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Sperm allocation in the three-spined stickleback

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Male three-spined stickleback *Gasterosteus aculeatus* have a fixed amount of sperm during the breeding season because spermatogenesis is inhibited at this time. A method was developed to estimate ejaculate size *in situ* by removing the sperm from the male's nest. The reliability of the method was tested using known numbers of sperm. In their first mating, males ejaculated $11{\cdot}64 \times 10^6$ sperm (median), representing *c.* 5% of the male's sperm store (median $27{\cdot}88 \times 10^7$ sperm). The amount of sperm in the testes was significantly reduced in males that had mated several times (median $8{\cdot}09 \times 10^7$). Additionally, ejaculate size was smaller in these experienced males (median $8{\cdot}79 \times 10^5$). Heavier and larger fish invested absolutely and relatively more sperm in a mating than did lighter and smaller fish. Ejaculate size did not correlate with the mass of the egg clutch.

Key words: ejaculate size; *Gasterosteus aculeatus*; *in situ* sperm collection; sperm depletion; sperm; testis mass.

INTRODUCTION

The small size of sperm relative to eggs suggests that a sperm is much cheaper to produce than an egg, but ejaculate size may be a better indicator of costs because males produce more sperm than there are eggs to fertilize (Dewsbury, 1982; Parker, 1982). When ejaculate size is taken as the unit, the costs of male gamete production are not negligible (up to 65% of that of females; Schärer & Robertson, 1999), and the number of ejaculates a male can produce within a given time is limited (Dewsbury, 1982; Nakatsuru & Kramer, 1982; Parker, 1990). Consequently, males are expected to allocate sperm carefully, and the number of sperm in an ejaculate should be influenced by the number of eggs available for fertilization (Parker, 1990; Shapiro & Giraldeau, 1996). Such sperm allocation has been reported in *Sparisoma radians* (Valenciennes) (Marconato & Shapiro, 1996) and *Thalassoma bifasciatum* (Bloch) (Shapiro *et al.*, 1994). In *T. bifasciatum,* males that expect a large number of matings, invest a low number of sperm per mating (Warner *et al.*, 1995) in accordance with the theory that males should maximize the number of fertilized eggs rather than fertilization rate (Shapiro & Giraldeau, 1996).

In fishes, ejaculate size has rarely been measured directly (Shapiro *et al.*, 1994; Fuller, 1998; Pilastro & Bisazza, 1999). Due to methodological constraints, male gamete investment is often assessed by counting the sperm in stripped volumes of

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semen (De Fraipont *et al.*, 1993; Stockley *et al.*, 1997). However, stripped sperm number may not generally represent ejaculate size, as fishes might be able to partition their available sperm (Rasotto & Shapiro, 1998). A method was therefore developed in the present study to measure ejaculate size directly *in situ*.

In male three-spined sticklebacks *Gasterosteus aculeatus* L., spermatogenesis occurs from September until January and is inhibited during the breeding season (Borg, 1982). Therefore, male sticklebacks have a fixed amount of sperm for allocation during the breeding season (*G. aculeatus*, Borg, 1982; *Culaea inconstans* (Kirtland), Ruby & McMillan, 1970). During the breeding season, males establish a territory and build a tunnel-shaped nest of plant material, into which females lay clutches of eggs (40–295 eggs per clutch, Wootton, 1984). During a breeding cycle males may collect up to twenty egg clutches (Kynard, 1978), or more (T. C. M. Bakker, unpubl. data) over a short period of time (median of 3 days, Kraak *et al.*, 1999*a*), and may complete several breeding cycles during the breeding season (Bakker & Mundwiler, 1994). Most three-spined stickleback populations (Van Mullem & Van der Vlugt, 1964) are annual and thus have only one breeding season in their lifetime.

The aim of the present study was to develop a method for a quantitative *in situ* assessment of ejaculate size in a nest-building fish. Because spermatogenesis in sticklebacks is inhibited during the breeding season, a further aim was to quantify the number of sperm in the testes (sperm store), the sperm allocated in a given mating, and the relationship between sperm store and ejaculate size. A final aim was to look for male traits that could predict ejaculate size, and whether males adjust ejaculate size to female size, mass and egg mass.

MATERIALS AND METHODS

STUDY ANIMALS AND REARING CONDITIONS

Three-spined sticklebacks, captured on Texel Island, The Netherlands, during their spring migration in March 2000, were transported to the University of Bern, Switzerland, where they were initially held in a storage tank (*c.* 320 l) which was supplied with running tap-water of 16–19° C. Later, the fish were distributed among two 200 l tanks supplied with running tap-water of 16 ± 1° C, were exposed to a 16L : 8D photoperiod, and fed to satiation with frozen chironomid larvae.

Males with developing breeding colouration were taken from the stock and placed into individual 15 l plastic aquaria containing a petri-dish (diameter 9 cm) with fine gravel, and 8 cm pieces of green cotton twine to serve as nesting material. The aquaria were separated by opaque grey partitions, supplied with running tap-water, but not aerated. Nests were constructed in 1–3 days and fish were used in tests within 2 days after nestbuilding.

Reproductively active females were moved from the two storage tanks to a 200 l female tank exposed to the same conditions as the storage tanks. Ripe females with open cloacae were used in the tests.

Experiments were performed between 18 May and 14 June 2000. Each fish was used only once.

IN SITU ASSESSMENT OF EJACULATE SIZE

The number of sperm released for fertilization was assessed using the method described by Shapiro *et al.* (1994) and adapted by Fuller (1998). Male standard length (L_S) and body mass were measured 1 h before the tests. Water flow to the aquaria was stopped during the tests. Ejaculate size was measured for two sets of males: (a) 18 males were

tested for their first and second mating in the breeding season (referred to as two matings males). Two females of known body mass were allowed to spawn with each male. The second test was performed 1–3 days after the first, when the male had rebuilt its nest; (b) during the same time, five males, which had experienced eight matings or more before were tested once (referred to as ≥eight matings males). These males came from the same sample as all other fish used but had been given the experience of 8, 10, 16, 34, and 38 matings, respectively. Before they were introduced into the experimental aquaria, they had been kept under similar conditions as the two matings males, except that the bottom of their tanks had been covered with a layer of fine gravel and they had had algae as nesting material (D. Mazzi, pers. comm.).

Before and after spawning, females were weighed in order to estimate egg mass (W_E,). Male condition factor after the experiments was calculated as $K=100 \times WL_S^{-2\cdot86}$, where the exponent is the slope of the regression of log (body mass W) on log (L_S) of the sample (Bolger & Connolly, 1989).

Immediately after the male had ' crept ' through the nest to fertilize the eggs, a lid was placed on the petri dish containing the nest, eggs, sperm and a small volume (*c.* 35 ml) of water. The contents of the petri dish were rinsed on a 30 μm filter using 500 ml water, and filtrated under vacuum. The resulting solution was filtered through a 20 μm filter, and the resulting filtrate was passed through a 0·2 μm Millipore filter to collect the sperm.

Sperm were stained using an adapted ' Christmas-tree ' staining (Oppitz, 1969) by treating the filter with 800 μl of ' nuclear fast red ' solution (50 g of aluminium sulphate and 1 g of nuclear fast red in 1000 ml distilled water) for 10 min, followed by washing with distilled water under vacuum. The filter was then treated with 700 μl of Picroindigocarmine (3·3 g of indigo carmine on 1000 ml of a saturated picric acid solution) for 15 s followed by washing with absolute ethanol. ' Nuclear fast red ' stains DNA with an intensive red colour, so sperm appear as distinct bright red dots under the microscope. Picroindigocarmine colours the cell plasma blue. The DNA stain of normal cells is therefore faded by the plasma colouration.

Four segments of the dried filter, each *c.* 0·125 of the filter's area, were mounted on a slide and cleared with immersion oil (Leong, 1989). The number of sperm within a field (0·002 mm^2) was counted for 25 fields of each segment, using a Sony Trinitron screen linked to a light microscope (magnification × 100). Segments were examined by stepwise screening so that no field was counted twice. The observer was blind with respect to the choice of the area because the microscope was unfocused while moving to the next area. Sperm estimates for the whole filter (S_F) (area 1452·2 mm^2) were then estimated as: S_F=(total no. of sperm counted) × [(100 fields × 0·002 mm^2)]$^{-1}$ × (1452·2 mm^2 of Millipore filter).

ASSESSMENT OF SPERM STORE

For all males in the experiment (two matings, $n=18$ and ≥eight matings, $n=5$) testes mass and the number of sperm in the testes (sperm store) was assessed on the day of the last experiment. The mass of the testes (W_T) was measured by placing them in a pre-weighed Eppendorff tube containing 200 μl isotonic medium (Fauvel *et al.*, 1999; NaCl, 3·5 g l^{-1}; KCl, 0·11 g l^{-1}; $MgCl_2$, 1·23 g l^{-1}; $CaCl_2$, 0·39 g l^{-1}; NaH_2CO_3, 1·68 g l^{-1}; glucose, 0·08 g l^{-1}). The gonadosomatic index (I_G) was calculated as: $I_G=100 \times W_T W^{-1}$. Testes were homogenized in the Eppendorff tube with a small pestle and the resulting suspension was vortexed and centrifuged for *c.* 2 s (Rotilabo-Mini-Centrifuge, 6000 rpm). Two μl of the supernatant were then diluted with 88 μl of a 4% formalin solution and vortexed again. One Neubauer haemocytometer chamber was then filled with a 12 μl sample of this solution. After 5 min the number of sperm was counted in 64 cells ($2\cdot5 \times 10^{-4}$ μl each) of the chamber. The total number of sperm (S_H) in the male's testes was calculated as: $S_H=4 \times 10^7 \times$ (mean number of sperm per cell).

RELIABILITY OF THE *IN SITU* SPERM COLLECTION METHOD

A series of 23 control trials was used to test the sperm collection method. Sperm collected from homogenized testes was assessed as described above, and fresh eggs were obtained by stripping ripe females. A portion, 0·30 g (± 0·04), of the egg clutch was

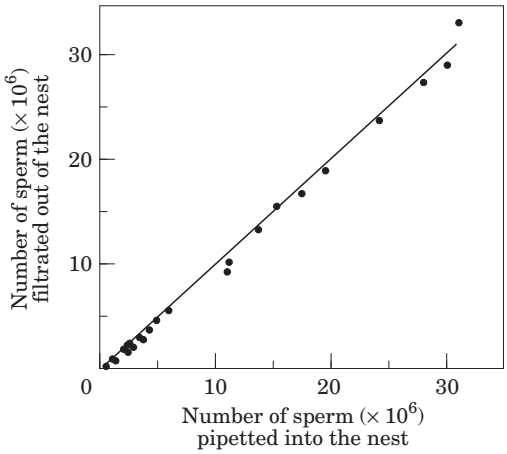

FIG. 1. Linear regression of estimates of sperm number (based on filtration after removal of the nest) on the number of sperm pipetted into the nest (calculated in a haemocytometer chamber) to test the *in situ* method ($y=1{\cdot}012x-282\,732$; $r^2=0{\cdot}99$, $F_{1,22}=5359{\cdot}2$, $P<0{\cdot}0001$).

placed into a stickleback nest, and then 10–30 µl sperm solution per trial were deposited inside the nest using a pipette. The nest and contents were then processed and the number of sperm estimated as described above. The sperm were motile when added into the nest; and stickleback sperm remain active for some hours in the isotonic saline, whereas in water they stop moving within seconds (M. Zbinden pers. obs.; cf. De Fraipont *et al.*, 1993).

The number of sperm that had been deposited in the nest was estimated from the haemocytometer count: Number of sperm added $=0.005\ S_H \times$ (volume of solution added).

STATISTICAL ANALYSES

The data were tested for normality using the Shapiro–Wilk W test, and some (body mass, testes mass, ejaculate size) were log transformed to give normal distributions. Normaly distributed data were tested with paired t-tests or linear regressions. When the distribution of the data was not normal, a Mann–Whitney U test was used. Analyses were performed using the JMP IN 3.2.1 (SAS Inst. Inc.) statistical package. When multiple tests were performed α-levels were corrected for, using a sequential Bonferroni-procedure (Rice, 1989). Given P values are two-tailed throughout.

RESULTS

IN SITU SPERM COLLECTION METHOD

The estimate of the number of sperm recovered from the nest correlated significantly with the estimate of the number added ($r_p=0{\cdot}99$, $F_{1,22}=5359{\cdot}2$, $P<0{\cdot}0001$; Fig. 1). The slope of the linear regression was not significantly different from 1 (t-test: $t=0{\cdot}85$, d.f.$=21$, $P>0{\cdot}2$; Fig. 1). The numbers of sperm pipetted into the nests ($1{\cdot}0\times10^6-33{\cdot}5\times10^6$) corresponded to the natural variation in ejaculate size (see below).

EJACULATE AND SPERM STORE SIZE

Within the two mating males, ejaculate sizes of the first spawn (median $11{\cdot}64\times10^6$ sperm, range $0{\cdot}91\times10^6-40{\cdot}44\times10^6$) and the second spawn

(median $10{\cdot}76 \times 10^6$, range $0{\cdot}65 \times 10^6 - 33{\cdot}85 \times 10^6$) were highly correlated ($r_p = 0{\cdot}88$, $F_{1,17} = 56{\cdot}56$, $P < 0{\cdot}0001$, second on first mating linear regression line of log transformed data: $y = 0{\cdot}987x + 0{\cdot}02$).

Sperm store after the second mating (S_H) of the two matings males was estimated to be $23{\cdot}60 \times 10^7 \pm 9{\cdot}98 \times 10^7$ (mean ± S.D.). The sum of S_H and the two ejaculate sizes yielded an estimate of the sperm store before any matings had taken place, i.e. how many sperm a male had available for the whole breeding season (S_0, $26{\cdot}18 \times 10^7 \pm 10{\cdot}83 \times 10^7$). S_0 was positively correlated with I_G and W_T (Table I, Fig. 2). By summing S_H and the second ejaculate size, an estimate of the sperm store before the second test was gained. Ejaculate size of the second mating did not significantly correlate with the available sperm before the mating, I_G, and W_T (Table I). Ejaculate size of the second mating showed positive relationships to L_S and W (Table I). The number of sperm ejaculated relative to the estimated sperm store before this mating measures the relative sperm investment for a given spawn. Males invested $4{\cdot}96 \pm 2{\cdot}89\%$ (mean ± S.D.) of their sperm store in the first and $5{\cdot}41 \pm 2{\cdot}95\%$ in their second mating. The mean relative investment for a male was significantly correlated with W and L_S (Table I). Therefore, heavier and larger fish invested relatively more in a mating than lighter and smaller fish. After Bonferroni correction, there was no significant relationship between the relative investment and I_G or W_T (Table I).

I_G and W_T did not significantly correlate with W ($r_p = 0{\cdot}33$, $F_{1,17} = 1{\cdot}97$, $P = 0{\cdot}18$ and $r_p = 0{\cdot}22$, $F_{1,17} = 0{\cdot}82$, $P = 0{\cdot}38$, respectively). Ejaculate size, S_0 and relative investment were not significantly related to a male's condition factor (all $P > 0{\cdot}1$). Neither $W_{\male}$, L_S, W_T, I_G, S_0 or ejaculate size covaried with the date of the experiment, the number of days until nest-building, and the number of days between nest-building and the experiment (all $P > 0{\cdot}1$).

Body mass of the females used for the first mating was not significantly different from that of the females in the second (paired t-test, data log transformed: $t = 0{\cdot}24$, d.f. = 16, $P = 0{\cdot}81$), nor was the mass of the egg clutches (paired t-test, data log transformed: $t = -1{\cdot}32$, d.f. = 16, $P = 0{\cdot}2$).

Neither ejaculate size nor relative sperm investment correlated significantly with female $W_{\female}$, L_S and W_E (all $P > 0{\cdot}1$). Also the difference in ejaculate size and relative investment between the two matings did not significantly correlate with differences in female traits between the matings (all $P > 0{\cdot}1$).

SPERM DEPLETION

The ≥eight matings males had a significantly reduced sperm store in comparison to the sperm store of the two matings males (Mann–Whitney U test, $n_1 = 5$, $n_2 = 18$, $z = -2{\cdot}58$, $P = 0{\cdot}01$, $\alpha = 0{\cdot}05$; Table II, Fig. 3). The two groups significantly differed (after sequential Bonferroni correction) in ejaculate size (Mann–Whitney U test, $n_1 = 5$, $n_2 = 18$, $z = -2{\cdot}27$, $P = 0{\cdot}023$, $\alpha = 0{\cdot}025$; Table II, Fig. 3), but not in relative sperm investment (Mann–Whitney U test, $n_1 = 5$, $n_2 = 18$, $z = -0{\cdot}71$, $P = 0{\cdot}48$, $\alpha = 0{\cdot}017$; Table II), W_T or I_G (Mann–Whitney U tests, $n_1 = 5$, $n_2 = 18$; $z = -0{\cdot}11$, $P = 0{\cdot}91$, $\alpha = 0{\cdot}013$ and $z = -0{\cdot}04$, $P = 0{\cdot}97$, $\alpha = 0{\cdot}01$, respectively; Table II). The two groups of males did not significantly differ in L_S and W ($P > 0{\cdot}2$).

TABLE I. Correlates of ejaculate size in the second mating, sperm store and relative sperm investment in the two matings males to sperm store (S_0, male's calculated available number of sperm for the breeding season)

	Sperm store prior to the second mating (S_0)				I_G				log W_T				L_S				log W			
	$F_{1,17}$	r_p	P	a	$F_{1,17}$	r_p	P	a	$F_{1,17}$	r_p	P	a	$F_{1,17}$	r_p	P	a	$F_{1,17}$	r_p	P	a
Ejaculate size (log)	3·7	0·4	0·07	0·02	0·2	− 0·12	0·63	0·01	2·7	10·38	0·12	0·01	14·1	0·68	**<0·01**	0·03	13·6	0·68	**<0·01**	0·05
Sperm store (S_0)					10·6	0·63	**<0·01**	0·03	25	0·78	**<0·01**	0·05	0·5	0·17	0·48	0·02	0·25	0·12	0·62	0·01
Relative investment					4·9	0·48	0·04	0·02	0·1	0·06	0·8	0·01	8·5	0·59	**0·01**	0·03	10·9	0·64	**<0·01**	0·05

Gonadosomatic index (I_G), testes mass (W_T), standard length (L_S) and body mass (W). r_p is the Pearson correlation coefficient, a-levels are set after sequential Bonferroni correction for multiple tests. Significant P values are in bold.

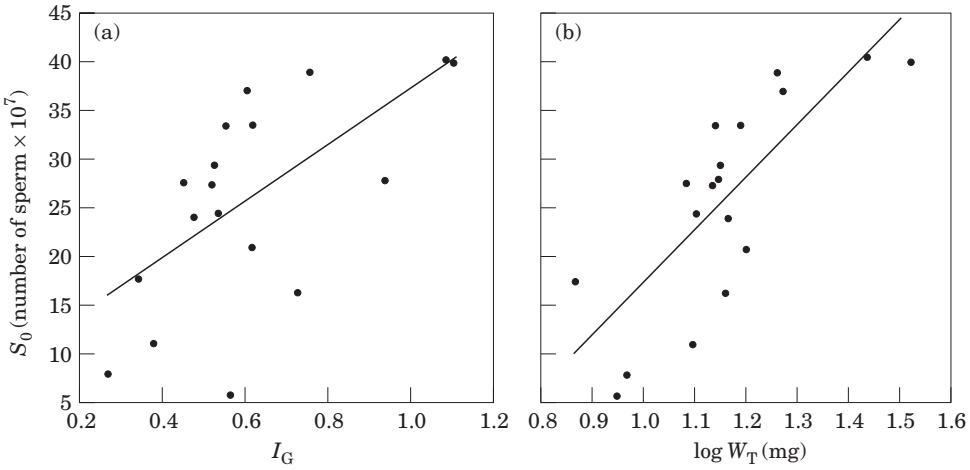

FIG. 2. Correlations of (a) the gonado-somatic index (I_G) (r_p=0·63, $F_{1,17}$=10·6, P<0·01) and (b) the log of testes mass (W_T) (r_p=0·78, $F_{1,17}$=25·0, P<0·01) and sperm store (S_0) in the two matings males. The lines are linear regressions.

DISCUSSION

The method developed for *in situ* estimation of ejaculate size in sticklebacks proved to be accurate in control tests (Fig. 1). A male's ' creeping ' through the nest to fertilize the eggs takes *c*. 2 s which corresponds to the time needed to experimentally deposit the sperm in the nest. The time needed to place the lid on the nest was probably slightly longer in the control than in the experiments, because pipetting and capping the nest was done by one person. Therefore the method was not expected to be less accurate in the experiments than in the control.

By using artificial nesting material and forcing the stickleback males to build their nest on a petri dish, a small volume of water could be analysed and the amount of small algae in the nest could be reduced that would hinder the count of sperm cells.

Freshly laid eggs are surrounded by mucus and some of the ejaculate will probably be trapped, so ejaculate size might be slightly (but systematically) underestimated by the method used. The estimate may further be biased by nuclei of destroyed cells. However, the number of nuclei having the same size and shape as sperm will be small.

Spermatogenesis in sticklebacks is inhibited during the breeding season (Borg, 1982) and accordingly males with eight or more matings had less sperm in their testes than males spawning for the first time. Males that had 34 and 38 matings, respectively ejaculated only *c*. 700 000 sperm (Fig. 3), but were nevertheless able to fertilize nearly all the eggs in their penultimate mating (D. Mazzi, pers. comm.). Therefore, males seem to invest several times more sperm than needed to fertilize the eggs in most matings. This finding is at odds with theories about male ejaculate size (Parker, 1990; Shapiro & Giraldeau, 1996), in which it is predicted that males should invest the minimum number of sperm in a given spawn in order to maximize the number of fertilized eggs over the breeding season. To minimize ejaculate size and thus taking the risk of having unfertilized

TABLE II. Measures of sperm release and sperm store in the first mating of the two matings males and in the mating of the ≥eight matings males

Males	n	Ejaculate size ($\times 10^6$)	S_0 ($\times 10^7$)	Relative investment (%)	W_T (mg)	I_G
Two matings	18	11·64 (0·91–40·44)	27·88 (61·16–407·94)	4·2 (1·0–6·2)	13·9 (7·4–32·9)	0·55 (0·26–1·10)
≥Eight matings	5	0·88 (0·69–10·09)*	8·09 (18·58–232·02)*	4·1 (0·3–11·0)	14·0 (8·7–16·0)	0·57 (0·40–0·67)

I_G, Gonadosomatic index. Data are shown as median and range. α-level is set after sequential Bonferroni correction. *Significant differences.

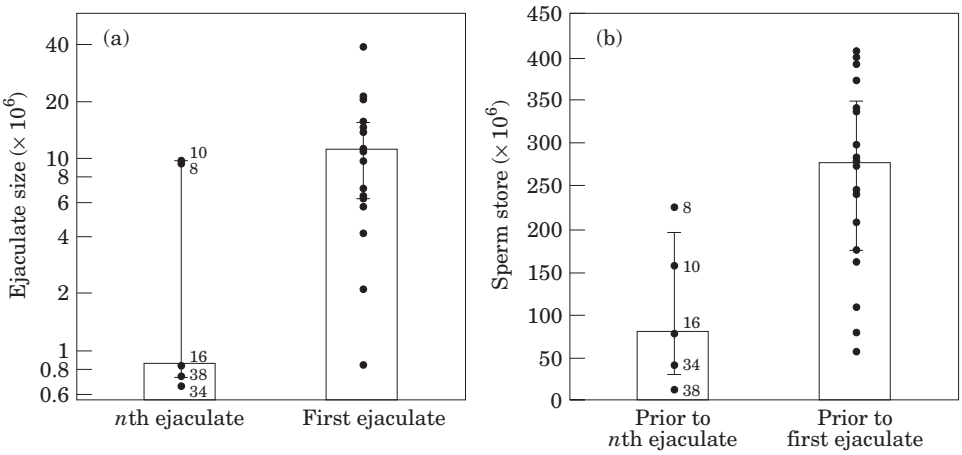

FIG. 3. (a) Ejaculate size (log scale) of the 5 ≥ eight matings males in their ultimate mating and of the 18 two matings males in their first mating and (b) the sperm store of these males before these matings. Plotted are individual data, and median and quartiles of both groups of males. The number of matings the ≥eight matings males have had before are indicated.

eggs in the nest may not be an optimal strategy for a stickleback. The eggs of successive females lie in closest proximity to each other in the nest. Unfertilized eggs are infected by fungi within a short time which can spread to neighbouring healthy eggs (M. Zbinden & T. C. M. Bakker, pers. obs.). A further indication that sticklebacks do not minimize their ejaculate size was the lack of a correlation between ejaculate size or relative investment and the mass of the egg clutch, which would be predicted by the model of Shapiro & Giraldeau (1996).

Ejaculate size was *c.* 5% of the stored sperm prior to the mating, irrespective of how many times a male had mated before. This suggests that sticklebacks ejaculate a certain percentage of the seminal fluid that (contrary to the sperm) may be refreshed between matings. By optimizing the volume of the ejaculate, sticklebacks may optimize the distribution of sperm over the eggs. An optimal distribution may be crucial since sperm have very short motility durations (between 300 and 450 s for three-spined sticklebacks; De Fraipont *et al.*, 1993).

The gonadosomatic index (I_G) is assumed to be a reliable measure of the investment in gametes. However, the relationship between I_G and sperm store within a species is not very well documented. In *S. radians,* testes mass is correlated with stripped sperm number (Marconato & Shapiro, 1996). In the present study, I_G and testes mass were well correlated with the number of sperm in the testes. Thus, the data support the use of the gonadosomatic index as a measure of sperm store in three-spined sticklebacks. Ejaculate size, sperm store and relative investment were not found to be correlated with male condition. Sperm store could be expected to be related to condition. However, condition during the breeding season may not reflect a fish's well being in autumn and winter when spermatogenesis occurs. Bigger males invested absolutely and relatively more sperm in a mating than smaller ones. There may be several possible explanations for this, including morphological constraints in sperm ejaculation that lead to larger ejaculate volumes in bigger fish or different tactic

decisions depending on body size. An association between body size and ejaculate size could be one of the reasons why larger males are more attractive to females (Kraak *et al.*, 1999*b*).

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