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Body size of virtual rivals affects ejaculate size in sticklebacks

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Sperm competition occurs when sperm of two or more males compete to fertilize a given set of eggs. Theories on sperm competition expect males under high risk of sperm competition to increase ejaculate size. Here we confirm this prediction experimentally in the three-spined stickleback (*Gasterosteus aculeatus*). In this species, sneaking (i.e., stealing of fertilizations by neighboring males) can lead to sperm competition. Sneaking males invade foreign nests, and the owners vigorously try to prevent this intrusion. In such fights, male body size is assumed to be an important predictor of success. Consequently, the risk of sperm competition may depend on the size of a potential competitor. We experimentally confronted males before spawning with either a large or a small computer-animated rival. We show that males ejaculated significantly more sperm after the presentation of the larger virtual rival than after the small stimulus. In addition, the time between the initiation of courting and the spawning was shorter in the large virtual male treatment. The results suggest that stickleback males tailor ejaculate size relative to the risk of sperm competition perceived by the size of a potential competitor. *Key words:* body size, computer animation, ejaculate size, *Gasterosteus aculeatus*, sperm competition. *[Behav Ecol 15:137–140 (2004)]*

A growing body of evidence for a variety of species and mating systems shows that sperm are invested strategically (see Wedell et al., 2002). Among other factors, the presence or absence of rival males has been shown to influence ejaculate size (Fuller, 1998; Gage, 1991; Gage and Barnard, 1996; Nicholls et al., 2001), as predicted by game theoretical models of risk as well as of intensity of sperm competition (Ball and Parker, 1997; Parker et al., 1996, 1997). Risk-models apply for species in which females usually mate with a single male and sperm competition only occurs in a proportion of the matings, and predict that males should increase ejaculate expenditure when there might be a competitor. For the case of an intensity of sperm competition (i.e., typically more than two males at a given mating), models predict a maximum sperm output if there are two competitors, but a decrease in ejaculate expenditure if the number of additional males increases.

In fish, territorial males of the rainbow darter (*Etheostoma caerulum*) ejaculated more sperm when other males (one or four) were present than when no other males or a female was there (Fuller, 1998). Pilastro et al. (2002) showed that sneaker males of two gobiid fishes, as predicted by the intensity models, have a maximum sperm output if there are two competitors but decrease their investment in sperm if the number of additional sneakers increases. Similarly, in the European bitterling, *Rhodeus seiceus*, dominant males invested most in ejaculations before oviposition if only one competitor was present (Candolin and Reynolds, 2002).

Male sticklebacks have a given amount of sperm available for the breeding season, because spermatogenesis is inhibited during this period (Borg, 1982). Consequently, multiply-mated males have a smaller sperm store in their testes than do virgin sticklebacks (Zbinden et al., 2001). This may lead to a limitation in sperm for sticklebacks, which are thus expected to allocate it carefully. Sperm competition in sticklebacks is induced through sneaking. Neighboring territorial males (mostly those without own eggs) attempt to steal fertilizations by spawning in foreign nests with freshly laid eggs (Goldschmidt et al., 1992; Jamieson and Colgan, 1992; Mori, 1995). In three studied populations, 15–25% of the nests contained sneaked eggs (Jones et al., 1998; Largiadèr et al., 2001; Rico et al., 1992). Sneakers approaching a foreign nest and trying to enter it are vigorously attacked by the nest owners. Generally, body size is an important factor in intrasexual conflicts in fish (see Alonzo et al., 2000; Mazzoldi et al., 2000). In sticklebacks, the success in fights for territories increases with male size (Dufresne et al., 1990; Rowland, 1989a). Empirical field observations suggest that nest owners' body size is a good predictor of whether sneaking attempts will be successful. Smaller males are more often victims of sneaking than are larger males (Largiadèr et al., 2001).

The ability to prevent sneaking may influence the assessment of, and the reaction to, sperm competition risk (Alonzo and Warner, 2000; Candolin and Reynolds, 2002). In addition, larger males have larger ejaculates (Zbinden et al., 2001), and larger sneakers may therefore imply more costs in terms of stolen fertilizations. The body size of a competitor could then influence the ejaculate size of nest owners. In this study, we experimentally manipulated the risk of sperm competition with a computer animation of a courting male stickleback. We tested whether ejaculate size changed in relation to the body size of the virtual males. After presenting either a small or a large virtual rival, males were allowed to mate, and the number of ejaculated sperm was counted.

METHODS

Biological material

Three-spined sticklebacks (*Gasterosteus aculeatus*) were collected at the end of March 2001 on the island of Texel (The Netherlands) and transported to the University of Bonn

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(Germany). The fish were kept in mixed-sex storage tanks and supplied with running tap-water (16°C–18°C). Before the experiment, males and females to be used in the tests were moved into a acclimatized room with a temperature of 17 ± 1°C, under a 16 h light/8 h dark regime. Fish were fed ad libitum with frozen chironomid larvae. The tanks were illuminated by fluorescent lamps (Osram, 36 W) mounted 25 cm above the water level.

The females were held in groups of between six and 10 individuals in aerated and filtrated 45-l tanks (50 × 30 × 30 cm). Males with developing breeding coloration were selected from the stock and placed individually into 15-l plastic aquaria (20 × 20 × 38 cm) that contained a Petri dish (diam = 9 cm) with fine gravel and 8 cm long pieces of green cotton twine, which served as nesting material. The tanks were separated by opaque gray partitions. Males completed their nest between 1 and 3 days after isolation and were then tested for the first time within the next 2 days.

Experiments

Each test started by placing the male's aquarium for half an hour alongside a computer screen that showed a virtual landscape (Künzler and Bakker, 1998) in which a computer-animated stickleback would court later in the experiment. The rest of the monitor (i.e., everything apart from the 800 × 600 pixel window) was covered by black nonreflecting paper. The other sides of the tank were covered by opaque dark-gray plastic, except the front side of the setup that was masked by a black curtain. Because the setup was well illuminated (fluorescent lamp Osram, 36 W, 35 cm above the aquarium) and the ambient light was low, the fish could be observed through the curtain. After the acclimatization time, a 1-l container of transparent plastic with a ripe female was placed from underneath the curtain in front of the male's aquarium for 5 min. After removal of the female, the male was shown one of the two computer-animation sequences (described below). To assess its aggressiveness, the male was video-recorded during the computer-animation sequence by using a webcam linked to a personal computer. As a measure of aggressiveness, we measured the time a male spent oriented toward the animation. Time was taken when the male's eye was less than 3 cm away from the wall of the tank in front of the animation and within a 14-cm range along this wall, covering the purview in which the virtual male courted. When the animation sequence was finished, a ripe female was carefully placed into the aquarium and allowed to spawn. The time from the initiation of male courting (first zig-zag display) until the female entered the nest was measured with a stopwatch. Body size and body mass before and after the test was assessed for every female in the experiment. None of these measures of the two females that spawned with a given male were significantly different (paired t tests, all $p > .7$). A total of 21 males were tested. The standard length of the males ranged from 50–62 mm (median = 56 mm). Median female size was 62.5 mm (57.75; 65.00 mm; quartiles).

Assessment of ejaculate size

Immediately after the male left the nest after spawning, we capped and removed the Petri dish containing the nest with eggs, sperm, and about 35 ml of water. Sperm were filtrated and stained on the filter. The methods used are described in detail in Zbinden et al. (2001). Forty-eight randomly chosen screenshots (4107 μm^2 each) distributed over four segments of the filter were recorded via microscope by a digital camera (Hitachi CCD, HV-C20A). The number of sperm on the pictures was counted after the experiment by using DISKUS software (Microscopic Image Acquisition and Documentation; Hilgers Technical Bureau). The sequences of pictures were randomized and prepared by an assistant, so we were blind with respect to treatment and a male's identity while counting. Sperm estimates for the whole filter (S_F) (area = 1452.2 mm^2) were based on the number of sperms in the screenshots.

When the male had rebuilt its nest (from 1–3 days after the first trial) the test was repeated with the other virtual stimulus. Throwing a coin decided the succession of the two computer animations for two males, where one had the opposite succession of the other.

Computer-animation sequences

The computer animations showed a male with moderate coloration approaching a nest site, fanning, and courting (zig-zag display) in the direction of the real male's aquarium side where the stimulus female had been shown before. The virtual scenery was modified from previous ones (Bakker et al., 1999; Künzler and Bakker, 1998; 2001; Mazzi et al., 2003), and can be viewed at http://www.unifr.ch/biol/ecology/ebert/group/zbinden/movie.html, using the free Quicktime player. The two animation sequences used differed exclusively in the standard body size of the virtual male, which was within the natural variation in standard length of the studied population. The large male was 63 mm long (herein large stimulus); the small one, 47 mm (small stimulus). To improve the three-dimensional effect and to give the test male an additional cue to assess and compare the sizes of the virtual fish, two virtual gray plastic tubes were placed as landmarks at different depth into the virtual landscape. These virtual tubes were rebuilt after real ones (diameter = 19 mm, height = 19 mm), of which every tested male got two in its aquarium. The perceived size of the virtual stimuli is unknown, because we do not know if and how sticklebacks assess depth (e.g., stereoscopic vision, monitoring the accommodation system, motion parallax). It has been shown that goldfish judge distances referring to the size of known objects (Douglas, 1996). We therefore think that the landmarks in our experiment may well be a cue sticklebacks use to assess the size of the virtual competitors. The refraction index of the transition air-plastic-water in our setup is estimated to be 0.8. Therefore, the virtual fish might seem to be smaller, further away, or both, but the difference between the two virtual fish and, consequently, the effect of our experimental treatment remains. Details on how the animation was manufactured, how and why the suppositions for a correct usage of computer animations are fulfilled in sticklebacks, and what the technical details of the used computer animations are has been described by Künzler and Bakker (1998; 2001).

Statistical analyses

Analyses were performed by using the JMP IN 3.2.1 (SAS Institute) statistical package. Data were tested for normality by using the Shapiro-Wilk W test, and consequently, parametric or nonparametric analyses and correlations were used. Paired t tests were performed if the differences between the pairs of data were normally distributed. All given p values are two-tailed.

RESULTS

Ejaculate size of males was significantly higher after having seen a large virtual competitor than after a small one (Wilcoxon signed-rank test, $N = 21$, $T = 67$, $p = .016$) (Figure 1). Ejaculate size (median and quartiles) measured 11.76×10^6 (8.82×10^6; 23.07×10^6) after the large stimulus, and 10.36×10^6 (6.4×10^6; 16.04×10^6) after the small stimulus. The order of the two stimuli (i.e., whether the large or the small male had

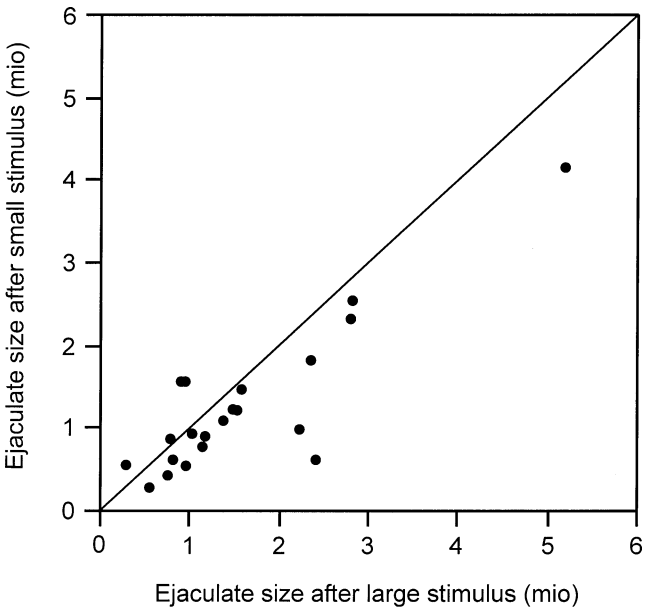

Figure 1
Number of sperm ejaculated in the two trials of each male. Before spawnings, either a large or a small courting computer-animated male was shown to each male. The bisecting line indicates the line of no difference in ejaculate size between the treatments.

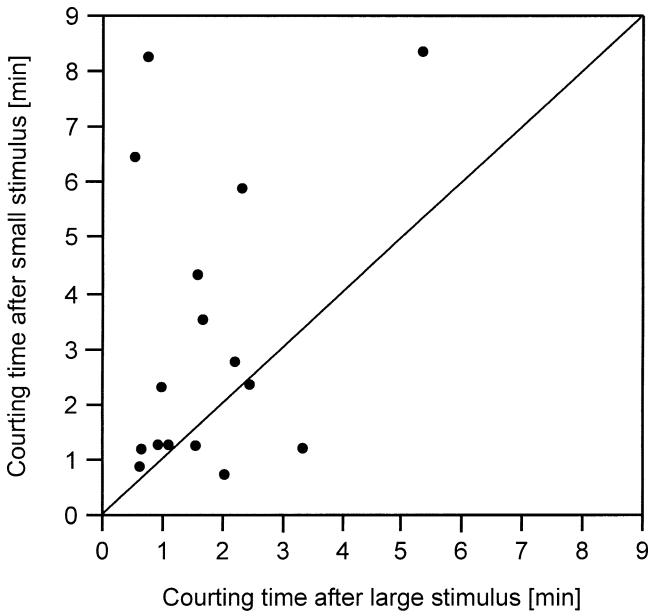

Figure 2
Time between the initiation of courting by the first zig-zag and the moment the female entered the nest in the two trials of each male. Before spawnings, either a large or a small courting computer-animated male was shown to each male. The bisecting line indicates the line of no difference in courting time between the treatments.

been shown first) had no significant effect on ejaculate size (Mann-Whitney U test, $N_{\text{small-large}} = 11$, $N_{\text{large-small}} = 10$, $z = .11$, $p > .9$; $z = -1.02$, $p > .3$, resp.), nor on the difference in ejaculate size between the two treatments (Mann-Whitney U test, $N_{\text{small-large}} = 11$, $N_{\text{large-small}} = 10$, $z = -1.23$, $p > .2$). The strength of the reaction on the stimuli, measured as the difference in ejaculate size between the treatments, was not significantly correlated with the standard length of real males ($N = 21$, $r_s = .3$, $p = .18$).

Ejaculate size was not affected significantly by female size, body mass before spawning, or egg mass, regardless of the treatment (all $p > .3$). Time spent oriented toward the animation in close proximity to the side of the aquarium was not significantly different between the two stimuli (paired t test, $t = .776$, df $= 14$, $p = .45$). However, the time between the first zig-zag until the moment the female entered the nest was significantly shorter after the large stimulus (1.59 min; .81, 2.3 min; median and quartiles) than after the small stimulus (2.37 min; 1.27, 5.55 min) (paired t test, $t = -2.4$, df $= 15$, $p = .03$) (Figure 2).

DISCUSSION

This study provides experimental evidence that male three-spined sticklebacks adjust ejaculate size according to the size of a neighboring male. They invest more sperm in a mating after having seen a large virtual male than after a small one (Figure 1). We interpret our results as a strategic investment in ejaculate size, which will depend on the perceived risk of sperm competition. In our scenario, larger males were perceived as a higher risk than were smaller males. Our results and interpretations are in accordance with theory about sperm competition (Parker et al., 1996, 1997), and with previous findings that male sticklebacks tailor their ejaculate size relative to the behavior of possible competitors, with a tendency for smaller males to react more strongly (Zbinden et al., 2003).

When manipulating the risk of sperm competition, one has to presuppose how this risk is assessed. This means that manipulation of the risk of sperm competition is actually done by manipulating factors assumed to be associated with sperm competition risk (e.g., number of possible competitors, female expected future reproduction, competitor body size). Therefore, possible alternative effects of the manipulation should be considered. In the present study, the differently sized stimuli could change the tested males' expected future reproduction due to female choice for larger males (Kraak et al., 1999; Rowland, 1989b). However, it is unlikely that the virtual male was assessed as better, as it was not brightly colored to reduce its attractiveness to females, and the real male finally got the only female available. The latter may actually increase the self-perceived attractiveness of the real male. In addition, if the expected future reproduction is assessed through the size of the competitors, it should have been the mean of the two successive stimuli in the second trial. We would thus expect an effect of succession on ejaculate sizes, which was absent in our data.

The risk of sperm competition could as well influence male aggressiveness (Candolin and Reynolds, 2002). Our experiment did not show such effect. However, as our measure of the time spent oriented toward the animation includes both aggressiveness and attentiveness, it may hide changes in aggressive behavior. To assess male aggressiveness, bites and bumps on the side of the tank have been measured (Rowland, 1984). This approach was not possible in our study, as many males hit the side so vigorously that single bites and bumps were impossible to distinguish.

The time between initiation of courting and the moment the female entered the nest was shorter after the large stimulus than after the small stimulus. Because females did not see the animation sequence, this effect must be due to the nest owner. It is possible that males were in different states of excitement after the two stimuli. Candolin (1999) showed that male-male interactions altered coloration intensity and courtship activity. Both these traits could not be measured accurately in our experiment, and the reasons why males needed less courtship

time in the presence of larger males remain unclear. Still it is interesting to discuss it in the light of sperm competition risk. This risk could be diminished if males mate without attracting the attention of possible competitors. Because gravid females that signal readiness to spawn may be conspicuous to surrounding males, fast spawning may be adaptive for males that are not able to prevent sneaking otherwise (i.e., smaller males).

To conclude, we experimentally showed that the size of a virtual competitor affects ejaculate size of male sticklebacks. Such a plastic reaction is adaptive when the risk of sperm competition is variable. Our results further suggest that assessment of future risk of sperm competition is based on more detailed cues than mere number of competitors. Finally, the present study illustrates the highly standardized experimental conditions offered by computer animations.

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REFERENCES

Alonzo SH, Taborsky M, Wirtz P, 2000. Male alternative reproductive behaviours in a Mediterranean wrasse, *Symphodus ocellatus*: evidence from otoliths for multiple life-history pathways. Evol Ecol Res 2:997–1007.

Alonzo SH, Warner RR, 2000. Allocation to mate guarding or increased sperm production in a Mediterranean wrasse. Am Nat 156: 266–275.

Bakker TCM, Künzler R, Mazzi D, 1999. Condition-related mate choice in sticklebacks. Nature 401:234.

Ball MA, Parker GA, 1997. Sperm competition games: inter- and intraspecies results of a continuous external fertilization model. J Theor Biol 186:459–466.

Borg B, 1982. Seasonal effects of photoperiod and temperature on spermatogenesis and male secondary sexual characters in the threespined stickleback (*Gasterosteus aculeatus*). Can J Zool 60:3377–3386.

Candolin U, 1999. Male-male competition facilitates female choice in sticklebacks. Proc R Soc Lond B 266:785–789.

Candolin U, Reynolds JD, 2002. Adjustments of ejaculation rates in response to risk of sperm competition in a fish, the bitterling (*Rhodeus sericeus*). Proc R Soc Lond B 269:1549–1553.

Douglas RH, 1996. Goldfish use the visual angle of a familiar landmark to locate a food source. J Fish Biol 49:532–536.

Dufresne F, FitzGerald GJ, Lachance S, 1990. Age and size-related differences in reproductive success and reproductive cost in threespine stickleback. Behav Ecol 1:140–147.

Fuller RC, 1998. Sperm competition affects male behaviour and sperm output in the rainbow darter. Proc R Soc Lond B 265:2365–2371.

Gage AR, Barnard CJ, 1996. Male crickets increase sperm number in relation to competition and female size. Behav Ecol Sociobiol 38: 349–353.

Gage MJG, 1991. Risk of sperm competition directly affects ejaculate size in the Mediterranean fruit fly. Anim Behav 42:1036–1037.

Goldschmidt T, Foster SA, Sevenster P, 1992. Inter-nest distance and sneaking in the three-spined stickleback. Anim Behav 44:793–795.

Jamieson IG, Colgan PW, 1992. Sneak spawning and egg stealing by male threespine sticklebacks. Can J Zool 70:963–967.

Jones AG, Östlund-Nilsson S, Avise JC, 1998. A microsatellite assessment of sneaked fertilizations and egg thievery in the fifteenspine stickleback. Evolution 52:848–858.

Kraak SBM, Bakker TCM, Mundwiler B, 1999. Sexual selection in sticklebacks in the field: correlates of reproductive, mating, and paternal success. Behav Ecol 10:696–706.

Künzler R, Bakker TCM, 1998. Computer animations as a tool in the study of mating preferences. Behaviour 135:1137–1159.

Künzler R, Bakker TCM, 2001. Female preference for single and combined traits in computer animated stickleback males. Behav Ecol 12:681–685.

Largiadèr CR, Fries V, Bakker TCM, 2001. Genetic analysis of sneaking and egg-thievery in a natural population of the three-spined stickleback (*Gasterosteus aculeatus* L.). Heredity 86:459–468.

Mazzi D, Künzler R, Bakker TCM, 2003. Female preference for symmetry in computer-animated three-spined sticklebacks, *Gasterosteus aculeatrus*. Behav Ecol Sociobiol 54:156–161.

Mazzoldi C, Scaggiante M, Ambrosin E, Rasotto MB, 2000. Mating system and alternative male mating tactics in the grass goby *Zosterisessor ophiocephalus* (Teleostei: Gobiidae). Mar Biol 137:1041–1048.

Mori S, 1995. Factors associated with and fitness effects of nest-raiding in the three-spined stickleback, *Gasterosteus aculeatus*, in a natural situation. Behaviour 132:1011–1023.

Nicholls EH, Burke T, Birkhead TR, 2001. Ejaculate allocation by male sand martins, *Riparia riparia*. Proc R Soc Lond B 268:1265–1270.

Parker GA, Ball MA, Stockley P, Gage MJG, 1996. Sperm competition games: individual assessment of sperm competition intensity by group spawners. Proc R Soc Lond B 263:1291–1297.

Parker GA, Ball MA, Stockley P, Gage MJG, 1997. Sperm competition games: a prospective analysis of risk assessment. Proc R Soc Lond B 264:1793–1802.

Pilastro A, Scaggiante M, Rasotto MB, 2002. Individual adjustment of sperm expenditure accords with sperm competition theory. Proc Natl Acad Sci USA 99:9913–9915.

Rico C, Kuhnlein U, FitzGerald GJ, 1992. Male reproductive tactics in the threespine stickleback: an evaluation by DNA fingerprinting. Mol Ecol 1:79–87.

Rowland WJ, 1984. The relationship among nuptial coloration, aggression, and courtship of male three-spined sticklebacks, *Gasterosteus aculeatus*. Can J Zool 62:999–1004.

Rowland WJ, 1989a. The effects of body size, aggression and nuptial coloration on competition for territories in male threespine sticklebacks, *Gasterosteus aculeatus*. Anim Behav 37:282–289.

Rowland WJ, 1989b. Mate choice and the supernormality effect in female sticklebacks (*Gasterosteus aculeatus*). Behav Ecol Sociobiol 24: 433–438.

Wedell N, Gage MJG, Parker GA, 2002. Sperm competition, male prudence and sperm-limited females. Trends Ecol Evol 17:313–320.

Zbinden M, Largiader CR, Bakker TCM, 2001. Sperm allocation in the three-spined stickleback. J Fish Biol 59:1287–1297.

Zbinden M, Mazzi D, Künzler R, Largiader CR, Bakker TCM, 2003. Courting virtual rivals increase ejaculate size in sticklebacks (*Gasterosteus aculeatus*). Behav Ecol Sociobiol 54:205–209.