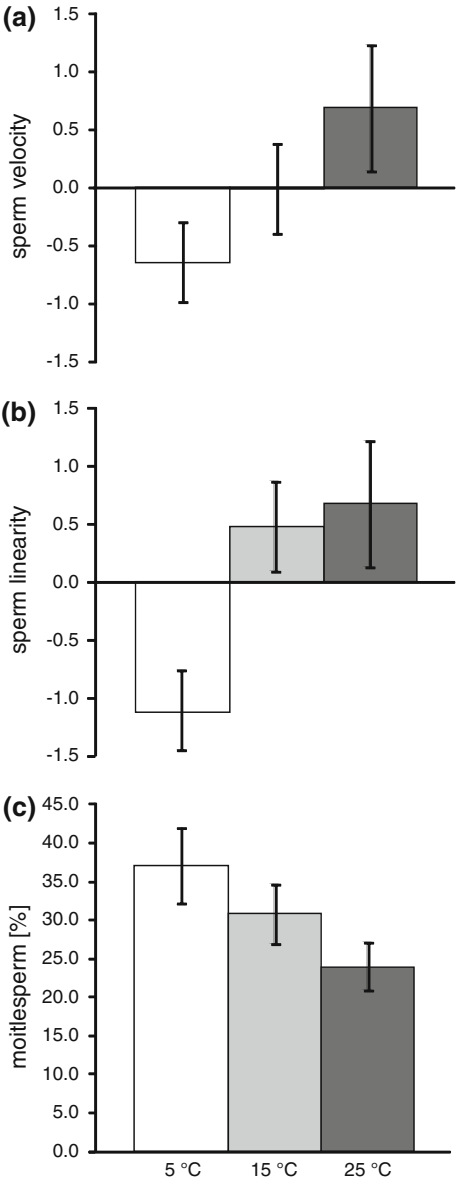

Fig. 2 Differences in **a** sperm velocity (PC1 of VCL, VAP and VSL), **b** linearity (PC1 of LIN, STR and WOB) and **c** percentage of motile sperm at different temperatures (*white* 5 °C, *gray* 15 °C, *dark gray* 25 °C). Shown are mean ± SE

$p = 0.014$), revealing an increased sperm swimming speed at higher temperatures (see Fig. 2a). There were also significant differences concerning sperm linearity ("lme": $N_{5\ °C} = 12$, $N_{15\ °C} = 12$, $N_{25\ °C} = 11$, $\Delta df = 2$, $\chi^2 = 20.210$, $p < 0.001$), with more linear sperm trajectories at higher temperatures (Fig. 2b). In addition, the percentage of motile sperm significantly declined with elevated test temperatures ("lme": $N_{5\ °C} = 12$, $N_{15\ °C} = 12$, $N_{25\ °C} = 11$, $\Delta df = 2$, $\chi^2 = 9.552$, $p = 0.008$, Fig. 2c), whereas sperm longevity did not differ significantly between the three temperature treatments (Kruskal–Wallis test: $N_{5\ °C} = 12$, $N_{15\ °C} = 12$, $N_{25\ °C} = 11$, $df = 2$, $\chi^2 = 2.043$, $p = 0.360$). For descriptive data see Supplementary Material Table S1b.

Discussion

Our study revealed that in relation to different sperm numbers the temperature of the surrounding water medium significantly influenced fertilization success in the three-spined sticklebacks. Furthermore, temperature also affected sperm swimming abilities. As all males were housed under the same temperature conditions prior to the experiments, and males of the different temperature treatments were random samples from the same population concerning their sperm morphology traits (e.g. tail length) it is likely that these effects were almost exclusively explained by sperm physiology. However, it cannot be completely ruled out that female and/or egg traits are of importance as well, like it was shown in other species (e.g. Nissling et al. 2006; Præbel et al. 2013). However, females used in our study (in vitro fertilization experiment) did not differ as to body length, body mass, body condition, egg mass, and clutch size, suggesting that the temperature effect on fertilization rate was rather due to sperm physiology than egg traits.

The observed temperature-dependent fertilization pattern strongly depends on sperm numbers that were used during the in vitro experiments. In detail, when using larger sperm numbers (i.e. more than 500,000 sperm per egg) a higher percentage of eggs were fertilized at the lower temperature (15 °C). On the other hand, relatively more eggs were fertilized when using smaller numbers of sperm per egg at higher temperatures (i.e. 25 °C). In a former study on sticklebacks, 50,000,000 sperm (the indicated sperm number was actually estimated too low by a factor two; see also Bakker et al. 2014) were used to fertilize on average 56 eggs, resulting in a sperm-egg ratio of about 1,000,000 (Bakker et al. 2006). Unfortunately, in this study no information is available concerning the temperature at which fertilization was done. However, fertilization rate saturated at about 90 % (Bakker et al. 2006), which compared to the present study might indicate a temperature of about 15 °C during fertilization. A recent study by Bombardelli et al. (2013) showed that in the catfish *Rhinelepis aspera* a ratio of 7,000 sperm per egg results in a high fertilization success of about 70 % whereas in African catfish (*Clarias gariepinusare*) 15,000 sperm per egg were needed to reach a fertilization rate of about 80 % (Rurangwa et al. 1998). Using such small sperm numbers in sticklebacks would result in a lowered fertilization success (less than 50 % at 15 and 25 °C, although no temperatures are indicated in the catfish studies). Thus, both the temperature and the sperm number per egg leading to high fertilization rates are species specific, and are probably based on the naturally occurring habitat conditions and/or the mating system. This makes it difficult to draw general conclusions about the conditions for maximal fertilization rates. However, in addition to the significance of sperm number the thermal effects on fertilization rate found in our in vitro fertilization experiment may be explained by the observed between-treatment differences in sperm swimming ability in our experiment on sperm performance.

In general, many factors are known to influence sperm swimming ability such as ion concentration, osmotic pressure, pH, and temperature of the surrounding medium (see Islam and Akhter 2011 for an overview). Sperm of three-spined stickleback males swam significantly faster at the higher temperature treatment (25 °C) when keeping all other factors constant. The energetic resources of fish spermatozoa are limited; an increased sperm velocity at higher water temperature may thus lead to a decreased longevity (e.g. Levitan 2000; Alavi and Cosson 2005). In accordance, although sperm longevity was not affected by the different temperature treatments in our study, the percentage of motile sperm was significantly lower at higher temperatures. Similar results were found in perches (*Perca fluviatilis*: Lahnsteiner 2011) and Siberian sturgeons (*Acipenser baeri*: Williot et al. 2000), where sperm motility duration was prolonged at low temperatures. But also with

respect to the effect of temperature on sperm motility the results are ambiguous among other species. In the sea urchin (*Heliocidaris erythrogramma*), for example, temperature affected swimming speed but not the percentage of motile sperm (Caldwell et al. 2011). In the streaked prochilod, *Prochilodus lineatus*, sperm motility showed an optimum at 17 °C but decreased at lower and higher temperatures (Romagosa et al. 2010). Our results indicated that there seems to be a trade-off between sperm velocity and the percentage of motile sperm, as the percentage of motile sperm continuously decreased at elevated temperatures whereas the sperm velocity increased (see also Bakker et al. 2014). However, a higher swimming speed at 25 °C does not necessarily equate to an improvement in male reproductive success, which in turn is confirmed by the results of the in vitro fertilization experiment, where the mean fertilization rate plateaued at 75 % in the 25 °C treatment.

Several decades ago, Parker postulated that under sperm competition in a 'fair-raffle' fertilization success would be equivalent to one's own sperm number in proportion to the total sperm number (Parker 1982, 1990). In general, three-spined stickleback males face an increased risk of sperm competition (e.g. Largiadèr et al. 2001) and they are sperm limited over the course of one breeding season (Borg 1982). Thus, careful sperm allocation is a critical component of reproductive success in this species (e.g. Zbinden et al. 2004) and a previous study showed that males from our study population ejaculated per mating about 5 % of their total sperm store (Zbinden et al. 2001). The present study showed that less sperm are needed to fertilize 50 % of the eggs at higher temperatures but complete fertilization of the clutch is not ensured, highlighting the ambiguous effects of elevated temperatures on reproductive performance at least in our study animal.

Sticklebacks breed in the littoral zone or in shallow tidal pools, where they are exposed to high and fluctuating temperatures changes during spring and summer. The occupation of breeding sites that provide optimal conditions for fertilization, embryo survival and development is one of the key determinants in life history. Multiple scenarios are plausible to explain how stickleback males might phenotypically adapt to acute changes of elevated temperatures at their breeding sites, in addition to potential genetic adaptations to the negative effects of higher temperatures. For example, they might establish territories in deeper water regions or might reproduce early or late in the day when ambient water temperatures are lower and thus more optimal for fertilization than at midday. However, if reproduction takes place in deeper water or at dawn/dusk visual communication during mate choice will be hampered. This could lead to severe consequences as visual signals such as the red breeding coloration of males (e.g. Bakker and Milinski 1993) as well as UV color patterns in both sexes (Rick et al. 2006; Rick and Bakker 2008) play important roles during inter- as well as intrasexual interactions. Another scenario of adaptation to rising temperatures might be that sticklebacks arrive earlier at their breeding sites so that the reproductive phase is generally shifted like in sticklebacks in warmer regions (Crivelli and Britton 1987; Poizat et al. 2002) or in other fish species (Cheung et al. 2013). Moreover, an increase in temperature due to global warming has the potential to reduce the habitable area for sticklebacks as is known for other species (Parmesan 2006). Nevertheless, future field work is needed, for example to assess long-term effects of thermal variation on male gamete function.

To conclude, we found a considerable impact of higher temperatures in relation to different sperm numbers on fertilization success in three-spined sticklebacks, which is likely due to changes in sperm swimming ability. The highest fertilization rates were reached when using more than 500,000 sperm per egg at 15 °C. In contrast, relatively more eggs were fertilized at 25 °C when using smaller sperm numbers per egg. However, fertilization rate never approached 100 % under these conditions, which could be

explained by a decrease in the percentage of motile sperm at higher temperatures accompanying the observed increase in sperm swimming ability. Thus, our findings may contribute to an overall better understanding of the impact of global warming on fitness relevant traits in animal mating systems suggesting adverse effects on life history.

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