Raffles, roles, and the outcome of sperm competition in sockeye salmon

Drew J. Hoysak, N. Robin Liley, and Eric B. Taylor

Abstract: In species with male alternative reproductive phenotypes, one phenotype is usually disadvantaged in mating competition. In salmonid fishes, large late-maturing males pair with nesting females and maintain close contact before and during spawning. Small early-maturing males have little contact with nesting females and, during spawning, begin to release sperm after the paired male. The effects of male phenotype and timing of ejaculation on success in sperm competition are not known. In this study, we determined paternity of offspring resulting from in vitro competitive fertilizations to examine these two aspects of sperm competition in sockeye salmon, *Oncorhynchus nerka* (Walbaum, 1792). When we fertilized eggs with mixtures of equal numbers of sperm from each of two male age classes, we found that success in sperm competition did not depend on male age. However, success in these competitive fertilizations did not conform to the fair raffle model of sperm competition, since paternity in most of the clutches was biased in favour of one male. When we added milt from two males sequentially to a batch of eggs, we found that sperm from the second male fertilized fewer eggs than sperm from the first male, but the difference was less than expected. In addition, a male's success when his milt was added first was not correlated with his success when his milt was added second.

Résumé : Chez les espèces qui possèdent des mâles reproducteurs de deux phénotypes différents, l'un des deux phénotypes est généralement désavantagé dans la compétition pour la reproduction. Chez les poissons salmonidés, les grands mâles à maturation tardive s'apparient avec les femelles au nid et gardent un contact serré avec elles avant et durant la fraye. Les petits mâles à maturation hâtive ont peu de contacts avec les femelles au nid et, durant la fraye, commencent à libérer leurs spermatozoïdes après le mâle apparié. Les effets du phénotype mâle et du moment de l'éjaculation sur le succès de la compétition spermatique ne sont pas connus. Nous avons déterminé la paternité des rejetons produits dans des fécondations compétitives in vitro afin d'étudier ces deux aspects de la compétition spermatique chez le saumon rouge, *Oncorhynchus nerka* (Walbaum, 1792). Lorsque les oeufs sont fécondés par des mélanges égaux de spermatozoïdes provenant de mâles de deux classes d'âge différentes, le succès de la compétition spermatique n'est pas relié à l'âge des mâles. Cependant, le succès de ces fécondations compétitives ne se conforme pas au modèle de la loterie équitable de la compétition spermatique, car la paternité de la plupart des masses d'oeufs est due de façon prépondérante à un seul mâle. Lorsque nous avons ajouté de la laitance de deux mâles en succession à une masse d'oeufs, les spermatozoïdes du second mâle ont fécondé moins d'oeufs que ceux du premier mâle, mais la différence était moindre que nous attendions. De plus, il n'y a pas de corrélation entre le succès d'un mâle dont la laitance est ajoutée en premier et le succès du même mâle quand sa laitance est ajoutée en second lieu.

[Traduit par la Rédaction]

Introduction

A large number of studies have examined the effects of sperm competition on the evolution of ejaculates (Birkhead and Møller 1998). Important theoretical developments in this field have come from the understanding that males do not always compete on a level playing field (Parker 1990*a*, 1990*b*); that is, sperm competition may be biased in favour of some males. Parker referred to this form of sperm competition as a loaded raffle (i.e., some individuals have a greater chance of winning by virtue of owning more tickets). This is in contrast to a fair raffle in which all individuals have the same number of tickets and have an equal chance of winning. The fairness of the raffle, in terms of sperm competition, may be influenced by the relative number of sperm from competing males as well as by temporal and spatial placement of ejaculates. An example in which some males may always have certain disadvantages in sperm competition occurs in breeding systems where males exhibit alternative reproductive phenotypes such as sneakers and guarders. In this type of system, it may be expected that sneakers are disadvantaged in sperm competition for three reasons. First, sneaker males are often smaller than guarders and this may result in sneaker males having fewer sperm available. Even if sneakers invest relatively more in sperm production than guarders, it may not be enough to make up for the disparity in body size (Gage et al. 1995; Foote et al. 1997; Leach and Montgomerie 2000). Second, sneaker males may mate in disadvantageous temporal positions in a sequence of matings. In internally fertilizing species like birds and insects, the last male to

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mate with a female usually sires most of the offspring (Birkhead 1998*b*; Simmons 2001). In externally fertilizing fish where there is direct and immediate contact between eggs and sperm, sneaker males often start releasing sperm after guarding males (Maekawa 1983; Gross 1985). Finally, sneaker males may mate in disadvantageous spatial positions when males release sperm simultaneously (Brockmann et al. 1994; Roberts et al. 1999). Because of these disadvantages to sneaking males, sperm competition may be best described as a loaded raffle in species with alternative male reproductive phenotypes.

The asymmetry between alternative male phenotypes is compounded by the fact that sneaker males always undergo sperm competition, whereas guarding males do not always compete with sneaker males. Therefore, sneaking phenotypes should experience strong selection for traits that improve success in sperm competition. This has led to the evolution of increased sperm production by sneaker phenotypes. In many fish species, sneaker males have larger testes relative to body size and greater numbers of available sperm (i.e., larger potential ejaculate size) relative to body size than do guarding males (Taborsky 1994; Gage et al. 1995; Ruchon et al. 1995; Foote et al. 1997; Scaggiante et al. 1999; Uglem et al. 2001). Similarly, sneaking males in the dung beetle *Onthophagus binodis* Thunberg, 1818 have relatively larger testes and larger ejaculate volume than guarding males (Simmons et al. 1999).

Much of the emphasis in studies of the evolutionary effects of sperm competition has been placed on the number of sperm produced, but the effect can be expressed more generally as an investment in sperm. Investment may be expressed in numbers of sperm, but it may also involve sperm "quality" (e.g., size, longevity, speed, etc.). Thus, when phenotypes differ in mating roles and (or) sperm competition risk, the resulting selection could lead to differences in sperm quality. This selection may also be important in the evolutionary maintenance of alternative phenotypes (Birkhead and Pizzari 2002). Differences in sperm traits between male phenotypes have been found in the dung beetle *O. binodis* in which sneaking males have longer sperm than do guarding males (Simmons et al. 1999). In Atlantic salmon, *Salmo salar* L., 1758 (Gage et al. 1995; Vladić and Järvi 2001), and corkwing wrasse, *Symphodus melops* (L., 1758) (Uglem et al. 2001), sperm from sneaker males have greater motility than sperm from guarding males. In addition, sperm from Atlantic salmon sneakers (parr) have been shown to fertilize a higher proportion of eggs than sperm from guarding (anadromous) males in noncompetitive in vitro fertilizations (Vladić and Järvi 2001). However, sperm from different corkwing wrasse phenotypes do not differ in their ability to fertilize eggs (Uglem et al. 2001), and other studies have shown no differences between male phenotypes in sperm traits or fertility (Leach and Montgomerie 2000; Hoysak and Liley 2001).

One of the problems with examining sperm characteristics is that, for most species, little is known about the relationship between the sperm traits and the ability of sperm cells to compete for fertilizations. An alternative method for comparing sperm is to examine directly the fertilization success of sperm from different males in a competitive environment. For example, females can be inseminated with a mixture of sperm from two males and paternity of the resulting offspring determined. For example, Evans et al. (2003) used this technique with guppies, *Poecilia reticulata* Peters, 1859, to show that sperm from relatively colourful males outcompete sperm from less colourful males. The main drawback of this approach is that it does not distinguish the effect of sperm competition from that of postcopulatory female choice (Birkhead 1998*a*). This problem may be reduced in externally fertilizing fish in which there can be no selection imposed by a female reproductive tract. In addition, in most fish species the first sperm to enter a single hole (micropyle) in the egg membrane fertilizes the egg (Yanagimachi et al. 1992). Although there is potential for eggs to manipulate sperm behaviour to some extent (Hart 1990), there is no evidence that manipulation can bias fertilization towards particular male phenotypes.

Salmonid fishes are particularly well suited for studies of sperm competition. In these species, males congregate at females' nests and compete to fertilize eggs (Fleming 1998). Large males typically fight and display for close access to a female, with the winner assuming the alpha position beside the female. The smallest males rarely fight for access to females, but instead adopt sneaking behaviour, using crypsis and refuges to remain close to a nesting female. During a spawning event, the female and alpha male simultaneously release gametes into the nest. Subordinate males may also rush into the nest and release sperm. Male behaviour is flexible in salmonids but is strongly influenced by life-history trajectories. In Pacific salmon (species of *Oncorhynchus* Suckley, 1861), males mature at different ages and all individuals die after breeding (Groot and Margolis 1991). Variation in age at maturity has both genetic and environmental origins (Iwamoto et al. 1984; Hankin et al. 1993; Heath et al. 1994; Wood and Foote 1996). Thus, different age classes represent alternative phenotypes that occupy different roles (sneaking and guarding) in mating.

Alternative male phenotypes of salmonids have not been compared for competitive sperm quality, but Foote et al. (1997) found that sneaker and guarding phenotypes of sockeye salmon, *Oncorhynchus nerka* (Walbaum, 1792), did not differ in fertilization success in semi-natural matings even though the sneaker males have fewer sperm available and they begin releasing sperm after guarding males. This result is consistent with the hypothesis that sneaker males have sperm that are competitively superior to sperm from guarding males. However, Neff et al. (2003) found that sperm from parental male bluegill sunfish, *Lepomis macrochirus* Rafinesque, 1819, tended to outcompete sperm from cuckolder males. This result contradicts the hypothesis that disadvantaged males have higher quality sperm than advantaged males.

In this study, we determined paternity of offspring resulting from in vitro competitive fertilizations to examine the effects of male phenotype, genotype, and timing of sperm release on success in sperm competition in sockeye salmon. In the first experiment, we placed sperm from alternative male phenotypes in competition to test the hypothesis that sperm from males in disfavoured roles should outcompete sperm from males in favoured roles. Males in the study population (see below) return to breed at 3, 4, or 5 years of age and females return to breed at 4 or 5 years of age (West

1978), and all individuals die after breeding. Because age classes correspond to alternative reproductive phenotypes and younger males have disfavoured mating roles, we predicted that sperm from younger males should outcompete sperm from older males.

This experiment also allowed us to test for morphological and genetic correlates of success in sperm competition. If success in sperm competition is determined by sperm quality, which in turn is influenced by phenotypic or genetic quality (Sheldon 1994), then we would expect success in sperm competition to be correlated with the size of secondary sex traits, body condition, and (or) heterozygosity (Sheldon 1994; Brown 1997). On the other hand, success in sperm competition may be influenced by inbreeding avoidance (Tregenza and Wedell 2000). If this is the case, we would expect males closely related to the female to be less successful than distantly related males.

In a second experiment, we examined the effect of the timing of sperm release on success in competitive fertilizations. Previous observations suggest that eggs are fertilized very quickly in this and other species (Hart 1990; Iwamatsu et al. 1991; Hoysak and Liley 2001; Liley et al. 2002). We therefore predicted that when sperm from two males of the same age are added sequentially to eggs, sperm from the first male would have a large advantage over those from the second male.

Materials and methods

The study was conducted at Fulton River, a tributary of Babine Lake, British Columbia, Canada. We captured fish at a counting fence (maintained by Fisheries and Oceans Canada) near the mouth of the Fulton River. They were handled in accordance with the principles and guidelines of the Canadian Council on Animal Care. Only fish without injuries, fungus, and fin wear were used to ensure that all individuals were of comparable physical condition and had not already participated in spawning. All fish selected had free-flowing gametes (easily collected with gentle pressure to the abdomen).

To obtain gametes, we anesthetized fish in 0.05% 2-phenoxyethanol, wiped the genital pore region dry with paper towels, and gently squeezed the abdomen to extrude milt and eggs into clean dry beakers. Care was taken to avoid contamination of gametes with faeces, urine, and blood. Gametes were stored on ice until the experiments were performed.

In both experiments, we controlled the number of sperm cells used from each male using spermatocrit as a measure of sperm abundance in the milt. Spermatocrit was measured for each milt sample by filling two capillary tubes with milt, centrifuging them for 10 min in a microhematocrit centrifuge (5900*g*), and measuring the proportion of the sample occupied by packed sperm cells. We used the average of two spermatocrit measurements for each male. This measure is a correlate of the number of sperm cells per unit volume of milt (Bouck and Jacobson 1976; D.J. Hoysak, unpublished data). Estimates of the correlation between spermatocrit and number of sperm cells per microlitre for our population range from 0.58 to 0.85 and repeatability of spermatocrit is 0.996 (D.J. Hoysak, unpublished data). The relationship between spermatocrit and sperm-cell count does not vary with male age (D.J. Hoysak, unpublished data), indicating that sperm size does not differ between age classes.

Experiment 1: effect of male phenotype and genotype

The purpose of the first experiment was to compare alternative phenotypes (age classes) in their success in sperm competition. We used three different individuals in each replicate: one 5-year-old male (hereinafter referred to as 5Y male), one 3-year-old male (hereinafter referred to as 3Y male), and one 4-year-old female. Age classes can be distinguished by body size (Hanson and Smith 1967). In this experiment, 3Y males ranged in hypural length, which is a measure of body size and is the distance from the posterior edge of the orbit to the posterior edge of the hypural plate, from 280 to 305 mm, 5Y males ranged from 485 to 530 mm, and females ranged from 410 to 475 mm.

Each replicate in this experiment consisted of three in vitro fertilizations. We fertilized one batch of approximately 150 eggs (range 105–199) with milt from a 3Y male. A second batch of approximately 150 eggs (range 115–186) was fertilized with milt from a 5Y male. We alternated the order of these fertilizations between replicates. The volume of milt used was that which yielded 0.5 mL of packed sperm cells as calculated from spermatocrit. These single-male fertilizations allowed us to assess the combined effects of fertilization success and survival of fertilized eggs for each male. We fertilized a third batch of approximately 300 eggs (range 176–377) with a mixture of milt from the two males. This mixture consisted of 0.5 mL of sperm from each male and was stirred thoroughly before being added to the eggs. We performed a total of 10 replicates using different individuals in each. We completed all fertilizations in a replicate within 45 min of starting to collect gametes. Neither eggs nor sperm lose viability during this time (Stoss 1983).

Fertilizations were performed by simultaneously adding milt and river water to the eggs. The volume of water used was 250 mL for the single-male fertilizations and 500 mL for the competitive fertilization. The gametes were swirled for several seconds and allowed to stand for 3 min. The eggs were then rinsed thoroughly and placed in perforated plastic tubes. The tubes were buried in gravel in a spawning channel and left to incubate.

Parental fish were given a lethal dose of anesthetic and body measurements were taken. These included hypural length (described above), mass, snout length (i.e., distance from the posterior edge of the maxilla to the tip of the upper jaw), and hump size (i.e., vertical distance between the lateral line and the edge of the body just anterior to the dorsal fin). Snouts and humps are secondary sexual characters that are important in male–male competition and female choice (Järvi 1990; Quinn and Foote 1994). Size-adjusted morphological measurements were calculated as the residual of the given measurement from a linear regression of log(trait size) vs. log(body size) for a sample of 36 males. A sample of liver tissue was stored in 95% ethanol for DNA analysis.

Approximately 2 months after the fertilizations, we removed all tubes from the spawning channel and placed them in a laboratory incubator. At this time, undeveloped and damaged eggs were removed and counted. We checked for further mortality every 2 or 3 days until the eggs hatched.

When the offspring reached the free-swimming fry stage, we gave them a lethal dose of anesthetic and preserved them in 95% ethanol.

Experiment 2: effect of timing

The purpose of the second experiment was to examine the effect of the timing of sperm release on paternity in competitive fertilizations. We again used two males and one female in each replicate, but in this experiment, we used only 5Y males matched for size. The maximum difference in hypural length was 30 mm.

As much as possible, we attempted to mimic conditions in a salmon nest in which water circulates continuously and in which the presence of fish and release of their gametes create additional turbulence. We placed a beaker containing 500 mL of river water and a magnetic stir bar on a stir plate and began stirring. We then added about 100 eggs to the stirring water. Approximately 8 s later, we added milt from one male. The volume of milt used was that which yielded 30 μL of packed sperm cells as calculated from spermatocrit. This is enough sperm to fertilize all of the eggs (Hoysak and Liley 2001). Using a metronome to control the timing, we added the same number of sperm from the other male 0.75, 1.5, or 3 s later. These times approximate the range of time differences between males in natural spawning events (D.J. Hoysak, unpublished data). To test for differences between males in competitive fertility, we performed a fourth treatment in which milt samples were added simultaneously.

For each of the three time treatments, milt from the two males was added in reciprocal order so that a total of seven fertilizations was performed in each replicate. The order of fertilizations was randomized within each replicate. We completed all replicates within 77 min of starting to collect gametes. Neither eggs nor sperm lose viability during this time (Stoss 1983). All fertilizations were videotaped so that we could measure the actual time difference between the addition of the two milt samples.

After each replicate, we gave the fish a lethal dose of anesthetic and collected a sample of liver tissue for DNA analysis. We performed a total of five replicates.

Eggs were incubated in Heath trays until offspring reached the free-swimming fry stage. At this point, we gave them a lethal dose of anesthetic and preserved them in 95% ethanol.

Paternity analysis

We determined paternity of the competitive fertilizations by examining variation in microsatellite DNA for 30 randomly selected fry in each replicate. In experiment 1, parents were typed at seven microsatellite loci (Table 1). In eight replicates, there was sufficient variability at a single locus to establish paternity unambiguously. In one replicate, a second locus was necessary, and in another replicate, three loci were required to unambiguously determine paternity for all fry. In experiment 2, parents were not typed at all loci. There was sufficient variability at a single locus to establish paternity unambiguously in all replicates.

We extracted DNA from adult liver tissue and whole fry with phenol (Taylor et al. 1996) or with a lysis buffer. The lysis buffer contained 40 mmol Tris/L at pH 8.3, 50 mmol KCl/L, 0.5% *v*/*v* Tween 20, and 200 μg proteinase K/mL. To extract DNA, a 6–10 mg sample of tissue was placed in 200 μL of lysis buffer, incubated at 65 °C for 2 h, and 95 °C for 15 min. The sample was then centrifuged to pellet the debris and the supernatant was used as the source of DNA template for the polymerase chain reaction (PCR).

Table 1. Characteristics of microsatellite loci used for paternity determination of sockeye salmon, *Oncorhynchus nerka*.

Locus	Repeat types	No. of alleles	Source
Omy77	di	7	Morris et al. 1996
Oneμ8	di	7	Scribner et al. 1996
Oneμ14	di	7	Scribner et al. 1996
Ots100	di, tetra	9	Nelson et al. 1998
Ots103	tetra	15	Beacham et al. 1998
Ots107	di, tetra	6	Beacham et al. 1998
Ssa85	di	16	O'Reilly et al. 1996

We amplified microsatellite loci using PCR with 10-μL reaction volumes. Each reaction contained 1× reaction buffer, 0.4 mmol dNTPs/L, 0.05 μmol of ^{32}P-endlabelled primer 1/L, 0.25 μmol of unlabelled primer 1/L, 0.6 μmol of unlabelled primer 2/L, 1.5 mmol $MgCl_2$/L, 0.5 U (1 U ≈ 16.67 nkat) of Taq polymerase, and 1.0 μL of the DNA template. After amplification, each reaction tube received 10 μL of loading buffer and was stored at –20 °C. Electrophoresis was performed in denaturing polyacrylamide (Long Ranger™; FMC Corp., Philadelphia, Pennsylvania) gels at 5% concentration. Samples were denatured at 95 °C for 5–10 min, placed on ice, and then loaded on the gel (5 μL per sample). Gels were vacuum-dried and exposed to autoradiographic film for 0.5–4 days. In gels where dams and putative sires were initially genotyped, *M13*-sequencing ladders were used as references to determine allele sizes. On gels where fry samples were genotyped, the dam and putative sires were included as references so that fry alleles could be compared directly with those of the parents.

Data analysis

To test for differences between 3Y males and 5Y males in experiment 1, we used a replicated goodness-of-fit test (Sokal and Rohlf 1981). In this test, the null hypothesis is that 3Y and 5Y males do not differ in competitive fertilization success so that each age class fertilizes 50% of the eggs, on average. If this hypothesis is false, the probability of rejecting it (power) depends on the true mean proportions fertilized by the age classes. For example, if one age class fertilizes 90% of the eggs, on average, while the other age class sires 10%, then the power of the test is likely to be much higher than if one age class sires 55%, on average, and the other age class sires 45%.

To examine the relationship between effect size and power of this test, we performed Monte Carlo simulations of the experiment using the statistical software R (Ihaka and Gentleman 1996). For this analysis, the effect size is the true proportion of eggs fertilized by 3Y males. We set the effect size and then generated random fertilization data based on the binomial distribution for 10 replicates of 30 eggs. We repeated this 100 000 times, performing the replicated goodness-of-fit test each time. The proportion of these simulations that resulted in rejection of the null hypothesis repre-

sents the power of the test accurate to two decimal places (Thomas and Juanes 1996). We repeated these simulations for effect sizes between 0.5 and 0.7 in increments of 0.01. When the mean proportion sired was set to values greater than 0.62, some of the simulations produced results in which one male fertilized all of the eggs in a replicate. When this occurred, the test statistic could not be calculated because it is based on the logarithm of the number of eggs fertilized, so we changed zero values to 0.00001.

The results of the power analysis are shown in Fig. 1. The power of the replicated goodness-of-fit test to reject the null hypothesis was 0.81 when the true mean proportion fertilized by one age class was 0.58 and power was 0.94 when the true mean proportion fertilized by one age class was 0.6. Thus, this experiment has sufficient power to detect even fairly small effect sizes.

The replicated goodness-of-fit test also allowed us to test two other hypotheses. First, a heterogeneity *G* value was calculated to test the hypothesis that proportions sired by the two age classes are homogeneous across replicates. Second, a total *G* value was used to test the hypothesis that the data as a whole fit the expectation of equal paternity between males.

Microsatellite data were used to test for genetic effects on male success in experiment 1. The genetic measures we used were male relatedness to the female (Queller and Goodnight 1989) and male heterozygosity (i.e., proportion of loci that were heterozygous). Both were based on genotypes at seven loci.

To examine the effect of timing of sperm release on paternity in experiment 2, we randomly chose one focal male from each replicate. For each focal male, we calculated P1 (i.e., the proportion of offspring that he sired when his milt was added first) for each of the three time treatments. We also calculated P2 (i.e., the proportion of fry sired by the focal male when his milt was added second) for each of the three time treatments. P1 represents a male's defensive success in sperm competition for a given time treatment and P2 represents a male's offensive success (Clark et al. 1995). Finally, we calculated the focal male's competitive fertility as the proportion of fry he sired when milt samples were added simultaneously. All three values were arcsine square-root transformed for analysis.

Fig. 1. Power of the replicated goodness-of-fit test to reject the null hypothesis of no difference between age classes in fertilization success of sockeye salmon, *Oncorhynchus nerka*, given that one age class actually fertilizes the proportion indicated, on average, on the *x* axis.

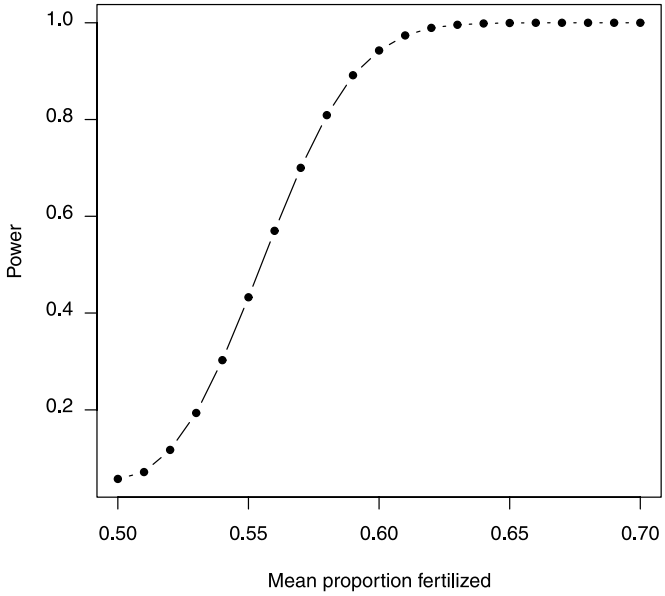


Table 2. Percentage of offspring sired by 3Y and 5Y male sockeye salmon in competitive fertilizations (n = 30 offspring for each replicate).

Replicate	3Y male	5Y male
1	53.3	46.7
2	70.0	30.0
3	70.0	30.0
4	43.3	56.7
5	36.7	63.3
6	63.3	36.7
7	63.3	36.7
8	20.0	80.0
9	66.7	33.3
10	46.7	53.3
Total	53.3	46.7

Results

Experiment 1: effect of male phenotype and genotype

An average of 84.3% of eggs was fertilized and survived to hatch (median 88.5; range 51.8–98.9) in the single-male and competitive fertilizations combined. For nine replicates, we were able to measure survival of eggs in single-male fertilizations (fry for one of the single-male fertilizations were lost). In single-male fertilizations, survival of clutches fertilized by 3Y males did not differ from survival of clutches sired by 5Y males (Wilcoxon's matched-ranks sign test, P = 0.570). There was a significant correlation between fry survival in 3Y-male fertilizations and fry survival in 5Y-male fertilizations (Spearman's rank correlation, r_S = 0.533, P = 0.032). Difference in fry survival between single-male fertilizations within replicates was not correlated significantly with difference in paternity in competitive fertilizations (r_S = 0.368, P = 0.298).

A replicated goodness-of-fit test revealed that 3Y and 5Y males did not differ in competitive fertilization success (G_{pooled} = 1.334, df = 1, P = 0.248). However, in most replicates paternity was biased toward one male (Table 2). The replicated goodness-of-fit test indicated significant deviation from the distribution expected if males had an equal probability of fertilizing eggs (G_{total} = 32.116, df = 10, P = 0.0001), and there was significant heterogeneity among replicates ($G_{heterogeneity}$ = 30.782, df = 9, P = 0.0001). The order of male capture did not influence paternity (replicated goodness-of-fit test, G_{pooled} = 2.617, df = 1, P = 0.106).

Success in sperm competition was not related to male morphology. Difference in paternity between males was not

Fig. 2. Scatterplot of the difference between competing male sockeye salmon in proportion of fry sired versus the difference in their relatedness with female sockeye salmon as measured by the kinship coefficient.

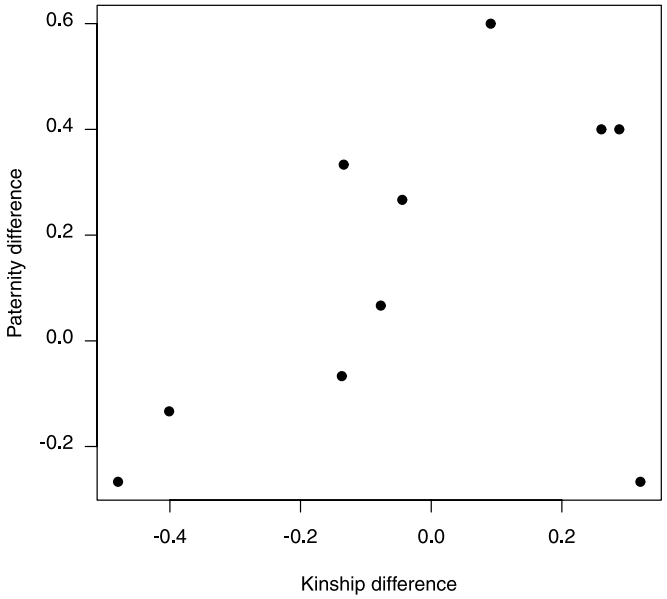
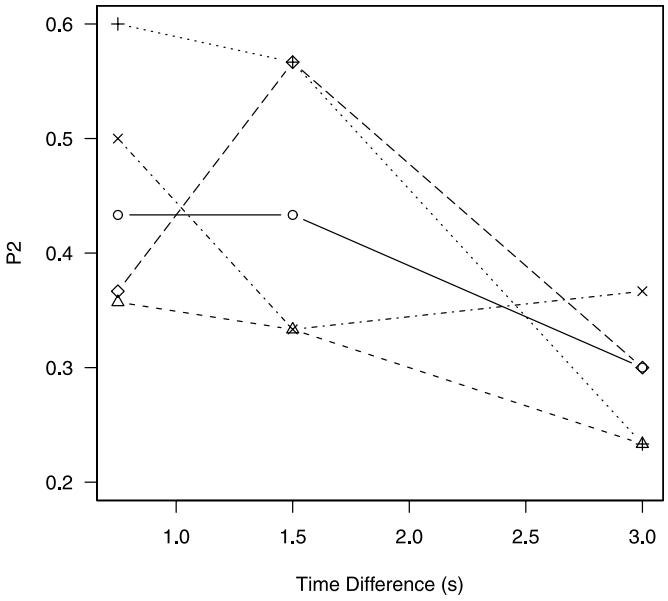


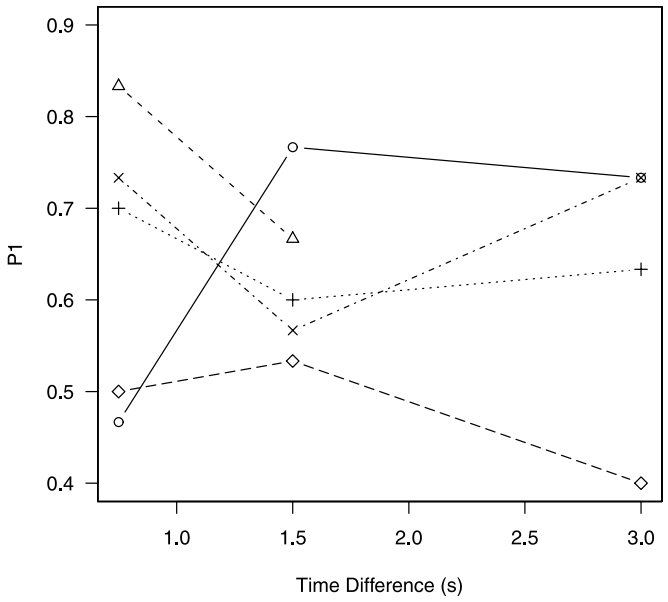
Fig. 3. Proportion of fry sired by the focal male sockeye salmon when his milt was added second (P2). Each replicate is given a different symbol and line type.



correlated with difference in residual body mass (r_S = 0.059, P = 0.868), residual hump size (r_S = 0.195, P = 0.458), or residual snout length (r_S = 0.189, P = 0.472).

We did not detect any effects of male genotype on success in sperm competition. Difference in paternity was not significantly correlated with difference in male heterozygosity (r_S = 0.182, P = 0.616). We also failed to detect a correlation between difference in paternity and difference in relatedness to females (r_S = 0.43, P = 0.194). The relationship between relatedness and success in sperm competition, while not significant, was in the opposite directions to that predicted (Fig. 2).

Fig. 4. Proportion of fry sired by the focal male sockeye salmon when his milt was added first (P1). Each replicate is given a different symbol and line type. Offspring were lost and paternity could not be determined in the 3-s treatment of one replicate (△).



Experiment 2: effect of timing

The actual time differences as measured by analysis of videotapes between addition of milt samples were very close to the intended values and were very consistent. The largest deviation from the intended time difference was 0.13 s and all but two were within 0.07 s. The repeatability of time differences was 0.999.

Focal males usually sired fewer offspring than their competitors when the focal males' milt was added second and P2 decreased as the time difference increased (Fig. 3). Repeated-measures ANOVA revealed that P2 was affected significantly by timing of sperm release ($F_{[1,8]}$ = 8.676, P = 0.019) but not by the focal males' success in simultaneous fertilizations ($F_{[1,3]}$ = 4.770, P = 0.117). The interaction between timing and simultaneous success was also not significant ($F_{[1,8]}$ = 0.064, P = 0.807).

When focal males' milt was added first, focal males usually sired more offspring than their competitors (Fig. 4). P1 was not significantly influenced by timing (repeated-measures ANOVA, $F_{[1,6]}$ = 0.087, P = 0.778), but there was a marginal positive effect of the focal males' success in simultaneous fertilizations ($F_{[1,2]}$ = 16.279, P = 0.056). The interaction between timing and simultaneous success was again not significant ($F_{[1,6]}$ = 0.565, P = 0.481). The focal males' P1 and P2 values were not correlated for any of the time treatments (all P values >0.4).

Discussion

Sperm from alternative phenotypes of sockeye salmon did not differ in competitive success in this study. This result was not what we predicted, but it is consistent with observa-

tions that sperm from 3Y and 5Y males are equally capable of fertilizing eggs in noncompetitive fertilizations (Hoysak and Liley 2001; this study). Thus, in this species, there is no evidence that competitive asymmetry between male roles has led to differences in sperm quality.

The power of our experiment was sufficient to detect a moderate to large effect size (i.e., if the true proportion of eggs fertilized by 3Y males was greater than 0.58). We cannot discount the possibility of a small difference in fertilizing ability between the age classes. However, in natural matings, small differences in sperm quality may be negated by more important factors like the number of sperm, spawning position, and timing of sperm release.

To our knowledge, the only other experimental study to examine the effect of male mating roles on success in sperm competition in fish is that of Neff et al. (2003). Contrary to the prediction that disadvantaged males have higher quality sperm, they concluded that sperm from parental male bluegill sunfish tended to outcompete sperm from cuckolder males. Their study had an advantage over ours in that males were tested more than once. However, only four males of each phenotype were used (as opposed to 10 of each in our study) and their measure of sperm number was less direct (based on size of testes). There is no reason to expect a systematic bias resulting from their methods, but further experiments are required to fully quantify sperm quality in bluegill sunfish, as well as in sockeye salmon.

We did not examine eggs immediately after insemination, so our measure of fertilization success included the combined effects of fertilization success and survival of embryos. However, embryo survival was not likely to have had a strong effect on our measure of male fertilization success. In addition to the fact that differences in paternity were not correlated with differences in survival, survival rates were high in most replicates. For example, replicates 2 and 3 had large differences in paternity (Table 2), but survival of both clutches was greater than 98%. Thus, the differences in paternity in these replicates could not have resulted from differences in embryo survival.

The volume of milt used in these fertilizations resulted in a high concentration of sperm relative to that necessary to fertilize all the eggs. Sperm concentration is likely to influence the rate at which eggs are fertilized (Iwamatsu et al. 1991), and if we extrapolate this observation, extreme sperm concentrations could result in virtually instantaneous fertilization of all eggs. In this extreme case, there could be no competitive advantage to either male. This was clearly not true in our experiment. However, it may be worth examining the effect of sperm concentration on the outcome of competition.

The results of this study leave unresolved the mechanism through which 3Y males obtain high success in semi-natural competitive matings (Foote et al. 1997). One possible solution is that the assumption that ejaculate size differs between male age classes is incorrect. Ejaculate size has not been measured in this species, and it is possible that measures of the number of sperm available for ejaculation are not correlated with ejaculate size. One way in which our experimental protocol differed from natural spawning was that milt samples were mixed before being added to the eggs. This resulted in milt samples being equalized in terms of spermatocrit and seminal chemistry, which may in turn have equalized sperm quality. An alternative explanation of the results from Foote et al. (1997) is that 3Y males may be able to use their small size to their advantage during spawning and obtain closer access to eggs being released by the female.

A factor that may have influenced both the results of the present study and those of Foote et al. (1997) is the social environment of the males. Male fish that are exposed to ovulated females undergo behavioural and physiological changes (referred to as the priming response) which include an increase in the volume of milt available for ejaculation (Liley et al. 1993; Liley and Kroon 1995), an increase in sperm motility (Miura et al. 1992; DeFraipont and Sorensen 1993), and in the case of goldfish (*Carassius auratus* (L., 1758)), an increase in the competitive ability of sperm (Zheng et al. 1997). When two males compete for access to a reproductive female, the priming response is stronger in the dominant male (Liley et al. 1993; Liley and Kroon 1995), but priming responses in alternative male phenotypes have not been examined. In the present study, males did not undergo priming, and it remains to be seen if the priming response differs among male phenotypes and if success in sperm competition is influenced by priming.

Another potential confounding factor is sperm age (Siva-Jothy 2000). There is little information on the effect of in vivo storage on sperm quality, but sperm are not active in the testes and only become motile upon exposure to water. Therefore, sperm quality is not likely to decline because of continuous metabolic activity. Nevertheless, males sampled at the end of the spawning season have lower fertility (Suquet et al. 1998; Dreanno et al. 1999) and sperm quality declines with in vitro storage (Stoss 1983). We do not know how long sperm had been stored in the testes of our study animals, but little spermiation occurs before males arrive at the spawning grounds (Liley et al. 1986; Liley et al. 1993). Therefore, there was unlikely to be significant variation in sperm age among males in our study.

We found no evidence that the outcome of sperm competition exerts directional selection on male phenotype, but the possibility remains that nondirectional selection occurs as a result of gamete interactions. In the sea urchin *Echinometra mathaei* (Blainville, 1825), eggs discriminate among sperm on the basis of sperm genotype at a surface protein locus (Palumbi 1999). Similarly, when female decorated field crickets, *Gryllodes supplicans* (Walker, 1859), are mated both to a sibling and an unrelated male, sperm from the unrelated male fertilize more eggs than do sperm from the sibling male (Stockley 1999). In both the genus *Drosophila* and the fowl *Gallus gallus* (L., 1758), male success in sperm competition differs across females (Clark et al. 1999; Birkhead et al. 2004). However, the process of fertilization in salmon is very different from that in sea urchins, insects, and birds. In the latter taxa, sperm cells swim a long distance, attach anywhere on the egg, and undergo an acrosome reaction. In salmon, gametes are mixed together at high concentration, fertilization is achieved quickly when a sperm cell swims into a small opening (micropyle) in the egg membrane, and there is no acrosome reaction. Observations of this process indicate that the first sperm to enter the micropyle fertilizes the egg (Yanagimachi et al. 1992). There

is also evidence that success in sperm competition is influenced by sperm swimming speed (Gage et al. 2004). Thus, if eggs discriminate among sperm, the mechanism would likely involve an influence of eggs either on the ability of sperm to locate the micropyle or on the swimming speed of sperm. Two studies in other fish species showed that a male's success in competitive in vitro fertilizations does vary across females (Gharrett and Shirley 1985; Rakitin et al. 1999), but males in a third study had consistent success across females (Gile and Ferguson 1995). A mechanism for egg discrimination of sperm has not been demonstrated in teleost fish, but ovarian fluid is known to influence sperm motility (Litvak and Trippel 1998), and if this effect varies among males then females could influence male fertilization success. In rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), male fertility in single-male fertilizations is correlated with the proportion of sperm possessing a specific antigen (Trummel et al. 1992), but neither the mechanism of this effect nor the effect of the antigen on competitive fertility are known.

We did not detect any phenotypic correlates of success in sperm competition, but we did find substantial differences between males in success. It should be noted that we cannot conclude that males differ in competitive success without replicated tests of individual males; we can only say that the particular milt samples which we collected showed significant variation in success. Nevertheless, we can conclude that the outcome of sperm competition is not a fair raffle in which success is determined only by the number of sperm (Parker 1990*a*).

More research is needed to find determinants of success in sperm competition, both in vitro and in natural matings. Sneaker males in sockeye salmon clearly achieve high reproductive success through some mechanism other than high sperm quality. A similar paradox occurs in bluegill sunfish in which parental males apparently have higher quality sperm than cuckolder males, but lower success in natural spawnings (Neff et al. 2003). Little is known about the dynamics of gamete release (e.g., relative positions of milt and egg release, timing of gamete release, rate of gamete release, and total ejaculate volume) in fishes and how these dynamics influence male reproductive success. In addition, potential relationships between genetic variables and success in sperm competition are worthy of further research.

The results of our second experiment were very surprising and indicate that the timing of sperm release may not be as important to a male's success as we expected. Previous studies in fish indicate that fertilization occurs very soon after gametes are mixed. For example, Hoysak and Liley (2001) found that nearly 50% of eggs are fertilized within 2 s of gamete mixing. In direct observations of eggs and sperm, Iwamatsu et al. (1991) found that many sperm entered the micropyle within 3 s of gamete mixing. However, the rate of fertilization may be influenced by sperm concentration (Iwamatsu et al. 1991) and turbulence (Petersen et al. 1992). Low sperm concentration and (or) high turbulence may reduce the rate of fertilization and, therefore, may reduce the advantage to the first male to release sperm.

Our results also indicated that offensive and defensive ability in sperm competition are not correlated in sockeye salmon. This is also true for the genus *Drosophila* in which seminal proteins play a key role in sperm competition (Clark et al. 1995, 1999). However, little is known about the physiological mechanisms underlying competitive ability in fish sperm. Given that the mating roles of alternative male phenotypes often involve one phenotype mating before another, it would be interesting to compare male phenotypes in their offensive and defensive ability in sperm competition.

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