*Molecular Ecology (2023, in press)*

# Sperm of more colorful males are better adapted to ovarian fluids in lake char (Salmonidae)

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## Author contributions

DN and CW designed the experiment. DN and LG organized and conducted the field work (with the help of CdG) and determined sperm characteristics. DN performed the two experiments, determined male colors, and genotyped the offspring for parental assignments. CdG did the ddRADseq library preparation and genomic analysis. LA stained the blood smears and classified the blood cells. DN & CW did the statistics and wrote the manuscript that was revised by all authors.

## Abstract

Fish often spawn eggs with ovarian fluids that have been hypothesized to support sperm of some males over others (cryptic female choice). Alternatively, sperm reactions to ovarian fluids could reveal male strategies. We used wild-caught lake char (*Salvelinus umbla*) to experimentally test whether sperm react differently to the presence of ovarian fluid, and whether any differential sperm reaction could be predicted by male breeding coloration, male inbreeding coefficients (based of 4,150 SNPs), or the kinship coefficients between males and females. Male coloration was positively linked to body size and current health (based on lymphocytosis and thrombocytosis) but was a poor predictor of inbreeding or kinship coefficients. We found that sperm of more colorful males were faster in diluted ovarian fluids than in water only, while sperm of paler males were faster in water than in ovarian fluids. We then let equal numbers of sperm compete for fertilizations in the presence or absence of ovarian fluids and genetically assigned 1,464 embryos (from 70 experimental trials) to their fathers. The presence of ovarian fluids significantly increased the success of the more colorful competitors. Sperm of less inbred competitors were more successful when tested in water only than in diluted ovarian fluids. The kinship coefficients had no significant effects on sperm traits or fertilization success in the presence of ovarian fluids, although parallel stress tests on embryos had revealed that females would profit more from mating with least related males rather than most colored ones. We conclude that sperm of more colorful males are best adapted to ovarian fluids, and that the observed reaction norms suggest male strategies rather than cryptic female choice.

**Key words:** sperm competition, loaded raffle, sperm velocity, male reproductive strategy, cryptic female choice, sperm reaction norm, inbreeding coefficient, kinship coefficient

## Introduction

Ejaculate economics predicts that males invest strategically into sperm according to the expected microecology they will experience after ejaculation (Parker & Pizzari 2010). This microecology is strongly defined by competitors and the level of sperm competition. Males are predicted to increase sperm number and/or sperm velocity with increasing risk of sperm competition (Parker & Pizzari 2010). This prediction is meanwhile well supported in many taxa (Magris 2021). Another aspect of the microecology that sperm have to deal with is the biochemical environment that females create around their eggs with their reproductive fluids. Such female reproductive fluids occur in internal and external fertilizers and include, for example, ovarian, follicular, oviductal or coelomic fluids (Gasparini *et al.* 2020). The functional significance of such female reproductive fluids is not well understood yet (Gasparini *et al.* 2020).

In externally spawning fishes, the fluids with which females expel their eggs are called "ovarian fluids" and consist of various inorganic and organic compounds (Lahnsteiner *et al.* 1995). Ovarian fluids have repeatedly been found to support sperm velocity, motility, and longevity (Zadmajid *et al.* 2019), i.e. key targets of sexual selection (Evans *et al.* 2013). They have also been discussed as allowing for certain forms of cryptic female choice (Firman *et al.* 2017). Their release with eggs can, for example, increase sperm competition by slowing down the fertilization process (Bakker *et al.* 2006) or specifically promote paternity of dominant males over parasitic spawners ("sneaker males") (Alonzo *et al.* 2016; Egeland *et al.* 2016; Makiguchi *et al.* 2016; Lehnert *et al.* 2017). The support of sperm function can be even more specific: Ovarian fluids of guppies (*Poecilia reticulata*) were found to slow down sperm of full-sibs as compared to unrelated males, potentially to reduce inbreeding (Gasparini & Pilastro 2011). The presence of ovarian fluids during spawning has also been found to reduce inbreeding by differentially supporting some sperm over others in Chinook salmon (*Oncorhynchus tshawytscha*) (Rosengrave *et al.* 2016; Gessner *et al.* 2017; Lehnert *et al.* 2017), while ovarian fluids of lake trout (*Salvelinus namaycush*) were found to promote sperm velocity of more related males (Butts *et al.* 2012), potentially to reduce outbreeding depression in this salmonid. Indeed, Yeates et al. (2013) found that ovarian fluids specifically support sperm of the same *Salmo* species over sperm of another *Salmo* species, suggesting a species-specific chemoattraction promoting reproductive isolation.

So far, likely effects of ovarian fluids on sperm have mainly been discussed in the light of possible adaptive female reproductive strategies. However, sperm characteristics can also reveal male reproductive strategies, as predicted by the theory of ejaculate economics (Parker & Pizzari 2010) and experimentally demonstrated in different taxa (Rudolfsen *et al.* 2006; Jeannerat *et al.* 2018). It is therefore possible that many of the observed effects of ovarian fluids on sperm function reveal variation in male reproductive strategies. If so, males would be expected to tailor sperm production and/or ejaculate characteristics to the likely exposure of their sperm to ovarian fluids, i.e., to the synchrony with, and the position to, the female during egg release (Egeland *et al.* 2016).

Salmonids are externally spawning fish whose ovarian fluids vary in amount and composition among species and even within populations (Lahnsteiner *et al.* 1995; Zadmajid *et al.* 2019). Viscosity of undiluted ovarian fluids is about 2 to 3 times higher than that of water (Zadmajid *et al.* 2019), but ovarian fluids will quickly be diluted when expelled with eggs

into water. During spawning, freshly spawned eggs and ovarian fluids are swirled around by male and female movements. Therefore, some eggs may no more be surrounded by significant amounts of ovarian fluids at the time of fertilizations. A likely predictor of how much sperm will be exposed to ovarian fluids is the position of the male during spawning. Arguably the closer a male is with its urogenital opening to a female's vent during egg release, the more likely its sperm will be exposed to ovarian fluids.

Salmonid males can play various roles during spawning (Esteve 2005), and many of these roles have been found to affect milt and sperm characteristics (Magris 2021). In migratory populations, for example, there is usually a fraction of males that avoid the costs of migration but then remain small and subdominant to their migratory rivals at spawning. They therefore reproduce mainly surreptitiously and invest disproportionally into sperm number and sperm velocity in order to increase the competitiveness of their sperm (Young *et al.* 2013). Evidence for strategic male investments has also been found within non-migratory populations. Male dominance at the spawning place is usually size dependent (Esteve 2005), and older males and larger male whitefish (*Coregonus muelleri)* (Selz & Seehausen 2023) have indeed been found to invest less into gonad weight and to have slower sperm than young and small males who may typically spawn further away from the female's vent (Rudolfsen *et al.* 2008). However, the links between male size and sperm traits can vary among populations (Perroud *et al.* 2021) and be plastic in response to the perceived social environment (Bartlett *et al.* 2017). When Rudolfsen et al. (2006), for example, controlled for size effects by confining pairs of size-matched male Arctic char (*Salvelinus alpinus*) in an enclosure and let them develop a dominance hierarchy, sperm characteristics changed quickly: as predicted from ejaculate theory, dominance led to reduced sperm velocity. Egeland et al. (2016) then used an analogous setup to show that sperm of dominant males swim better in ovarian fluids. One possible explanation for this increased performance is that ovarian fluids interact with milt of dominant males and specifically promote their sperm (cryptic female choice). An alternative hypothesis is that dominant males can expect to be close to the females during gamete release and may therefore produce sperm that do well when exposed to ovarian fluids. If so, any other male trait that is likely to affect the distance between male and female during spawning would be predicted to affect sperm reaction to ovarian fluids. Here we use wild-caught lake char (*Salvelinus umbla*) from Lake Geneva (Switzerland) to test the reaction of sperm of different of males to the presence of ovarian fluids.

The lake char is a non-migratory salmonid endemic to Alpine lakes. It spawns in winter in lek-like mating system where male competition is typically intense and male mating success can be expected to be positively correlated with their sexual ornamentation (Fiske *et al.* 1998). The lake char is closely related to the Arctic char (Kottelat & Freyhof 2007) that has often been used to study effects of ovarian fluids on sperm motility (e.g.Turner & Montgomerie 2002; Urbach *et al.* 2005; Egeland *et al.* 2016). Both species develop spawning colorations. Arctic char often develop strong red colorations in both sexes (Janhunen *et al.* 2011). Male lake char of our study population are mostly yellow during spawning season while females hardly develop any spawning coloration. This sex difference and the color differences within males are, to the best of our knowledge, not sufficiently understood yet.

In other fish families, sexual ornaments have sometimes been found to be positively linked to aspects of sperm performance, e.g., the carotenoid-based skin colors in three-spined

sticklebacks (*Gasterosteus aculeatus*) (Mehlis & Bakker 2013), European minnows (*Phoxinus phoxinus*) (Kekäläinen *et al.* 2014), or cherry barbs (*Puntius titteya*) (Kortet *et al.* 2004; Fukuda & Karino 2014). A common argument here is that both types of traits can react to induced stress, suggesting that high quality sperm in well ornamented males is an indicator of health and vigor rather than of alternative mating strategies. The pattern in char seems less clear. It has been suggested that skin colors in Arctic char reveal health and vigor and hence dominance (Skarstein & Folstad 1996; but see Backström *et al.* 2015). Rudolfsen et al. (2006) found in wild Artic char that dominant males have reduced sperm velocity. However, Janhunen et al. (2009) found in a captive population of Arctic char that the intensity of male coloration was positively correlated with sperm velocity when tested in water only.

Here we use hematological analyses (Seibel *et al.* 2021) to first test whether male coloration or their inbreeding coefficient ($F_{beta}$) are indicators of current health and vigor in wild-caught lake char. High leukocyte counts and especially relative lymphocytosis (percentage of lymphocytes among leukocytes) have been associated with acute infections (Haenen *et al.* 2010) or other forms of stress (Meuthen *et al.* 2020; Oluah *et al.* 2020). Thrombocytosis (elevated platelet counts) is another possible indicator of an acute infection (Rose *et al.* 2012; Yan *et al.* 2013). We also test whether male coloration are correlated to inbreeding coefficients ($F_{beta}$).

In a first series of experiments, we then test whether (i) male size, skin coloration, or $F_{beta}$ predicts sperm performance in water, (ii) sperm of different types of males react differently to the presence of ovarian fluids, and (iii) ovarian fluids vary in their effects on sperm. In a second series of experiments, we use sperm competition trials and genotyping of the resulting offspring to test whether (iv) the competitive success can be predicted by male color, male $F_{beta}$, or their kinship coefficient to the female that would determine the average inbreeding coefficient of the offspring, and (v) the presence of ovarian fluids change the outcome of sperm competition.

## Methods

*Sampling and handling of fish, gametes, and ovarian fluids*

Wild char were caught in December 2017 and 2018 from three spawning sites in Lake Geneva (Ripaille, 46.3957N-6.4718E; Locum, 46.4049N-6.7588E; Meillerie, 46.4113N-6.7216E) by local fishermen using gill nets (48 mm mesh size, set over night) and transported to a research facility (INRA, Thonon-les-Bains, France) were they were kept in a 1,000 L circular tank with free-flowing lake water before further processing. In 2017, 10 males and 4 females were haphazardly chosen for the experiment. Males were processed on the first day after catching and females on the second day. Each fish was anaesthetized in 10 L of water containing 3 mL of Eugenol (clove oil in ethanol at a 1:9 ratio), immediately photographed in a custom-made photobox under standardized conditions (17 mm, f/5.0, 1/200 s, ISO 400, WB 4000 K, JPG 24 Mpx) together with a standard color scale (Tiffen, USA), and stripped to collect gametes. Tissue samples were taken from the anal fin and stored in 70% ethanol at -20°C for further analysis. In 2018, further 24 males were sampled and handled as in 2017 to test again for correlations between skin colorations and sperm characteristics.

Milt was directly stripped into 50 mL conical tubes (Falcon, BD Biosciences, Allschwil, Switzerland), carefully avoiding contamination by urine, feces, or water. Between

2 and 5 mL of milt were obtained per male. These samples were stored at 4°C (< 2 hours) until further use. When all males had been stripped, 1.5 mL of each milt sample was diluted 1:9 in Storfish (IMV Technologies, France; an inactivating medium) in 15 mL tubes (Falcon, BD Biosciences, Allschwil, Switzerland). These tubes were then placed on ice and are referred to as 'diluted milt'.

Eggs and ovarian fluids were stripped into individual plastic containers. About 20 mL of ovarian fluid each was separated from the eggs with a syringe and stored in 50 mL tubes at 4°C. Eggs were kept (<1h) in the dark at 4°C in about 20 mL Ovafish (IMV Technologies, France) to prevent drying.

*Color measurement*

Custom-made macros in Fiji (Schindelin *et al.* 2012) were used to analyze male skin colorations. First, the white balance of each image was standardized with the help of the white and black values of the color scale. Skin gray value was then determined as the overall body gray value in the RGB color space (range from 0 = black, to 255 = white; using a mean of all pixels in each of the RGB channels). Redness and yellowness were measured from 3 squares (about 1% of the total body area each) in the pectoral region, the ventral region and the anal region, as described by Parolini et al. (2018) (Fig. S1). Redness and yellowness were measured as the $a^*$ and $b^*$ components of the CIE-$L^*a^*b^*$ color space, respectively (the a* axis for greenness-redness and the b* axis for blueness-yellowness both range from -100 to 100). All measures of yellowness were significantly correlated (Pearson's $r > 0.92$, $p$ always $< 0.001$). Similarly, all measures of redness were significantly correlated (Pearson's $r > 0.75$, $p < 0.013$). Therefore, the means of the three measurements per fish were used for each color. Gray values were correlated with yellowness (the yellower the lighter) and with redness (the redder the darker; Fig. S1) and were therefore ignored in further analyses.

*Sperm characteristicss*

Sperm characteristics of all males caught in 2017 and 2018 were measured on the day of sampling with Qualisperm® (AKYmed, Switzerland). Briefly, 20 µL of the diluted milt was activated in a microtube with 980 µL ice-cooled standardized water (OECD 1992) and vortexed for 5s. Then, 2 µL of this activated solution was pipetted into the well of a 4-well chamber slide (Leja, AKYmed, Switzerland), on a cooling station set at 4.5°C (the expected water temperature at the spawning ground). Sperm concentration, sperm velocity (average path velocity), and percentage of immotile sperm (sperm motility) were recorded under a phase contrast microscope at 20X magnification 20 s after activation in 2017 and 25 s after activation in 2018. Sperm longevity was recorded with a stopwatch from the time of activation until no progressive sperm motion could be observed in the frame of capture. Two replicated measures were taken for each sperm sample to test for consistency and means of these measurements were used for statistics. The concentration of active spermatozoa was calculated for each male by multiplying the total sperm concentration with the rate of motile sperm (e.g., 2,000 Mio/mL times 80% motile sperm = 1,600 Mio/mL active spermatozoa).

*Hematological analyses*

In order to determine leukocyte counts, relative lymphocytosis, and thrombocytosis, a blood sample (2-3 mL) was drawn from the caudal vasculature of the 10 males sampled in 2017 with a 3 mL syringe mounted with a 23 G needle heparinized with heparin 1 IU/µL. The blood was emptied in 2 mL microtubes, centrifuged 10 min at 10,000 g and 4°C. The plasma was then carefully collected, disposed in 1.5 mL microtubes and frozen in the vapor of liquid nitrogen for transportation and stored at -80°C in the lab for further analysis. A drop a blood from the syringe was used to make two blood smears per individual, on microscope frosted slides. Blood smears were dried in the field and fixed for 3 minutes in absolute ethanol back in the lab. Slides were stained for 20 minutes in Giemsa stain and rinsed twice in PBS. From each slide, 10 photographs (i.e. 20 per individual) were taken on an EVOS XL Core inverted light transmission microscope (Thermofisher, Switzerland) at 40x magnification. Counts of thrombocytes, lymphocytes, and granulocytes were done manually by a naïve observer (Fig. S2). Counts of total number of cells were done automatically with a custom-made macro in Fiji (Fig. S2). They were used to determine "leukocyte counts" (% of all blood cells), "relative lymphocytosis" (% of lymphocytes among leukocytes), and "thrombocytosis" (thrombocytes counts per 100 blood cells).

*Experiment 1: Sperm characteristics in response to ovarian fluids*

Experiments 1 and 2 were done with the 2017 sample. Sperm characteristics were determined again one day after the fish had been sampled from the wild, to determine each male's sperm characteristics in each female's ovarian fluid (40 combinations of milt samples and ovarian fluids) and in water (controls, 2 replicates each; Table S1). To do so, 10 µL of the diluted milt were activated either with 490 µL standardized water or with 490 µL ovarian fluid solution. The concentration of ovarian fluid in this solution was 50% as this would reflect a possible concentration of ovarian fluid in the water when sperm swim towards the egg (Rosengrave *et al.* 2016).

*Experiment 2: Competitive fertilization trials and embryo monitoring*

The 10 males were haphazardly assigned to 5 dyads (pairs of competitors) whose sperm then competed for fertilization of 24 eggs/female, either activated in water only or in water with ovarian fluid. Each possible combination of dyad x female was tested in both environments twice, resulting in 80 sperm competition trials (5 dyads x 4 females x 2 treatments x 2 replicates; Table S2) that involved in total 1,920 eggs. Due to an accident during handling, 10 of these 80 experimental cells were lost (all of the same female, prepared to be exposed to sperm activated in water only, see Table S2), reducing the number of eggs that could be monitored to N = 1,680.

In preparation of these sperm competition trials, the eggs of each female were first washed twice with 200 mL Ovafish® (IMV Technologies, France) to remove ovarian fluids. Then, 20 batches of 24 eggs each were placed in wells of 6-well plates (Falcon, BD Biosciences, Allschwil, Switzerland). Ten mL of diluted milt of 2 males each was prepared such that each male was represented with the same concentration of active sperm (25 Mio/mL) in the mix. One mL of each mix was then used to fertilize the 2 batches of 24 eggs/female. This 1 mL of mix was activated in a separated tube with either 4 mL of

standardized water or 4 mL of standardized water with ovarian fluid (ratio ovarian fluid to water = 1:2) and vortexed for 5s. The solution was poured in a well with eggs. Two minutes later, i.e., after fertilization could be expected to have happened, standardized water was added to fill the wells (16.8 mL) and the eggs were left undisturbed in a dark environment for 2 hours to allow hardening.

Each batch of 24 supposedly fertilized eggs was transferred to a 50 mL tube filled with standardized water and transported on ice to a climate chamber where they were rinsed for 30s under running tap water (4 L/min) in a sterilized tea strainer. The eggs were then distributed singly to wells of 24-well plates (Falcon, BD Biosciences, Allschwil, Switzerland) filled with 1.8 mL of autoclaved standardized water (OECD 1992). Embryos were raised at 4.5°C in a 12h:12h light-dark cycle. Eggs were classified as non-fertilized if no embryo could be found at the neurula stage. Embryo mortality, the timing of hatching, hatchling size, and hatchling growth was monitored from then on in the course of two parallel studies, one that combines these data with further stress experiments (by a pathogen and a chemical pollutant) on embryos produced in a regular full-factorial breeding design to test for parental effects on embryo performance (Garaud *et al.* 2023), and another one on sex-specific stress tolerance (Nusbaumer *et al.* 2021). Embryos and larvae that died during the observational period were transferred to individual microtubes and stored in 96% ethanol at -20°C for further analysis. Larvae were euthanized 14 days after hatching with a spike of 100 µL of Koi Med® Sleep 4.85% (Koi & Bonsai Zimmermann, Germany) in the well before being transferred to individual microtubes.

*Genotyping and paternity assignment based on microsatellite markers*

In the case of larvae and adults, DNA was extracted from up to 25 mg tissue with an extraction robot and the DNAeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following manufacturer's instructions. Unhatched embryos were homogenized and DNA was extracted using the Qiamp Fast DNA Stool mini kit protocol (Qiagen, Germany) as in Wilkins et al. (2015). A first multiplex PCR of 3 polymorphic microsatellite markers (Savary *et al.* 2017) turned out not sufficient to assign all embryos and larvae to their fathers (Bernatchez & Duchesne 2000). A second multiplex PCR of 3 further microsatellite markers (Savary *et al.* 2017) was therefore used for to assign the remaining offspring (Table S3). In the first multiplex PCR, the *sdY* sex marker (Yano *et al.* 2013) was included to sex offspring (Table S3) for a parallel study on sex-specific stress tolerance (Nusbaumer *et al.* 2021). Multiplex PCR were run on a Biometra thermocycler in 20 µL reaction volume using HotStarTaq DNA Polymerase (Qiagen, Germany) reagents (i.e. 2 µL PCR buffer 10X, 4 µL QSolution 5X, 0.4 µL HotStar Taq DNA Polymerase), 0.4 µL of dNTPs 10 mM and 0.4 µL of each primer). PCR cycles were run as follow: initial heat activation of 15 min at 95 °C; 35 cycles of 30 s denaturation at 94°C, 90 s annealing at 57°C and 60 s extension at 72°C; followed by 30 min of final extension at 72°C and 10 min at 4°C. DNA extracted from unhatched embryo was amplified using the same protocol, but in 10 µL reaction volume and with 40 cycles. PCR products were diluted 2X and run on an ABI3100 sequencer (Applied Biosystems, USA). Genotypes were read using Genemapper v4.0 (Applied Biosystems, USA). Genotyping of 3 dead embryos was not successful possibly due to poor DNA quality, bringing the final number of genotyped offspring to N = 1,475.

Adults were genotyped twice (no discrepancies observed). Paternity was assigned using CERVUS v3.0 (Kalinowski *et al.* 2007) as recommended for categorical assignments in controlled experiments (Jones *et al.* 2010). Input simulation parameters were set with 6 loci, 10,000 offspring, 2 candidate fathers with a proportion of sampling of 1 and a proportion of typing of 1, a minimum of 2 typed loci and an allelic dropout/mistyping rate of 0.05 as recommended by Wang (2004), and an assignment confidence of 95%.

*Double-digested RAD sequencing and SNPs calling*

DNA from all males and females of experiment 2 was used to also determine their multi-locus heterozygosity (MLH) and the genomic similarity ("kinship coefficient") between males and females of all possible combinations (N = 40). DNA concentration was measured using Qubit 2.0 (Thermo Fisher Scientific, Waltham**,** Massachusetts) while its integrity was verified on a 1% agarose gel. Each individual's DNA concentration was then standardized to 20 ng/µL. Two sequencing libraries were produced using all 10 males and 4 females each following the Brelsford et al. (2016) protocol adapted from Parchman et al. (2012). Briefly, DNA was digested using the enzymes EcoRI-HF and MspI (New England Biolab, Ipswich, Massachusetts, United States) and a unique EcoRI barcode was ligated to each individual. After library purification, PCR amplification were performed and fragments size-selected in between 400-550bp. Libraries were then single-end genotyped on 2 lanes of Illumina HiSeq 2500 with fragments of 125 bp length at Lausanne Genomic Technologies Facility (University of Lausanne).

After the quality control on the resulting fastq files done with FASTQC v0.11.7 (Andrews 2010), reads were trimmed to 110bp given the low per-base quality of the sequences (<20 Phred score). The resulting RAD data were analysed using Stacks 1.48 (Catchen *et al.* 2013) using the default parameters unless otherwise specified. Individuals sequences were demultiplexed using *process_radtags* (Stacks 1.48) and reads mapped to the *Salvelinus spp* reference genome (NCBI accession number SRP101753) using BWA (Li & Durbin 2010). *Pstacks* (Stacks 1.48) was done selecting a minimum stack depth of 5 (*-m* 5) and using the bounded SNP model. The catalogue of loci was created using *Cstacks* (Stacks 1.48) allowing 2 mismatches (*-n* 2) between loci. *Populations* (Stacks 1.48) was used to generate the VCF file considering only loci present in 100% of the individuals and markers heterozygosity of 0.5 (*--max_obs_het* 0.50). None of the individuals was excluded based on genotyping rate. Further filtering was done using *vcftools*, v0.1.15 (Danecek *et al.* 2011). To reduce incorrect heterozygosity call and remove paralogs, loci were filtered for a minimum coverage of 10X (*--min-meanDP* 10) and a maximum coverage of 50X *(--max-meanDP* 50; two times the mean coverage). Only loci under Hardy-Weinberg equilibrium were retained with a P-value treshold of 0.05 (*--hwe* 0.05). Finally, all the loci that were not shared between all individuals were discarded (*--max-missing* 1). No filtering was made based on minor allele frequencies. A total of 4,150 SNPs was retained after filtering with a mean coverage per individual of 29X.

Individual MLH, i.e. the number of heterozygous loci on the genotyped portion of the genome, was calculated using *vcftools* (*--het*) (Danecek *et al.* 2011). The kinship coefficient, i.e. the expected inbreeding coefficient for the progeny of one family, was estimated using the *beta.dosage* function of the package *Hierfstat* (Goudet 2005). This function generates a

genomic matrix based on allele dosage to estimate kinship coefficients considering the proportion of alleles shared between individuals (Goudet *et al.* 2018). The inbreeding coefficients for females and males, $F_{beta}$, were obtained by extracting the diagonal of the genomic matrix obtained with *beta.dosage*. Fig. S3 shows how MLH (range in males: 0.242 to 0.259) relates to $F_{beta}$ (range in males: 0.029 to 0.092). Further analyses were focused on $F_{beta}$.

*Statistical analysis*

Analyses were done in R (R Development Core Team 2015) and in JMP® 15.2.1. Pearson correlation coefficients (r) were used to test for correlations when visual inspection of the plots suggested that the conditions for parametric statistics were not significantly violated. Linear and logistic regression models were run on continuous and categorical variables, respectively. In experiment 2, the likely effects of ovarian fluids were tested on one dataset but for three different types of focal males within the dyads. Therefore, the Holm-Bonferroni method was used to adjust the critical p-values when testing for effects of ovarian fluids.

## Results

*Male phenotypes, $F_{beta}$, leukocyte counts, and milt characteristics*

Colorful males were mostly yellow (Fig. 1) and in few cases also slightly reddish. However, 19 of 34 males had negative $a^*$ values and the mean (±95% CI) $a^*$ value for the males was 0.95±1.78). Because redness was also positively correlated with yellowness ($r = 0.45$, $p < 0.005$), it was ignored in further analyses.

Yellowness increased with male size in both years (Fig. 1A; linear regression, effect of yellowness: $F = 5.6$, $p = 0.02$, year: $F = 0.3$, $p = 0.59$, yellowness x year: $F = 0.1$, $p = 0.74$) but was not significantly correlated with $F_{beta}$ (Fig. 1B; $r = 0.16$, $p = 0.65$). While the correlation between leukocyte counts and skin coloration was not significant ($r = -0.47$, $p = 0.17$), skin coloration turned out to be a good predictor of relative lymphocytosis (Fig. 1C; $r = -0.73$, $p = 0.02$) and of thrombocytosis (Fig. 1D; $r = -0.74$, $p = 0.01$). $F_{beta}$ was not corrected to any immune parameters (Fig. S4; leukocyte count: $r = 0.03$, lymphocytosis: $r = 0.18$, thrombocytosis: $r = -0.12$, p always $> 0.61$).

Yellowness did not correlate with sperm concentration (linear regression, effect of yellowness: $F = 0.8$, $p = 0.38$, year: $F = 1.0$, $p = 0.32$, yellowness x year: $F = 0.1$, $p = 0.72$). When their milt was activated in water only, yellowness did also not significantly correlate with sperm velocity (Fig. 1E; effect of yellowness: $F = 3.8$, $p = 0.06$, year: $F = 1.7$, $p = 0.20$, yellowness x year: $F = 0.1$, $p = 0.74$), the rate of immotile sperm when controlling for significant year effects (Fig. 1F; effect of yellowness: $F = 2.1$, $p = 0.16$, year: $F = 6.4$, $p = 0.02$, yellowness x year: $F = 0.3$, $p = 0.61$), nor sperm longevity (Fig. 1G; $r = -0.22$, $p = 0.30$).

Male size different between sampling years (Fig. 1A; $F = 4.9$, $p = 0.03$). When controlling for this year effect, none of the sperm traits could be predicted by male size (linear regressions, effect of male size: F always $< 1.8$, p always $> 0.19$). $F_{beta}$ was no significant predictor of sperm velocity (Fig. S4; $r = -0.25$, $p = 0.48$), but males with higher $F_{beta}$ had higher counts of immotile sperm (Fig. S4; $r = 0.74$, $p = 0.01$).

*Experiment 1: Sperm performance in ovarian fluid*

Activating sperm with or without ovarian fluids did not significantly affect average sperm velocity, but sperm velocity differed between males, and sperm of different males reacted differently to the presence of absence of ovarian fluids (Fig. 2A; linear regression, effect of activation medium: $F = 1.2$, $p = 0.28$, effect of male identity: $F = 10.5$, $p < 0.001$, activation medium x male: $F = 2.6$, $p = 0.01$). Activating sperm with ovarian fluids generally reduced the rate of immotile sperm (Fig. 2B; effect of activation medium: $F = 5.6$, $p = 0.02$, male: $F = 21.7$, $p < 0.001$, activation medium x male: $F = 1.6$, $p = 0.14$) and increased sperm longevity (Fig. 2C; effect of activation medium: $F = 263.8$, $p < 0.0001$, male: $F = 1.7$, $p = 0.11$, activation medium x male: $F = 0.9$, $p = 0.55$).

Table 1 focuses on sperm traits after activation with ovarian fluids. Males significantly varied in all sperm traits that were monitored here (main effects of male identity in Table 1). Females significantly varied in how their ovarian fluids affected average sperm velocity and longevity but not the percentage of immotile sperm (see main effects of female identity in Table 1). Sperm of different males responded differently to the ovarian fluids of different females: average sperm velocity and longevity but not the percentage of immotile sperm depended on the combination of male and female identities (male × female interaction terms in Table 1; see also Fig. S5).

The male-specific responses to ovarian fluids could partly be predicted by male skin coloration. With increasing yellowness, sperm velocity increased more in the presence of ovarian fluids (Fig. 2D; regression on means per male: $r = 0.65$, $n = 10$, $p = 0.04$). Male yellowness did, however, not predict the reduced rate of immotile sperm that ovarian fluids caused (Fig. 2E; $r = 0.42$, $p = 0.23$), nor did it predict some of the increase in sperm longevity linked to the presence of ovarian fluids (Fig. 2F; $r = -0.36$, $p = 0.31$). None of the analogous correlations to male $F_{beta}$ nor to the average kinship between males and females were significant ($r$ always $< 0.55$, $n = 10$, $p$ always $> 0.10$), i.e., the effects that ovarian fluids had on sperm traits were not linked to male $F_{beta}$ or the average kinship to the donors of ovarian fluids.

*Experiment 2: Males success in sperm competition*

In total 1,475 offspring were genotyped of which 1,464 could be assigned to one of the two competitors within the dyads (99.3% success). In the following, three types of focal males within the dyads are considered: the most yellow, the least inbred, and the least related to the respective female. Male yellowness did not correlate with their $F_{beta}$ (Fig. 1B; see above) nor with the kinship with the females (Fig. S6A; $r = 0.13$, $n = 40$, $p = 0.42$), and kinship could not be predicted by male $F_{beta}$ (Fig. S6B; $r = 0.12$, $n = 40$, $p = 0.47$).

Figure 3 shows the outcome of the individual sperm competition trials when run in water only or with ovarian fluids of the different females, plotted each for the three types of focal males within the dyads (see Table S4 for the identities of the focal males in the different scenarios). Overall, the average (SD) success rates of focal males were 0.534 (0.180), 0.562 (0.169), and 0.532 (0.179) if the focal male was the most yellow, the least inbred, or the least related to the female, respectively (Fig. 3). Dyad identity always had a strong effect on the focal male's success rate (Table 2).

If the focal male was the yellower of the two competitors, its fertilization success was significantly enhanced in the presence of ovarian fluid as compared to water only (Table 2A, Fig. 3A) while female identity played no significant role in this scenario (Table 2A). If the focal male was the one with the lower $F_{beta}$, its fertilization success was higher when sperm were activated with water only instead of diluted ovarian fluids (Table 2B, Fig. 3B). As before, female identity played no significant role (Table 2B). In both previous scenarios, the different effects that ovarian fluids had on sperm velocity contributed significantly to the outcome (Table 2A,B). No effects of ovarian fluids could be observed if the focal male was the least related to the egg donor (Table 2C, Fig. 3C). In the latter scenario, male success was best explained by female identity and the difference in sperm velocity and longevity between the rivals (Table 2C).

**Discussion**

The mating systems of salmonid fish typically lead to intense male-male competition (Fiske *et al.* 1998; Esteve 2005) that is likely to affect male reproductive strategies (Parker & Pizzari 2010; Magris 2021). Previous studies on male reproductive strategies have often focused on traits that may only partly determine fertilization success under ecologically relevant conditions (Dougherty *et al.* 2022). In fish, for example, it is still largely unclear whether and to what degree sperm reaction to ovarian fluids may reveal male adaptations to the microecology that their sperm are likely exposed to during fertilization. Here we asked whether male skin coloration or their inbreeding coefficient $F_{beta}$ reveal factors that may matter in male-male competition, and whether sperm traits and especially the reaction of sperm to ovarian fluids can be predicted from male coloration or $F_{beta}$.

Secondary sexual traits like conspicuous skin colors are expected to reveal general health and vigor (Andersson 1994), and they have repeatedly been observed to do so in other species (Milinski & Bakker 1990; Johnson & Fuller 2015). In our study, paler males suffered from increased relative lymphocytosis and thrombocytosis, two potential indicators of acute infections or other physiological stress (Haenen *et al.* 2010; Rose *et al.* 2012; Yan *et al.* 2013) (Johansen *et al.* 2019), than yellower males. We therefore conclude that male yellowness is a good predictor of current male health condition. We also found a positive correlation between male yellowness and body size that often determines male dominance in fish (Jacob *et al.* 2007; Jacob *et al.* 2009). However, neither male yellowness nor body size correlated significantly with any milt or sperm trait when sperm were activated with water only. Our study therefore provides no support of the phenotype-linked fertility hypothesis of mate selection (e.g. Mehlis *et al.* 2013; Monteiro *et al.* 2017).

Theory also predicts a correlation between an individual's inbreeding coefficient and general health and vigor (Allendorf & Luikard 2007), and such links have indeed been found in other studies (Fox & Reed 2011). We did not find any significant correlations between male $F_{beta}$ and immune parameters, but this may need to be studied again in larger samples that provide more statistical power. There was also no significant correlation between male $F_{beta}$ and milt or sperm traits when tested in water only, except that the rates of immotile sperm increased with increasing male $F_{beta}$. This latter observation supports the hypothesis that sperm motility can still be an indicator of certain aspects of health and vigor (Kowalski & Cejko 2019).

$F_{beta}$ and skin colorations were not significantly correlated in our sample. In previous studies, such a correlation was sometimes found (e.g. Zajitschek & Brooks 2010; Herdegen *et al.* 2014) and sometimes not (e.g. Frommen *et al.* 2008; Marsh *et al.* 2017), including a study on Arctic char (Janhunen *et al.* 2011). One possibility is that skin coloration is a plastic trait that quickly reacts to acute stress (Johnson & Fuller 2015) while $F_{beta}$ affects more the overall tolerance to environmental stress. If so, the link between $F_{beta}$ and skin colorations is expected to change with changing environmental conditions.

Our first experiment was designed to test whether and how ovarian fluids affect sperm traits, and whether such effects differ for different types of males. We found that ovarian fluids reduced the percentage of immotile sperm and nearly doubled mean sperm longevity. There was no significant link between these supportive effects of ovarian fluids and male yellowness, their $F_{beta}$, nor to the kinship between males and females. With regard to these sperm traits, all types of males profited similarity from the presence of ovarian fluids.

Sperm velocity was not generally increased by ovarian fluids, apparently contrary to some previous findings in other fishes (Zadmajid *et al.* 2019). However, when comparing the effects of ovarian fluids on sperm characteristics of different types of males, it turned out that the yellower a male, the more does the velocity of its sperm increase when in contact with ovarian fluids. Interestingly, sperm of paler males were faster in water only than in diluted ovarian fluids. Such a link between the effects of ovarian fluids and male coloration suggests that sperm of yellower males are better prepared to ovarian fluids while sperm of paler males are better prepared to the absence of ovarian fluids. Our findings suggest that yellower males are typically closer to the female vent during spawning than paler males. This could be due to male-male dominance, female preference, or both.

Females varied in how much their ovarian fluids generally increased sperm velocity and sperm longevity, but not in how much their ovarian fluids reduced the rate of immotile sperm. Females also varied in how much their ovarian fluids specifically increased velocity and longevity of sperm of certain males over others, i.e., we found the corresponding females x male interactions to be significant (but again not in the case of sperm motility). However, effects of ovarian fluids on sperm traits were not significantly linked to male $F_{beta}$ or the average kinship between males and females.

Our second experiment was to see whose sperm benefit most from exposure or non-exposure to ovarian fluids when in sperm competition, and whether the most successful males are best characterized by their skin coloration, their $F_{beta}$, or their kinship to a given female. We found that the presence of ovarian fluids led to enhanced fertilization success of the more yellow of the two competitors. This result further supports the conclusion from the first experiment that sperm of more yellow males seem better adapted to the presence of ovarian fluid than sperm of pale males. If the focal male was the one with the lower $F_{beta}$, its fertilization success turned out to be higher when sperm were activated with water only than with diluted ovarian fluids. No significant effects of ovarian fluids were observed if the focal male was the least related to the female.

Competition trials typically reveal a limited range of possible situations. In our case, the overall sperm to egg ratios were so high that variation in sperm longevity may not have affected the outcome of the sperm competition, while late fertilizations may often be relevant under natural conditions. However, no significant correlations between sperm longevity and

male yellowness, $F_{beta}$, or kinship to the donor of the ovarian fluid was observed in the first experiment. We therefore conclude that the competitive advantage that sperm of more yellow males have over sperm of pale males depends on the presence of ovarian fluids.

In a parallel study, Garaud et al. (2023) used an extended sample of our study population to test whether females would profit from mating with more yellow, less inbred, or less related males. They used full-factorial *in vitro* fertilizations (crossing 10 male and 6 females in all possible combinations) and single rearing of large numbers of offspring to study embryo development under different environmental conditions. Contrary to expectations from 'good-genes' hypotheses of sexual selection (Andersson 1994), offspring of yellower males were on average smaller and less tolerant to pathogen stress than offspring of paler males. These results confirm previous findings on Arctic char (Janhunen *et al.* 2011) and suggest that females would not profit from giving yellower males a selective advantage during sperm competition. Interestingly, Garaud et al. (2023) found significant effects of the genetic relatedness between males and females: Embryos grew smaller with increasing kinship of their parents.

In conclusion, male yellowness correlated positively with health and vigor and body size, i.e., with traits that are likely to determine the outcome of male-male competition. Yellower males may therefore typically be closer to the females during egg release than pale males. If so, yellow males could expect their sperm to be more likely exposed to ovarian fluids than pale males. We conclude from two different types of experiments that sperm of yellow males profit more from the presence of ovarian fluid than sperm of pale males. No such beneficial effects of ovarian fluid could be seen with regard to male $F_{beta}$ or the kinship between males and females, while parallel breeding experiments with the same study population concluded that females would profit most from supporting sperm of least related males. Combined, these results suggest that the increased performance of sperm of yellower males in ovarian fluids is an adaptation to the microenvironment that the sperm are likely to be exposed to, rather than cryptic female choice by ovarian fluids. Studies on sexual selection at the gamete level (e.g., testing for cryptic female choice) should therefore always distinguish between likely effects of male strategies and likely effects of female strategies.

**Ethics**

This work complied with the national, cantonal and university regulations where it was carried out. The handling and transport of adults and transport of embryos was approved by the French authorities (INTRA.FR.2017.0109258).

**Data accessibility**

The data used for this study have been deposited in the Dryad depository (de Guttry *et al.* 2022; Nusbaumer *et al.* 2022).

**Funding**

This project was supported by the Swiss National Science Foundation (31003A_159579 and 31003A_182265).

**Acknowledgments**

We thank G. Levray from the Pistolet fishery for catching the fish, the APERA association and the *pisciculture des Rives* for access to the fish, L. Adhia Eya, A. Atherton, C. Berney, J. Bideaux, F. Dolivo, L. Espinat, S. Kreuter, E. Longange, L. Marques da Cunha, and N. Sironi for assistance in the field or in the lab, F. Schütz for discussion on statistics, E. Lasne for discussion and access to the INRA facilities, and handling editor M. M. Hansen and four reviewers for helpful comments.

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**Tables**

**Table 1.** Experiment 1: Sperm characteristics of different males after activation with diluted ovarian fluid of different females. Significant *P*-values are highlighted in bold.

| Factor | d.f. | F | *p* |
|---|---|---|---|
| *(A) Sperm velocity* | | | |
| Male | 9 | 7.1 | **<0.001** |
| Female | 3 | 6.2 | **0.002** |
| Male x female | 27 | 1.9 | **0.03** |
| | | | |
| *(B) Sperm motility* | | | |
| Male | 9 | 27.9 | **<0.001** |
| Female | 3 | 0.6 | 0.64 |
| Male x female | 27 | 0.8 | 0.74 |
| | | | |
| *(C) Longevity* | | | |
| Male | 9 | 446.5 | **<0.001** |
| Female | 3 | 1185.3 | **<0.001** |
| Male x female | 27 | 145.8 | **<0.001** |

**Table 2.** Experiment 2: Correlates to fertilization success of the focal male in the sperm competition trials, if the focal male is (A) the more yellow one of the two competitors, (B) the one with the lower inbreeding coefficient $F_{beta}$, or (C) the least related to the female. Logistic regressions with female (egg donor), dyad (pair of competing males), ovarian fluid (present of absent), and the difference in mean sperm velocity, rates of immotile sperm, and sperm longevity between rivals (focus – opponent). These differences in sperm traits had been determined in experiment 1 both in water and in ovarian fluid and were therefore nested in the factor "ovarian fluid". The table shows the outcome after stepwise removal of non-significant effects of sperm traits. Significant effects of ovarian fluids (after Holm-Bonferroni correction for multiple comparison) are highlighted in bold.

| Factor | d.f. | $\chi^2$ | *p* |
|---|---|---|---|
| *(A) Focal male: more yellow* | | | |
| Female | 3 | 6.3 | 0.10 |
| Dyad | 4 | 156.4 | <0.001 |
| Ovarian fluid | 1 | 7.8 | **0.005** |
| Difference in sperm velocity[1] | 2 | 7.8 | 0.02 |
| | | | |
| *(B) Focal male: lower $F_{beta}$* | | | |
| Female | 3 | 6.0 | 0.11 |
| Dyad | 4 | 122.1 | <0.001 |
| Ovarian fluid | 1 | 5.8 | **0.02** |
| Difference in sperm velocity[1] | 2 | 7.9 | 0.02 |
| | | | |
| *(C) Focal male: lower kinship* | | | |
| Female | 3 | 20.4 | <0.001 |
| Dyad | 4 | 26.7 | <0.001 |
| Ovarian fluid | 1 | 1.8 | 0.18 |
| Difference in sperm velocity[1] | 2 | 6.2 | 0.04 |
| Difference in sperm longevity[1] | 2 | 39.0 | <0.001 |

[1] Nested in "ovarian fluid"

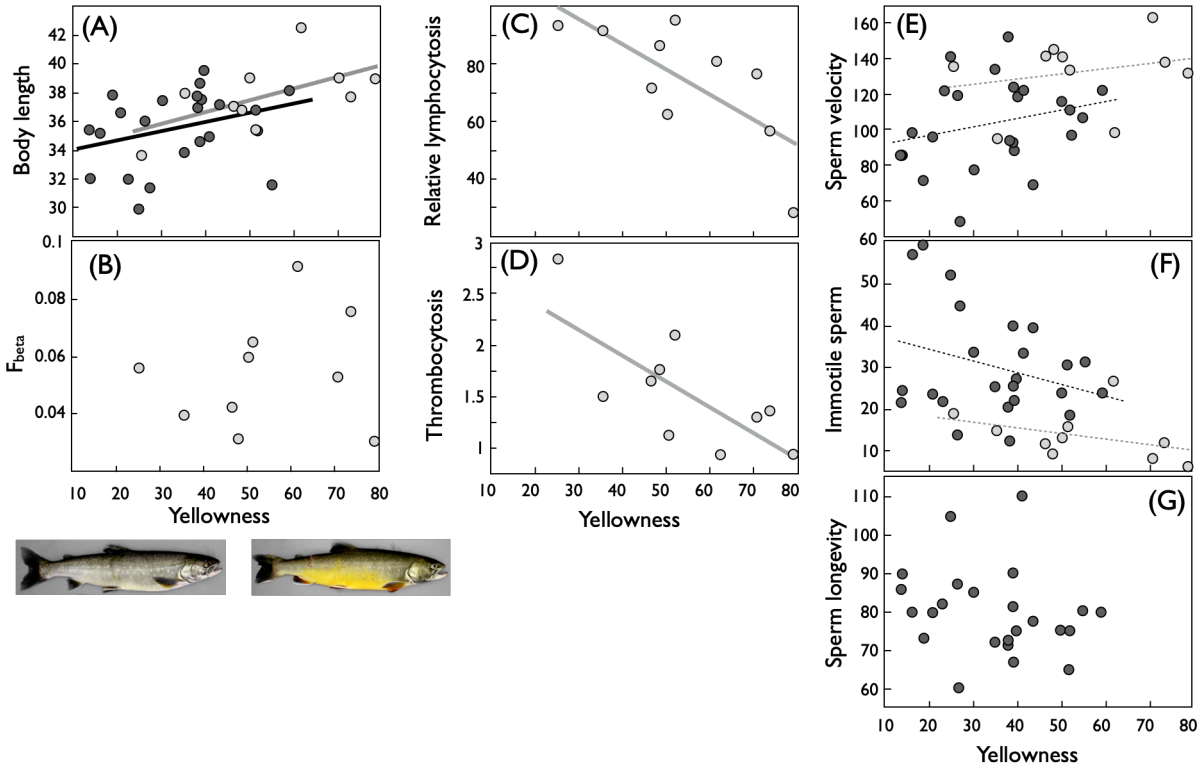


**Figure 1. Male yellowness vs size, inbreeding coefficient, immune parameters, and characteristics of sperm when activated in water only.** The two fish illustrate the two extremes of observed yellowness in 2017. Yellowness versus (A) body size (cm), (B) inbreeding coefficients ($F_{beta}$), the immune parameters (C) relative lymphocytosis (% of lymphocytes among leukocytes) and (D) thrombocytosis (thrombocytes counts per 100 blood cells), and the sperm characteristics (E) velocity (µm/s), (F) rate of immotile sperm (%), and (G) longevity (sec). Non-hatched and hatched regression lines illustrate significant and non-significant relationships, respectively, after potentially confounding effects of sampling year were taken into account. Symbols and regressions in light grey indicate samples collected in 2017, darker symbols and regression lines those from 2018. See text for statistics.

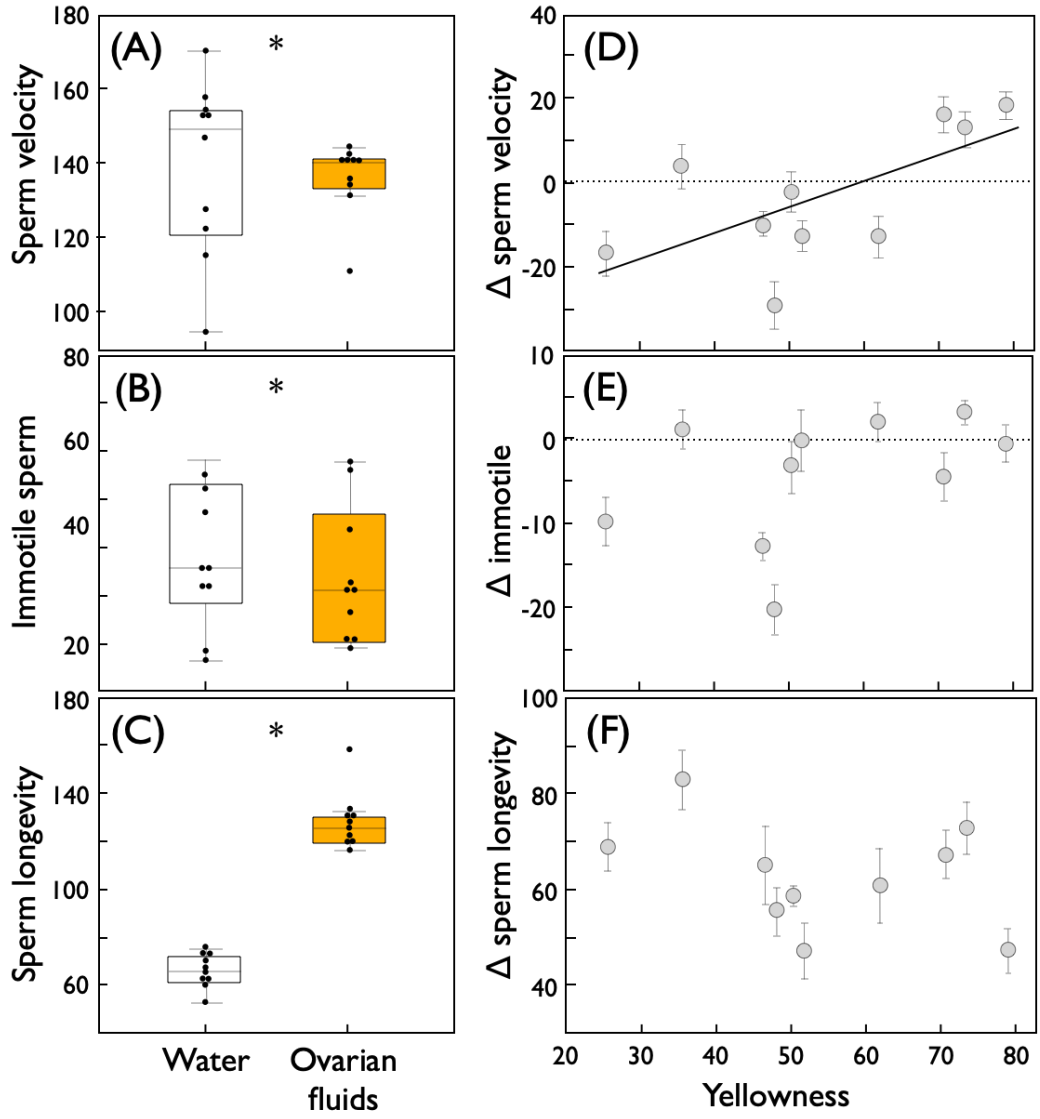


**Figure 2. The effects of ovarian fluids on sperm of different types of males (experiment 1).** Panels A-C show average sperm characteristics (Turkey box plots with quartiles and whiskers) for each of the 10 males after their sperm were activated in water only or in diluted ovarian fluids: (A) sperm velocity (μm/sec), (B) rate of immotile sperm (%), and (C) sperm longevity (sec). Panels D-F give the mean (±SD) differences between of sperm characteristics in ovarian fluid and in water (8 measurements per male in ovarian fluids compared to the respective mean in water each) relative to male yellowness: (D) differences in sperm velocity, (E) differences in rates of immotile sperm, (F) differences in longevity. The regression line illustrates the significant correlation, the dotted line in (D) and (E) mark zero difference. The asterisks indicate a significant effect of the activation medium, either as main effect (B, C) or in interaction with male identity (A). See text for statistics.

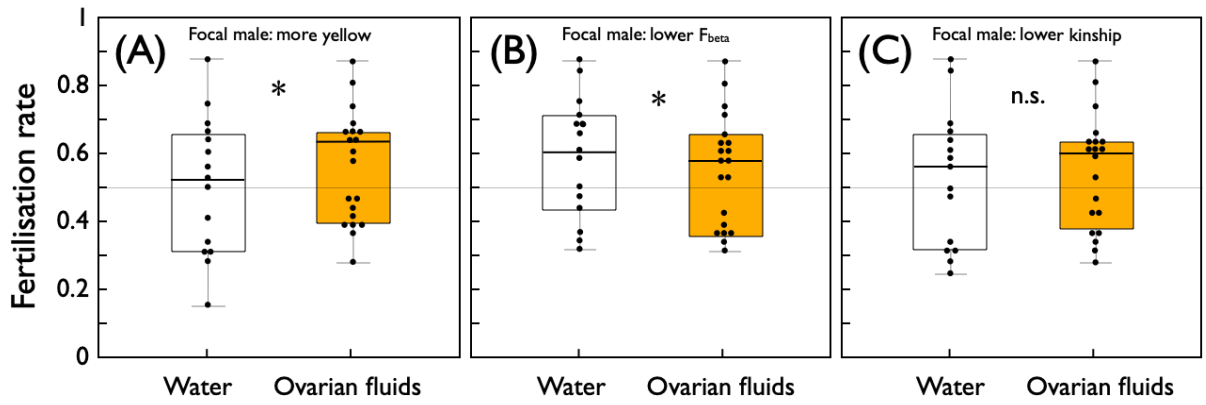


**Figure 3. Outcome of the sperm competition trials (experiment 2).** The mean fertilization rates per dyad, female, and whether or not ovarian fluids were present of (A) the yellower male of the two competitors each, (B) the less inbred male each, and (C) the male each that is least related to the female whose ovarian fluids the sperm was exposed to. Turkey box plots with quartiles and whiskers. The asterisks indicate a significant difference linked to the activation medium, n.s. = not significant. See Table 2 for statistics.

**Supplemental Information for:**

# Sperm of more colorful males are better adapted to ovarian fluids in lake char (Salmonidae)

David Nusbaumer, Laura Garaud, Christian de Guttry, Laurie Ançay & Claus Wedekind

## Table of Contents:

**Table S1.** Experiment 1: Determining sperm characteristics of males #1700 to #1709 in various activation media, i.e., diluted ovarian fluid of one of 4 females (#GAA to #GAD) or water only. The entries give the number of independent sets of measurements.

| | Male | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Activation medium | #1700 | #1701 | #1702 | #1703 | #1704 | #1705 | #1706 | #1707 | #1708 | #1709 |
| Ovarian fluid of #GAA | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Ovarian fluid of #GAB | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Ovarian fluid of #GAC | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Ovarian fluid of #GAD | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Water only | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |

**Table S2.** Experiment 2: The sperm competition trials, with 2 times 24 eggs per experimental cell. Asterisks indicate experimental cells that were accidentally lost.

| | | Dyad | | | | |
|---|---|---|---|---|---|---|
| Female | Activation | #1700 vs #1701 | #1702 vs #1704 | #1703 vs #1705 | #1706 vs #1707 | #1708 vs #1709 |
| #GAA | ovarian fluid | 2 x 24 | 2 x 24 | 2 x 24 | 2 x 24 | 2 x 24 |
| #GAA | water | 2 x 24 | 2 x 24 | 2 x 24 | 2 x 24 | 2 x 24 |
| #GAB | ovarian fluid | 2 x 24 | 2 x 24 | 2 x 24 | 2 x 24 | 2 x 24 |
| #GAB | water | 2 x 24* | 2 x 24* | 2 x 24* | 2 x 24* | 2 x 24* |
| #GAC | ovarian fluid | 2 x 24 | 2 x 24 | 2 x 24 | 2 x 24 | 2 x 24 |
| #GAC | water | 2 x 24 | 2 x 24 | 2 x 24 | 2 x 24 | 2 x 24 |
| #GAD | ovarian fluid | 2 x 24 | 2 x 24 | 2 x 24 | 2 x 24 | 2 x 24 |
| #GAD | water | 2 x 24 | 2 x 24 | 2 x 24 | 2 x 24 | 2 x 24 |

**Table S3**. The 6 microsatellite markers used for paternity assignment, and the sex marker that was added to the first multiplex.

| Marker | Multiplex | Sequence forward primer | Sequence reverse primer | Dye | $N_{alleles}$ | Range [bp] |
|---|---|---|---|---|---|---|
| Cocl3-N | 1 | 5'-TTCAGGTTTGGTAAGCAAG-3' | 5'-AGTGTAATAAATCACCCGAG-3' | atto550 | 8 | 226-270 |
| OtsG253b | 1 | 5'-GAGCAGGCCGAGCAGGTGTCT-3' | 5'-AATTGGGTCATTAAGGCTCTGTGG-3' | fam | 16 | 88-118 |
| Ssa456 | 1 | 5'-CTTCCCAGGAGTCATCATAAATCT-3' | 5'-TAAACCCCACTGCTTGTTGAGTGT-3' | hex | 4 | 209-215 |
| Sfo8 | 2 | 5'-CAACGAGCACAGAACAGG-3' | 5'-CTTCCCCTGGAGAGGAAA-3' | hex | 12 | 248-275 |
| Sfo23 | 2 | 5'-GTGTTCTTTTCTCAGCCC-3' | 5'-AATGAGCGTTACGAGAGG-3' | atto550 | 11 | 136-217 |
| Ssa85 | 2 | 5'-AGGTGGGTCCTCCAAGCTAC-3' | 5'-ACCCGCTCCTCACTTAATC-3' | fam | 11 | 156-189 |
| Sdy[1] | 1 | 5'-CCCAGCACTGTTTTCTTGTCTCA-3' | 5'-CTTAAAACCACTCCACCCTCCAT-3' | atto620 | 1 | 226-226 |

[1]Sex marker, used for a parallel study on sex-specific embryo development (Nusbaumer *et al.* 2021).

**Table S4.** Identity of focal males (ID = #1700 to #1709) if it is the more yellow than its competitor, less inbred, or least kin to a given female (ID = from #GAA to #GAD).

| | | | Least kin to female | | | |
|---|---|---|---|---|---|---|
| Dyad | More yellow | Less inbred | #GAA | #GAB | #GAC | #GAD |
| 1 | #1701 | #1701 | #1701 | #1701 | #1701 | #1700 |
| 2 | #1702 | #1704 | #1704 | #1702 | #1704 | #1702 |
| 3 | #1703 | #1705 | #1703 | #1705 | #1705 | #1705 |
| 4 | #1706 | #1707 | #1707 | #1707 | #1707 | #1707 |
| 5 | #1709 | #1708 | #1709 | #1708 | #1709 | #1709 |

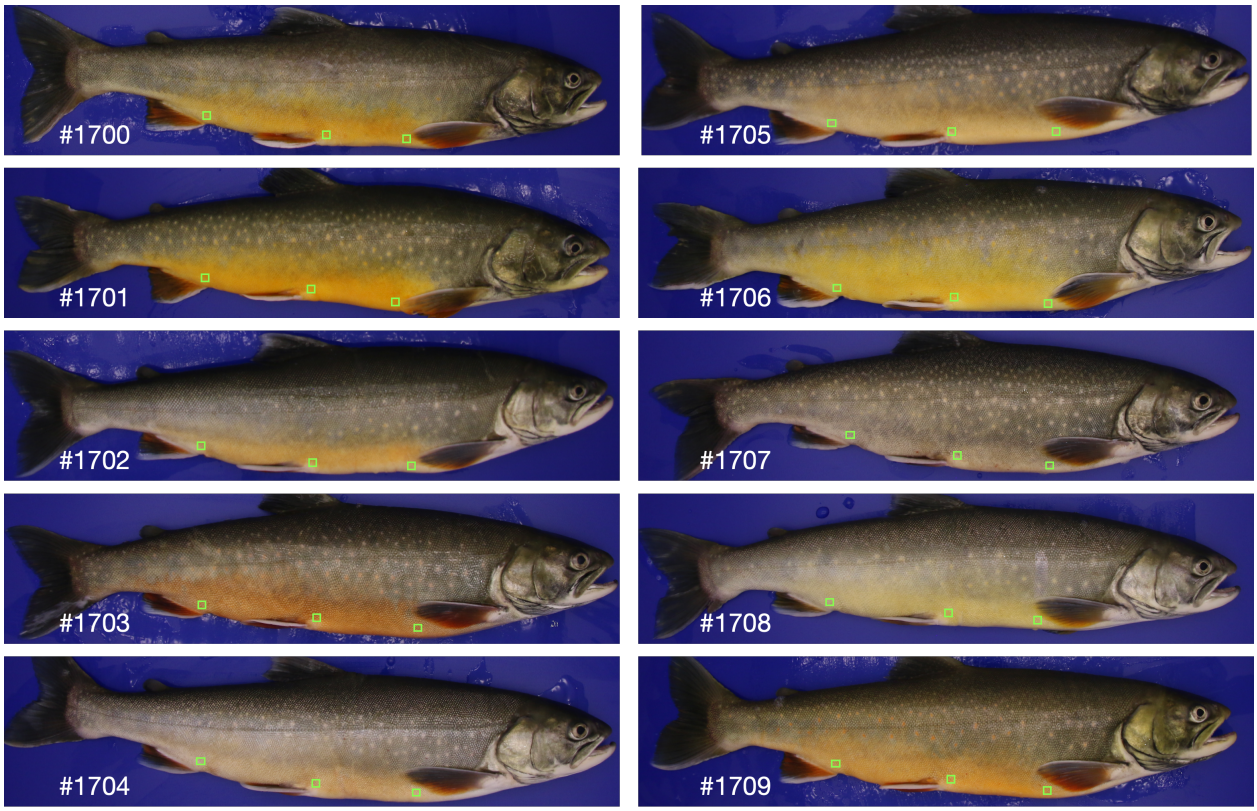


**Figure S1.** The 10 males sampled in 1017 and used for experiments 1 and 2, and the locations of the 3 squares (in green) from which color measurements of the ventral area (yellow and red) were taken. When including the 24 males sampled in 2018, mean gray value was positively correlated with yellowness, i.e., the yellower the lighter ($r = -0.47$, $p < 0.005$) and negatively with redness, i.e., the redder the darker ($r = -0.66$, $p < 0.001$).

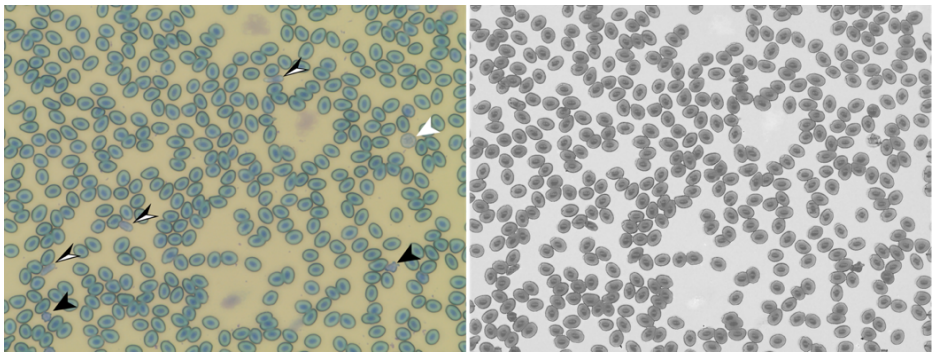

**Figure S2.** Example of a blood smear image as taken under the microscope (left) and its output of automated cell count (right). On the left image, black arrow heads point towards lymphocytes, the white arrow towards a granulocyte, and the black and white arrow towards thrombocytes. The rest are erythrocytes.

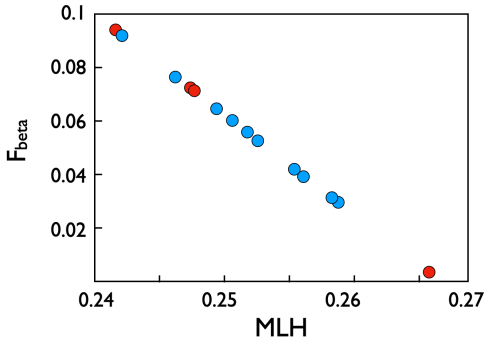


**Figure S3.** The link between multi-locus heterozygosity (MLH) and individual inbreeding coefficients ($F_{beta}$) for the males (blue) and females (red) sampled in 2017 and used in experiments 1 and 2.

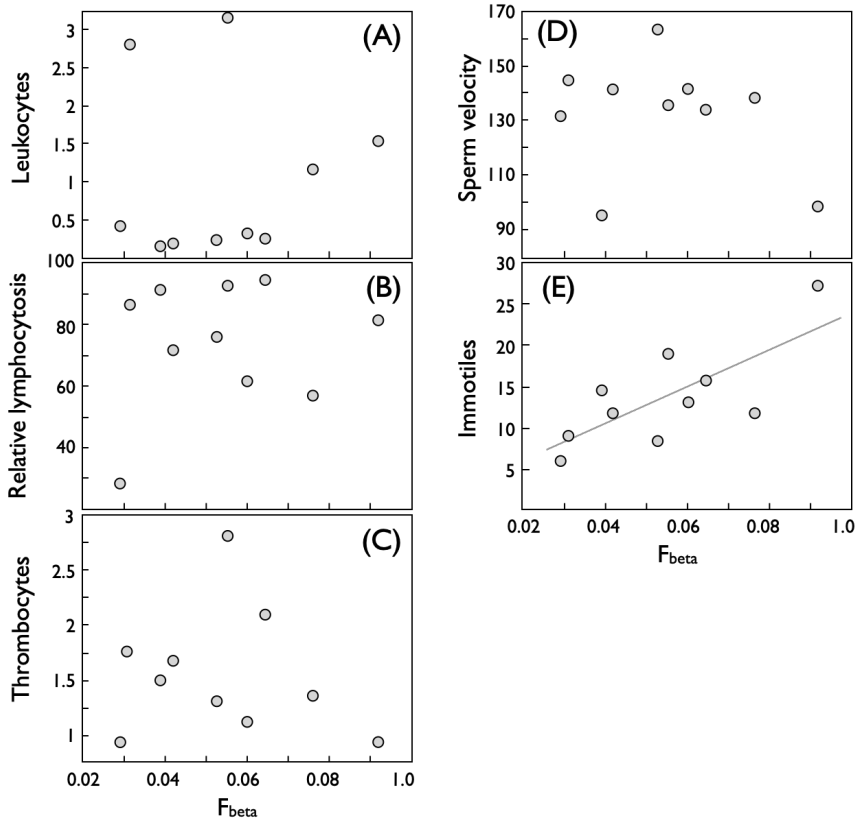


**Figure S4.** Immunological and sperm characteristics of males relative to their inbreeding coefficient ($F_{beta}$): (A) leukocytes (% of all blood cells, (B) relative lymphocytosis (% of lymphocytes among leukocytes), (C) thrombocytes per 100 blood cells, (D) sperm velocity (µm/sec), (E) percentage of immotile sperm. Sperm longevity was not measured in 2017. The regression line is given for the significant relationship. See text for statistics.

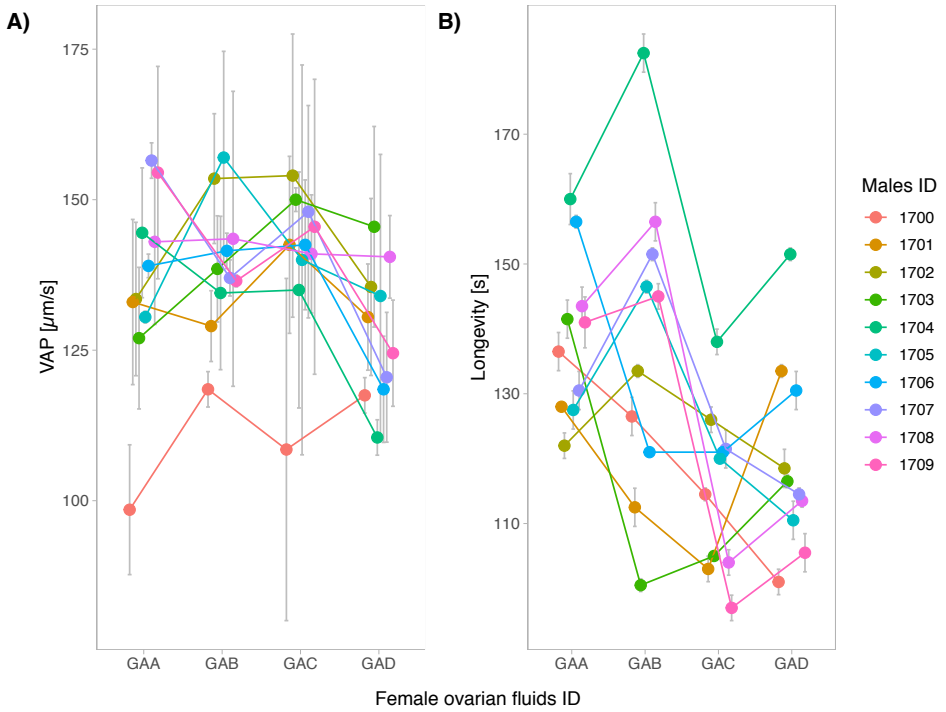


**Figure S5.** Interactions effects between females' ovarian fluids and males' identities on A) sperm velocity (VAP, in µm/s) and (B) sperm longevity (s). Symbols depicts means of within subject repeated measures with their 95% confidence intervals. See Table 1 for statistics.

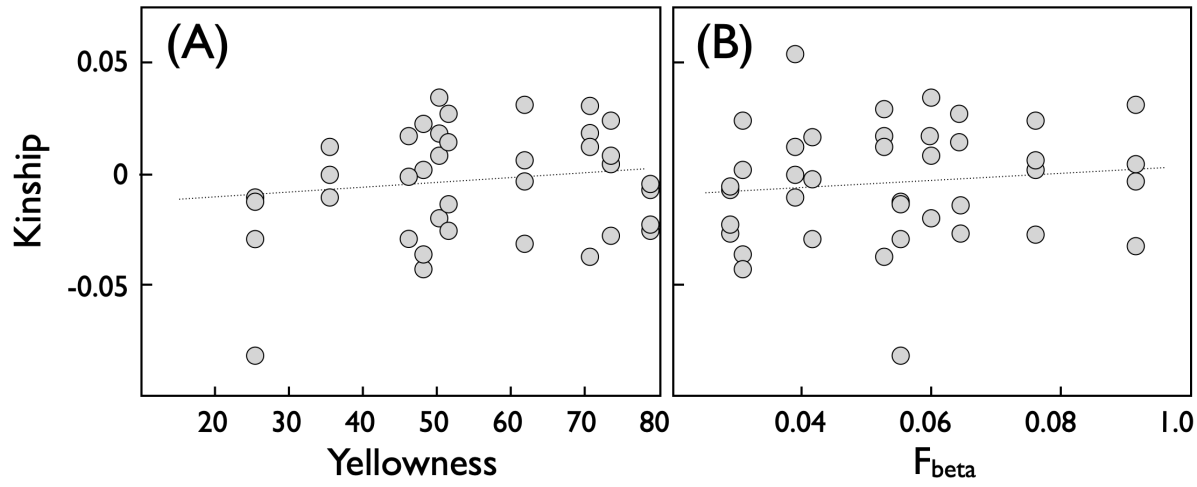


**Figure S6.** Relatedness between males and females (kinship) that were used in the sperm competition trials (4 females x 10 males = 40 combinations) versus (A) male skin colorations (yellowness) and (B) individual male inbreeding coefficients ($F_{beta}$). The dotted lines give the non-significant regressions.