Theriogenology 86 (2016) 2189–2193

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Reproductive investment patterns and comparison of sperm quality in the presence and absence of ovarian fluid in alternative reproductive tactics of masu salmon, *Oncorhynchus masou*

Yuya Makiguchi [a,*], Mitsuru Torao [b], Takahito Kojima [a], Trevor E. Pitcher [c,d]

[a] College of Bioresource Sciences, Nihon University, Fujisawa, Kanagawa, Japan
[b] Doto Research Branch, Salmon and Freshwater Fisheries Research Institute, Hokkaido Research Organization, Nakashibetsu, Hokkaido, Japan
[c] Department of Biological Sciences, University of Windsor, Windsor, Ontario, Canada
[d] Great Lakes Institute for Environmental Research, University of Windsor, Windsor, Ontario, Canada

ARTICLE INFO

Article history:
Received 31 January 2016
Received in revised form 13 June 2016
Accepted 10 July 2016

Keywords:
Postcopulatory
Sperm competition
Gonadosomatic index
External fertilization

ABSTRACT

In most teleost fish species, sperm competition is a key factor in determining male reproductive success, leading to selection on males to increase their reproductive investment in gonads and ejaculate competitiveness. In this study, reproductive investment patterns were assayed by examining the relative investment in gonads and sperm quality metrics (in river water and in the presence of ovarian fluid) of masu salmon, *Oncorhynchus masou*, representing two fixed male alternative reproductive tactics (ARTs; small sneaking parr males and large dominant anadromous males). Although anadromous males were significantly larger in body size compared to parr males, the latter invested significantly more in relative gonad mass than the former. Sperm velocity and motility were significantly higher, and longevity was significantly lower in parr males than in anadromous males in river water. However, no difference in any of these sperm quality metrics was detected between the ARTs in the presence of ovarian fluid. Sperm velocity and motility were not affected by the presence of ovarian fluid compared to river water for parr males, but both traits increased significantly for anadromous males in ovarian fluid relative to river water, whereas longevity significantly increased in the presence of ovarian fluid compared to river water for both ARTs. We interpret these findings in light of potential cryptic female choice mechanisms and the sneak-guard model of sperm competition that is based on differences in sperm competition risk and alternative investment possibilities among ARTs.

1. Introduction

Sperm competition theory predicts that in external fertilizing teleost fish species, small sneaker males will invest relatively more energy into reproduction (e.g., testes development) and produce faster swimming sperm than large guard males to compensate for disadvantageous timing and spawning positions [1]. Numerous empirical studies have investigated sperm competition between alternative reproductive tactics (ARTs) and found that small sneaker males often have more competitive sperm (e.g., larger testis size, larger ejaculate size and density, faster swimming velocity, greater proportion of motility

* Corresponding author. Tel./fax: +81-466-84-3687.
E-mail address: yuya.makiguchi@gmail.com (Y. Makiguchi).

0093-691X/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2016.07.009

Table 1
Body mass, testes mass, gonadosomatic index (GSI), and spermatocrit for male alternative reproductive tactics in masu salmon, *Oncorhynchus masou*.

Traits	Parr males (N = 15)		Anadromous males (N = 15)		*df*	*t* test	
	Mean ± SE	Range (min–max)	Mean ± SE	Range (min–max)		*t*	P
Body mass (g)	44.27 ± 5.87	18–100	798.13 ± 56.67	392–1210	28	13.20	<0.001
Testes mass (g)	3.27 ± 0.51	1.00–8.50	23.69 ± 3.59	6.90–53.80	28	5.64	<0.001
GSI (%)	6.94 ± 0.35	3.59–8.96	2.97 ± 0.38	0.95–5.38	28	−7.64	<0.001
Spermatocrit (%)	38.24 ± 2.11	24.63–59.69	27.16 ± 3.12	4.68–41.52	28	−2.94	0.01

sperm, and greater sperm adenosine 5'-triphosphate contents) compared to large guard males because sperm performance leads directly to competitive fertilization success [2]. In addition, sperm performance including sperm velocity, motility, and longevity are correlated with both fertilization success and sperm competition success in fish [3]. Recent studies have shown that sperm performance of external fertilizing fish is potentially affected by ovarian fluid expelled by females at the time of spawning, where sperm competition takes place [4–6]. However, to date, sperm performance has been evaluated typically in aqueous environments without the presence of ovarian fluid, which does not realistically simulate a natural spawning microenvironment [3,7].

Masu salmon, *Oncorhynchus masou*, exhibits typical fixed male ARTs (i.e., a male is only one of the two tactics in his lifetime and both male morphs are the same species); small precocious parr males which are resident and sexually mature in the river for a 1- or 2-year period and large anadromous males which migrate to sea and return to their natal stream to spawn after three or 4 years [8]. The large anadromous males fight before spawning to achieve dominant access to the females, whereas the small parr males adopt a sneaker tactic to dart into spawning events and surreptitiously position themselves under ovipositing females to release milt near the female as she releases her eggs and ovarian fluid. Sperm competition theory predicts that the small parr males should be expected to face higher levels of sperm competition intensity than the anadromous males because they are presumed to have disadvantageous spawning position and timing relative to the anadromous males. The objectives of this study are to (1) compare relative investment in reproduction and sperm quality metrics between the male ARTs and (2) investigate the effects of ovarian fluid on sperm quality metrics for each of the ARTs, in masu salmon.

2. Materials and methods

Masu salmon were collected from September 16 to 18, 2014, in the Tawaramappu River (43°54′N, 144°96′E), a branch of the Shibetsu River, Hokkaido, Japan. The fish were anesthetized with 1.0 mL/L concentration of 2-phenoxyethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and the external urogenital pore was wiped dry to avoid contamination from water, urine, feces, and blood. Milt was collected from 15 parr and 15 anadromous males (Table 1 and Fig. 1). Ovarian fluid was collected from 15 gravid females (mean body mass ± SD, 858.4 ± 136.8 g). We collected ovarian fluid (using 10-mL pipettor tips) from the external urogenital pore of females, and then, each sample was separately used for the experiment (i.e., one female's ovarian fluid was used for one pair of males; one parr male and one anadromous male). The samples were individually placed in coolers (4 °C–6 °C) until sperm quality analyses took place in the laboratory (within 5 hours). Body mass (±1 g) and testes mass (±0.1 g) were measured for all males. To compare relative investment in

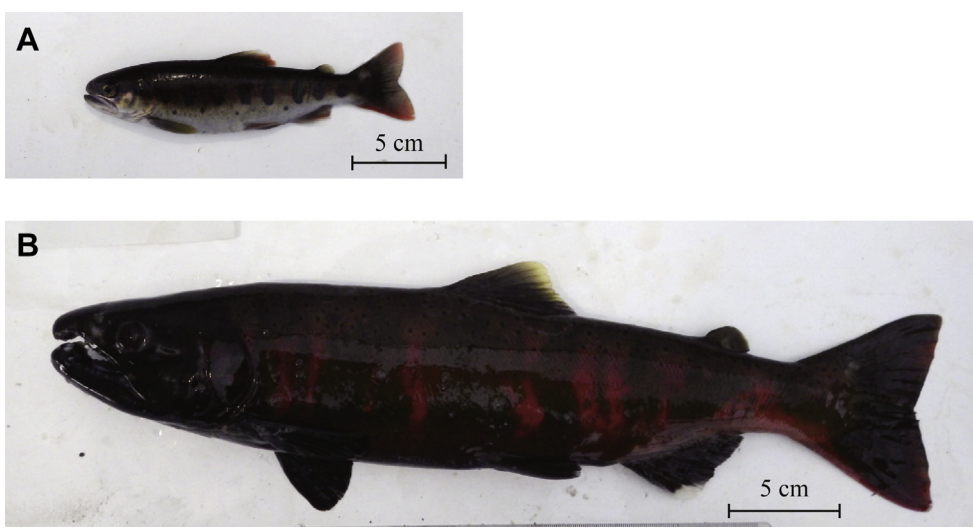


Fig. 1. Photographs of representative masu salmon (*Oncorhynchus masou*) male alternative reproductive tactics (with 5 cm scale). Small parr males (A) and large anadromous males (B).

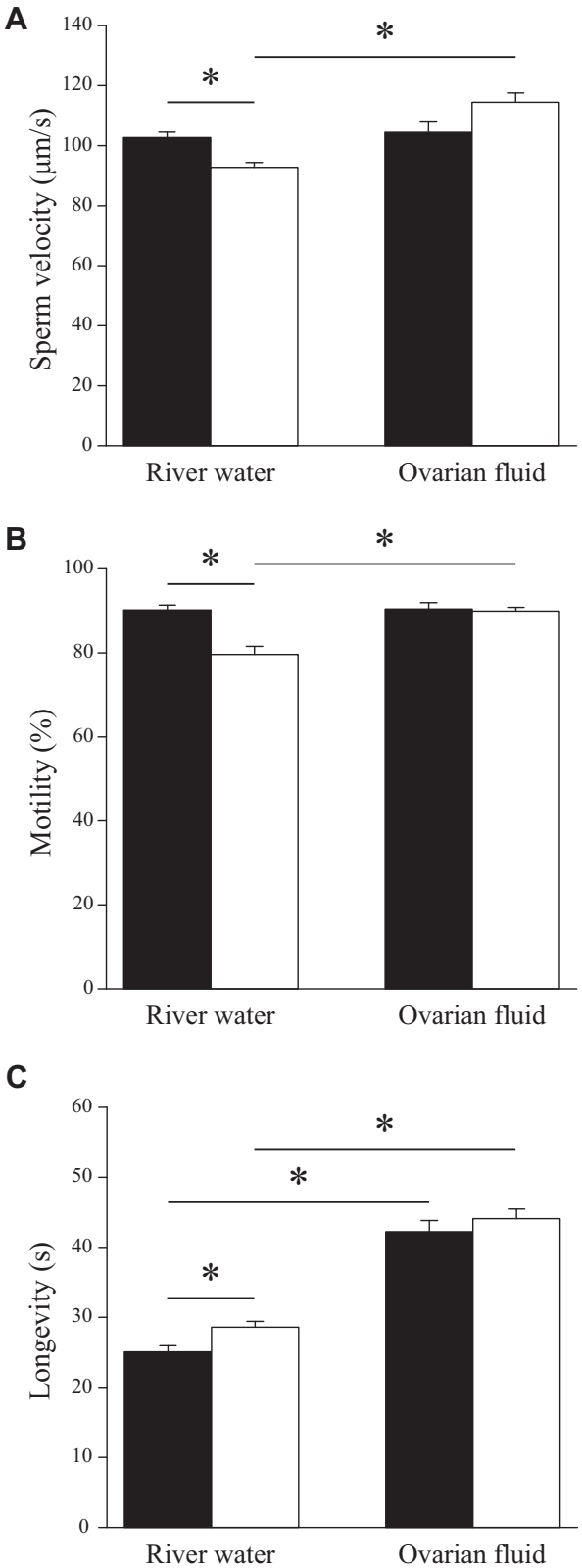


Fig. 2. Mean (±SE) sperm velocity at 5 seconds postactivation (A), sperm motility at 5 seconds postactivation (B), and longevity (C) for parr males (solid bars, N = 15) and anadromous males (open bars, N = 15) in river water and in the ovarian fluid solution. (See text for details.) Comparisons are made between sperm quality metrics of the two alternative reproductive tactics and between the sperm metrics in river water and the ovarian fluid solution. (See Results and Discussion for details.) Asterisks indicate a significant difference ($P < 0.05$).

testes between the ARTs, gonadosomatic index (GSI) was calculated as a percentage by dividing testes mass by the body mass for each male. To compare sperm density between the ARTs, spermatocrit was estimated by calculating the percentage sperm cells per total volume of milt (*via* centrifugation of each milt sample in capillary tubes at 2000 *g* for 5 minutes [9]). To examine sperm performance, a small amount of milt (less than 0.5 μL) was applied into the chamber of a 2X-CEL glass slide (Hamilton Thorne, Beverly, MA, USA) covered with a glass coverslip, and then, the sperm were activated with 15 μL of river water at 8.0 °C (similar to the river water temperature) or an ovarian fluid solution composed of 10% ovarian fluid in river water (hereafter ovarian fluid solution) at 8.0 °C [4,10]. Sperm quality metrics were recorded using a digital video camera (HDR-CX590 V, Sony Corporation, Tokyo, Japan) at 29 frames/s, mounted on an inverted microscope (CKX31, Olympus, Tokyo, Japan) at ×80 magnification. To compare sperm quality metrics between the ARTs, sperm velocity (μm/s) and motility (proportion of sperm moving forward in the field of view) were measured at 5 seconds post-activation (when most fertilizations are believed to occur) using 2D-Motion Analysis software (Move-tr/2D, Library Co., Ltd., Tokyo, Japan). These metrics were averaged for each individual. Longevity, the time from sperm activation until ~95% of the sperm cells stop moving in the field of view [10], was also measured for each individual in river water and the ovarian fluid solution.

To examine the relative investment in reproduction (i.e., GSI), independent-samples *t* tests were used to compare the respective traits between the ARTs. Sperm velocity, motility, and longevity were analyzed using a mixed-model repeated-measures model containing the ART (anadromous and parr), the activation medium (river water or ovarian fluid solution), and the ART by activation medium interaction. Alternative reproductive tactics and activation medium were considered fixed factors. Variation associated with each female was controlled for statistically by incorporating female as a random factor. Significance levels were set at $P < 0.05$ for main effects and interactions. When interactions were detected, reduced models were run separately at each level of activation medium and ART to facilitate their interpretation (independent-samples *t* test or paired *t* tests, as appropriate because these data used in these tests were normally distributed according to Kolmogorov–Smirnov tests). Most of the comparisons were homoscedastic (21 of 28 comparisons) and some were not homoscedastic ($n = 7$ comparisons). Despite this fact, we used the same statistics for all the comparisons because *t* tests are known to be relatively robust to this kind of minor violation when the sample size is the same. In the case of a nonsignificant interaction, main effects were interpreted. The statistical analysis were conducted using the R software version 3.2.1 [11] and lme4 package. Values are expressed as mean ± SE.

Table 2
The mixed-model repeated measures test the effect of male alternative reproductive tactics (ARTs), activation medium (AM), and the ART by AM interaction.

Factor	Velocity		Motility		Longevity	
	5 s postactivation		5 s postactivation			
	t	P	*t*	P	*t*	P
ARTs	−2.480	0.016	−5.080	<0.01	2.286	0.027
AM	−2.036	0.047	−2.115	0.039	3.641	<0.01
ARTs × AM	3.527	<0.01	3.421	0.001	−0.762	0.450

Degree of freedom (*df*), *t* value, and P value for each postactivation time in velocity, motility, and longevity of sperm are shown.
$df_{ART} = 1$, $df_{AM} = 1$, $df_{ART \times AM} = 1$, $df_{error} = 56$, $df_{total} = 59$.

3. Results and discussion

The GSI and spermatocrit were significantly greater in parr males compared to anadromous males; the parr males had on average 2.3 times greater GSI and 1.4 times greater spermatocrit values than anadromous males (Table 1). These results demonstrate that parr males (i.e., sneaker males) invest relatively more energy into gonad development and sperm density than anadromous males in masu salmon, which is consistent with sperm competition theory related to ARTs [1,4] and previous studies in other species with intense sperm competition and male ARTs [10,12] including the focal species [13].

We found significant differences in sperm velocity, motility, and longevity depending on which ART the male belonged to and whether ovarian fluid was present (Fig. 2, Table 2). For the velocity and motility mixed-model repeated-measures models, the ART by activation medium interactions were both significant (Table 2). Therefore, the models were revised into separate models at each level of ART and activation medium. Sperm velocity and motility were significantly higher in parr males compared to anadromous males in river water (velocity: $t_{14} = -4.095$, P = 0.001, Fig. 2A; motility: $t_{14} = -4.036$, P = 0.001, Fig. 2B). However, sperm velocity and motility did not differ between the ARTs in the ovarian fluid solution (velocity: $t_{14} = 1.600$, P = 0.132, Fig. 2A; motility: $t_{14} = -0.264$, P = 0.796, Fig. 2B). Our findings that sperm velocity and motility of parr males were not influenced by the presence of ovarian fluid indicate that sperm of parr males adopting a sneaking tactic are likely selected by high levels of sperm competition compared to anadromous males because they dart into the nest from behind the females and release sperm later than anadromous males [14]. Ovarian fluid significantly increased sperm velocity and motility in anadromous males compared to their respective paired values in river water (velocity: $t_{14} = 9.402$, P < 0.001; motility: $t_{14} = 4.787$, P < 0.001, respectively), but no significant difference existed between trait values in river water and ovarian fluid for the parr males (velocity: $t_{14} = 0.391$, P = 0.702; motility: $t_{14} = 0.109$, P = 0.915). Our finding that sperm velocity and motility are higher in the presence of ovarian fluid than river water is consistent with numerous studies looking at the effects of ovarian fluid on sperm velocity in fishes [15,16]. Anadromous male sperm should be selected to function well in the presence of ovarian fluid because these males monopolize access to females, enter the nest first at the time of spawning, and release their sperm in close proximity to where eggs and higher concentrations of ovarian fluid are expected to be expelled by females. Unlike previous studies that only examined one of the male ARTs [16], we also examined sperm from males of the ART (parr males) and found that sperm velocity of parr males was not significantly different in river water versus the ovarian fluid solution. This result could be explained by the fact that parr sperm must function well in both river water and in the presence of ovarian fluid because they enter spawning bouts later than anadromous males and as such are forced to release their gametes later and presumably in lower concentrations of ovarian fluid [17,18].

For the longevity mixed-model repeated-measures model, the ART by activation medium interaction was not significant, but the ART and activation medium factors were significant (Table 2). Longevity was significantly higher in anadromous males than in parr males in river water ($t_{14} = 2.889$, P = 0.012, Fig. 2C). However, longevity did not differ between the ARTs in the ovarian fluid solution ($t_{14} = 1.001$, P = 0.332, Fig. 2C). Our findings that sperm performance of anadromous males increases in the presence of ovarian fluid indicate that the reproductive success of anadromous male should be selected for by ovarian fluid expelled by females because they typically have the closest proximity to the female during the spawning event and release sperm under relatively high concentrations of ovarian fluid at the moment of spawning. Ovarian fluid significantly increased longevity for both the male ARTs (parr males: $t_{14} = 10.108$, P < 0.001; anadromous males: $t_{14} = 8.530$, P < 0.001), a result that is consistent with a previous study showing that ovarian fluid enhances sperm longevity in Arctic charr (*Salvelinus alpinus*) [15] and Chinook salmon (*O. tshawytscha*) [16], likely due to the rich ionic composition of ovarian fluid that nourishes sperm activity [19].

3.1. Conclusion

Our study demonstrated that ovarian fluid differentially affects sperm performance between small parr males and large dominant anadromous males in masu salmon. Future studies are needed to investigate the possibility that ovarian fluid biases paternity in competitive fertilization contexts and thus plays a role in cryptic female choice [17] and also determine how the constituents of ovarian fluid such as ionic, biochemical, protein, and genetic components influence the reproductive success for the male ARTs of this species in the wild. Also, a high-throughput proteomics approach would be useful to identify known and novel seminal plasma proteins [20] from the two ARTs of masu salmon, to determine whether both tactics use the same mechanisms to achieve reproductive success. Overall, our experiment has serious ramifications for studies investigating reproductive success in fishes; using ovarian fluid in experiments is essential to obtain realistic data regarding their fertilization dynamics.

Acknowledgments

The authors thank Fumiaki Kobashi, Kotoe Ishikawa, Toshiki Kubota, Takahisa Shimasaki, and Shion Ito of the College of Bioresource Science, Nihon University, Japan, for their assistance with the experiments. They also thank Fumio Ito of the Hokkaido Natural Fisheries Research Institute and the staff of Hokkaido Salmon Propagation Association, Hokkaido, Japan, for collection of experimental fish. This work was supported by JSPS KAKENHI grant 15K07229 and College of Bioresource Sciences, Nihon University.

Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.theriogenology.2016.07.009.

References

[1] Parker GA. Sperm competition games—raffles and roles. P R Soc B 1990;242:120–6.
[2] Taborsky M. Alternative reproductive tactics in fish. In: Oliveira RF, Taborsky M, Brockmann HJ, editors. Alternative reproductive tactics. New York: Cambridge University Press; 2008. p. 251–99.
[3] Gage MJG, Macfarlane CP, Yeates S, Ward RG, Searle JB, Parker GA. Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. Curr Biol 2004;14:44–7.
[4] Butts IAE, Johnson K, Wilson CC, Pitcher TE. Ovarian fluid enhances sperm velocity based on relatedness in lake trout, *Salvelinus namaycush*. Theriogenology 2012;78:2105–9.
[5] Galvano PM, Johnson K, Wilson CC, Pitcher TE, Butts IA. Ovarian fluid influences sperm performance in lake trout, *Salvelinus namaycush*. Reprod Biol 2013;13:172–5.
[6] Urbach D, Folstad I, Rudolfsen G. Effects of ovarian fluid on sperm velocity in Arctic charr (*Salvelinus alpinus*). Behav Ecol Sociobiol 2005;57:438–44.
[7] Gage MJG, MacFarlane C, Yeates S, Shackleton R, Parker GA. Relationships between sperm morphometry and sperm motility in the Atlantic salmon. J Fish Biol 2002;61:1528–39.
[8] Koseki Y, Maekawa K. Sexual selection on mature male parr of masu salmon (*Oncorhynchus masou*): does sneaking behavior favor small body size and less-developed sexual characters? Behav Ecol Sociobiol 2000;48:211–7.
[9] Vladic TV, Jarvi T. Sperm quality in the alternative reproductive tactics of Atlantic salmon: the importance of the loaded raffle mechanism. P R Soc B 2001;268:2375–81.
[10] Flannery EW, Butts IAE, Slowinska M, Ciereszko A, Pitcher TE. Reproductive investment patterns, sperm characteristics, and seminal plasma physiology in alternative reproductive tactics of Chinook salmon (*Oncorhynchus tshawytscha*). Biol J Linn Soc 2013;108:99–108.
[11] Team RC. R: A language and Environment for Statistical Computing Vienna: R Fundation for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2015.
[12] Neff BD, Fu P, Gross MR. Sperm investment and alternative mating tactics in bluegill sunfish (*Lepomis macrochirus*). Behav Ecol 2003;14:634–41.
[13] Koseki Y, Maekawa K. Differential energy allocation of alternative male tactics in masu salmon (*Oncorhynchus masou*). Can J Fish Aquat Sci 2002;59:1717–23.
[14] Watanabe M, Takamura S, Maekawa K. Effects of timing of nest entry and body size on the fertilization success of alternative male reproductive phenotypes of masu salmon (*Oncorhynchus masou*). Can J Zool 2008;86:1121–30.
[15] Turner E, Montgomerie R. Ovarian fluid enhances sperm movement in Arctic charr. J Fish Biol 2002;60:1570–9.
[16] Rosengrave P, Gemmell NJ, Metcalf V, McBride K, Montgomerie R. A mechanism for cryptic female choice in Chinook salmon. Behav Ecol 2008;19:1179–85.
[17] Yeates SE, Diamond SE, Einum S, Emerson BC, Holt WV, Gage MJG. Cryptic choice of conspecific sperm controlled by the impact of ovarian fluid on sperm swimming behavior. Evolution 2013;67:3523–36.
[18] Yanagimachi R, Cherr G, Matsubara T, Andoh T, Harumi T, Vines C, et al. Sperm attractant in the micropyle region of fish and insect eggs. Biol Reprod 2013;88.
[19] Rosengrave P, Taylor H, Montgomerie R, Metcalf V, McBride K, Gemmell NJ. Chemical composition of seminal and ovarian fluids of Chinook salmon (*Oncorhynchus tshawytscha*) and their effects on sperm motility traits. Comp Biochem Physiol A Mol Integr Physiol 2009;152:123–9.
[20] Nynca J, Arnold GJ, Frohlich T, Otte K, Flenkenthaler F, Ciereszko A. Proteomic identification of rainbow trout seminal plasma proteins. Proteomics 2014;14:133–40.

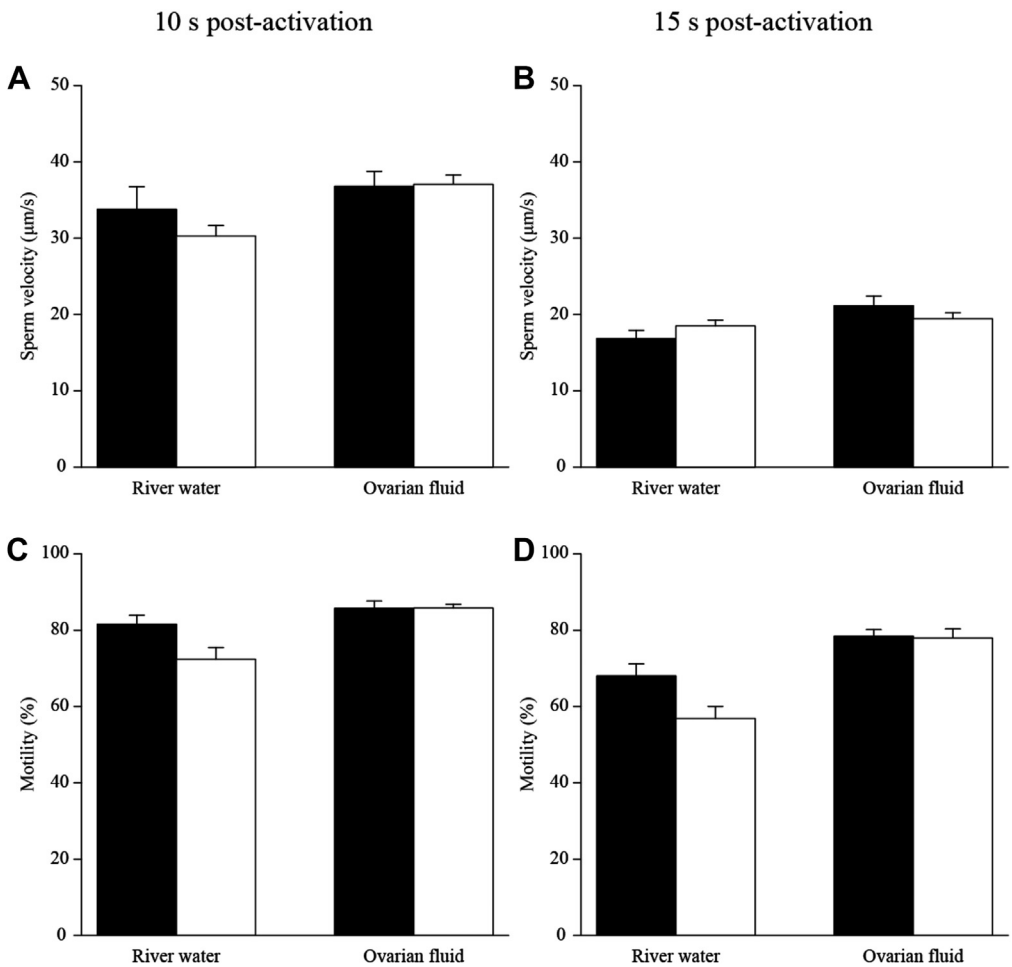
Appendix A. Supplementary data

The results of the mixed model are in the Supplementary Table 1. Sperm velocity and motility 10 seconds postactivation were analyzed using a mixed-model repeated-measures model containing the alternative reproductive tactic (ART; anadromous and parr), the activation medium (river water or ovarian fluid solution), and the ART by activation medium interaction. ART and activation medium were considered fixed factors. Variation associated with each female was controlled statistically by incorporating female as a random factor. Significance levels were set at $P < 0.05$ for main effects and interactions. When interactions were detected, reduced models were run separately at each level of activation medium and ART to facilitate their interpretation (independent samples *t* test or paired *t* tests, as appropriate). In the case of a nonsignificant interaction, main effects were interpreted. The statistical analyses were conducted using the R software version 3.2.1 and lme4 package. Values are expressed as mean ± standard error (SE.)

For the sake of brevity and because it is believed most fertilizations occur shortly after spawning, we focus on sperm quality metrics at 5 seconds postactivation in the main text of the manuscript. In the following section, we provided additional results (using the same Methods outlined in the main body of the text) regarding sperm quality metrics at 10 and 15 seconds postactivation in river water and in the ovarian fluid solution.

There was no significant difference in sperm velocity between the ARTs at 10 seconds ($t_{14} = -1.097$, $P = 0.291$) and 15 seconds ($t_{14} = 1.108$, $P = 0.286$) postactivation in river water. Similarly, there was no significant difference in sperm velocity between the ARTs at 10 seconds ($t_{14} = 0.104$, $P = 0.919$) and 15 seconds ($t_{14} = -1.008$, $P = 0.331$) postactivation in the ovarian fluid solution.

There was no significant difference in motility between the ARTs at 10 seconds ($t_{14} = -1.848$, $P = 0.086$), whereas there was a significant difference at 15 seconds ($t_{14} = -2.134$, $P = 0.051$) postactivation in river water. Furthermore, there was no significant difference in motility between the ARTs at 10 seconds ($t_{14} = 0.021$, $P = 0.984$) and 15 seconds ($t_{14} = -0.156$, $P = 0.878$) postactivation in the ovarian fluid solution.



Supplementary Fig. 1. Mean (±SE) sperm velocity at 10 s (A) and 15 s post-activation (B) in river water and in the ovarian fluid solution and sperm motility at 10 s (C) and 15 s post-activation (D) in river water and in the ovarian fluid solution for parr males (solid bars, N = 15) and anadromous males (open bars, N = 15).

Supplementary Table 1
The mixed-model repeated measures test the effect of male alternative reproductive tactics (ARTs), activation medium (AM), and the ART by AM interaction.

Factor	10 s postactivation		15 s postactivation	
	t	P	*t*	P
Velocity				
ARTs	−1.193	0.238	1.124	0.266
AM	−0.112	0.911	2.357	0.022
ARTs × AM	0.906	0.369	−1.633	0.108
Motility				
ARTs	−2.848	<0.01	−2.854	<0.01
AM	−0.703	0.485	−0.028	0.978
ARTs × AM	2.024	0.048	1.928	0.059

Degree of freedom (*df*), *t* value, and P value for 10 and 15 seconds post-activation time in velocity and motility of sperm are shown.
$df_{ARTs} = 1$, $df_{AM} = 1$, $df_{ARTs \times AM} = 1$, $df_{error} = 56$, $df_{total} = 59$.