

In vitro study on sperm competition in common carp (*Cyprinus carpio* L.)

by

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ABSTRACT. - Sperm competition occurs when sperm from different males compete for fertilization. The aim of this *in vitro* fertilization study was to compare the competitive success of five males using heterosperm with an equal number of sperm from each male and four different sperm/egg ratios (5,000, 10,000, 20,000 and 100,000 spermatozoa per egg) in order to better understand variations of competitive fertilization. The roles of sperm motility and velocity were studied. Fertilization and hatching rates of the 5 males studied, with 100,000 spermatozoa per egg, were between 23.7-94.8% and 23.7-92.2%, respectively. Sperm velocity and percentage of motile sperm ranged from 85.0 to 137.6 $\mu\text{m s}^{-1}$ and 2.0 to 93.5% at 15 sec post sperm activation, respectively. The contribution of individual males to the progeny when using heterosperm was explored by DNA fingerprinting and was very diverse. Males with very low level of sperm motility (M3 4.45%, M4 1.95%) were represented by low contribution in all groups of progenies, but significant differences in the contribution of males were also found among individuals with a similar percentage of motile spermatozoa. The overall number of spermatozoa per egg strongly influenced the fertilization/hatching rate when using heterosperm, but had no impact on the relative number of progenies sired by each of the 5 competing males. Several models were tested in order to attempt to explain the contribution of individual males to the progeny.

Key words. - Aquaculture - Artificial reproduction - Paternity assignment.

Introduction

Sperm competition occurs when sperm from different males compete for fertilization (Parker *et al.*, 1990). Sperm competition has been explored in many behavioural studies on several fish species, however there have been few studies investigating sperm competition under controlled conditions of *in vitro* fertilization in important aquaculture species (Gage *et al.*, 2004, Linhart *et al.*, 2005). In the case of the Atlantic salmon, paternity analysis based on microsatellite DNA fingerprinting revealed that relative sperm velocity was the primary determinant of fertilization success and that sperm longevity correlated negatively with competition success (Gage *et al.*, 2004). Linhart *et al.* (2005) identified progenies in the common carp (*Cyprinus carpio* L.) by colour markers, using equal numbers of sperm per male, in 30 male-to-male individual competition tests. The mean percentage of offspring sired was strongly influenced by the individual males. The observed parameters of sperm motility and velocity, only partly explained the variation in male competitive success.

The aim of this study was to evaluate competitive success and spermatozoal parameters of five males in a competitive trial, using equal numbers of spermatozoa from each of the males in the trial, with different sperm/egg ratios for an *in vitro* fertilization (5,000, 10,000, 20,000 and 100,000 spermatozoa per egg).

Methods

Broodstock handling and collection of gametes

Based on preliminary genotyping of four microsatellite loci, one female and five males were selected for the experiment, as their genotypes ensured 100% paternity assignment in their progeny from the four loci tested. Artificial fertilization was carried out according to Linhart *et al.*, (2005).

The sperm of five males was collected individually and sperm concentrations were estimated using a Burker cell hemocytometer. Following the sperm concentration estimates, part of the sperm was stored individually for fertilization and hatching control tests and the rest was used in preparation of the heterosperm, where quantities of sperm from each of the 5 males were adjusted to ensure an equal representation of each male in the pool, each male representing 20% of the total number of spermatozoa in the pool.

Fertilization

Five grams of ova (800 ova.g⁻¹) were placed into four dishes on an orbital agitator, and heterosperm was added to each of these, with volumes corresponding to the following ratios of spermatozoa number per egg: 5,000 (G1), 10,000 (G2), 20,000 (G3) and 100,000 (G4). Gametes were activated on the agitator (200 rpm, 10 mm deflection) by adding 5 ml of hatchery water. After 2 min of mixing, approximately 100 ova were transferred to Petri dishes and placed into an experimental incubator supplied

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with UV-sterilized and dechlorinated tap water (22°C, 9 mg l⁻¹ O₂).

Fertilization and hatching control for individual males

Samples of 5 g of ova were fertilized with individual sperm from each of the five males using 100,000 spermatozoa per egg as described above. This was repeated in duplicate for each of the males. Live and dead eggs were counted during the time of incubation, dead eggs were removed and hatched fry were counted in each incubator.

Sperm motility and velocity recording and evaluation for individual males

Sperm activity was video-recorded using dark-field microscopy and velocity and motility were assessed 15 s after activation. Velocity in $\mu\text{m s}^{-1}$ and percentage of motility were analysed (see Linhart *et al.*, 2005).

Sampling and paternity analysis

Fry from the four progeny groups (G1-G4) were individually sampled into Eppendorf tubes filled with 96% ethanol, soon after hatching. Genomic DNA was extracted using the EZNA. Tissue DNA Kit (Peqlab GmbH). Microsatellite DNA fingerprinting of the four microsatellite loci *MFW1*, *MFW6*, *MFW7* and *MFW28* (Crooijmans *et al.*, 1997) was used for paternity analysis. 54 (G1) to 91 (G4) progenies per group were genotyped.

Statistical analysis

Descriptive statistics and ANOVA of fertilization and hatching control rates, sperm motility and velocity measurement were performed using Statistica 6.0 (StatSoft).

The effects of different sperm/egg ratios were evaluated by a likelihood-ratio chi-square test for homogeneity, where we evaluated if the contributions of each male under each of the conditions were consistent with the global contribution of each male under all conditions, corrected by the sample size for each condition. As some expected cell counts were lower than 5, we used a Monte Carlo estimate (100,000 samples) of the p-value for the chi-square tests (SAS-Freq).

On the global dataset (all sperm/egg ratio conditions merged), we tried to evaluate the possible effects of explanatory variables on male representation. The following four models were assumed:

Model 1, that predicts uniform distribution of progenies sired by each of the males ($p_{1i} = 0.2$ for each male i).

Model 2, that includes number of motile spermatozoa ($p_{2i} = M_i / \sum M_i$) with M_i the motility of sperm i in the first 15 s. With model 2, the number of offspring sired is expected to be proportional to the number of motile spermatozoa per male

Model 3, where velocity of motile spermatozoa is included ($p_{3i} = M_i V_i / \sum M_i V_i$) with V_i the velocity of spermatozoa from male i in the first 15 s. With model 3, the number of offspring sired is expected to be proportional to the cumulat-

ed distance covered in the first 15 s by the motile spermatozoa of each male.

Model 4, that includes the hatching rate of eggs fertilized by each male in a separate fertilization trial ($p_{4i} = H_i / \sum H_i$) with H_i the hatching rate of eggs fertilized by male i . With model 4, the number of offspring sired is expected to be proportional to the hatchability of eggs separately fertilized by each male.

The models were compared in a Bayesian approach using log-likelihood ratios. The log-likelihood ratio between models a and b was calculated as:

$$\log \left(\frac{P(X|\text{Model}_A)}{P(X|\text{Model}_B)} \right) = \sum_i N_i \log_{10} \left(\frac{p_{ai}}{p_{bi}} \right)$$

with X the observed data set, N_i the observed number of offspring from male i , \log_{10} the decimal logarithm and p_{ai} and p_{bi} the expected proportions of offspring sired in models A and B, respectively. The differences between models were estimated according to the scale given by Goodman (1999) for the Bayes factor, considering equal prior probability for each model: a value higher than 2 for the log-likelihood ratio was considered as strong evidence that Model A is more likely than Model B. Conversely, a value lower than -2 was considered as strong evidence that Model B was more likely than Model A.

Results and discussion

The males were chosen according to their genotypes to facilitate paternity assignment, and this resulted in choosing males with strongly differing sperm quality traits. Fertilization and hatching rates of control tests ranged from 23.67 to 94.82% and from 23.67 to 92.22%, respectively. Analysis of variance ($\alpha = 0.05$) did not show significant differences in this parameter for three of the spawners – M1, M2 and M5 (Fig. 1). Differences were reported in the sperm motility,

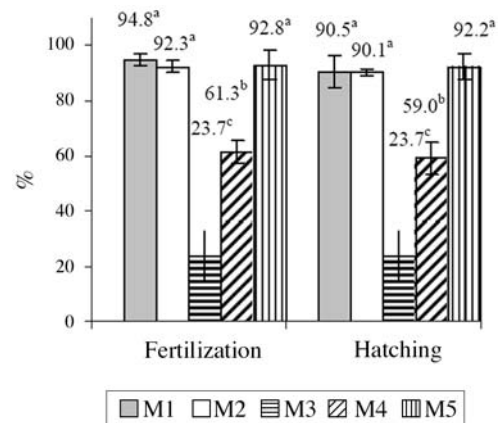


Figure 1. - Individual fertilization and hatching control rates of males M1-M5 when the rate of 100,000 spermatozoa per egg was used for fertilization. Groups of values with the same superscript do not differ significantly ($p > 0.05$).

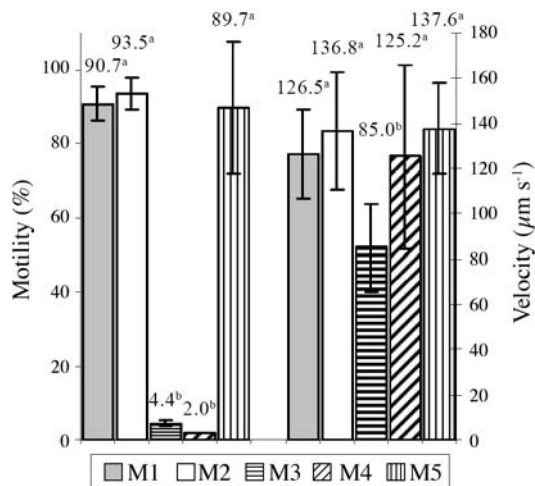


Figure 2. - Sperm motility and velocity of the individual males M1-M5 Groups of values with the same superscript do not differ significantly ($p > 0.05$).

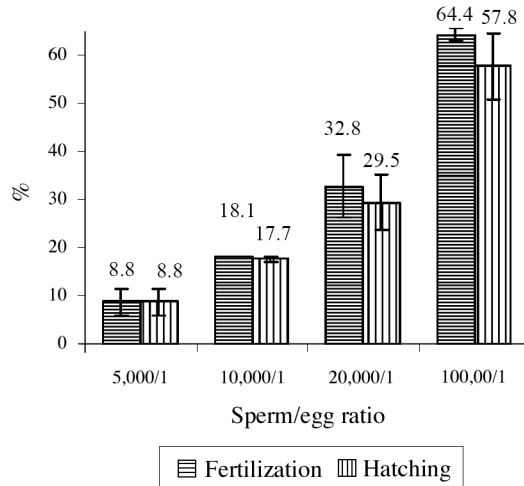


Figure 4. - Fertilization and hatching control rates for a heterosperm containing 20% of the spermatozoa from each of males M1-M5.

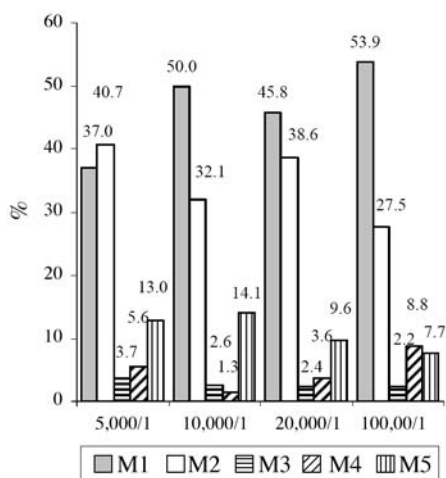


Figure 3. - Contribution of males in four groups of progenies constructed using four different heterosperm/egg ratios. Heterosperm was composed from 20% of the spermatozoa from each of five males, M1-M5.

ance did not report significant differences in four of the males – M1, M2, M4 and M5 (Fig. 2). Contribution of males in four groups of progenies is shown in figure 3. Fertilization and hatching rates of heterosperm for the 5 sperm/egg ratios tested are shown in figure 4.

Microsatellite genotyping of a total of 306 progenies across groups G1 to G4 showed that all of them could undoubtedly be assigned to one of the five males. Male contributions were highly disequilibrating in all fertilization conditions ($p < 0.0001$, Tab. I). The homogeneity test for contribution of males was not significant ($\chi^2 = 12.5$, d.f. = 12, $p > 0.4$), showing that the different number of spermatozoa per egg had no effect on the representation of the five males.

Likelihood ratios for comparison of the tested explanatory models for male representation after competitive fertilization are given in table II. The results show, not surprisingly, that all models (motility, motility and velocity, hatchability) are better than the uniform representation model, which, as we saw before, does not fit the data at all. Among all models,

sperm of two males showing a very low percentage of motile spermatozoa – M3 4.44%, M4 1.99%. Spermatozoa velocity ranged from 85.03 to 137.56 $\mu\text{m}\cdot\text{s}^{-1}$ and the analysis of vari-

Table I. - Chi-square tests for equi-representation of the 5 males in the four heterosperm/egg ratios tested.

	N spermatozoa/egg				all
	5000	10000	20000	100000	
N offspring scored	54	78	83	91	306
χ^2 value	33.6	67.6	70.3	81.7	241.5
d.f.	4	4	4	4	4
Probability	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Table II. - Log-likelihood ratios for the comparison of explanatory models for success of individual males, M1-M5. Model 1: Uniform distribution of progenies sired by each of males M1-M5 predicted; Model 2: Effect of sperm motility of individual males M1-M5; Model 3: Effect of motility and velocity of individual males M1-M5; Model 4: Effect of hatchability when equal sperm number (10,000 spermatozoa per egg) was used for fertilization from individual males M1-M5.

	Model 2	Model 3	Model 4
Model 1	29.7	26.6	27.0
Model 2	-	-3.2	-5.1
Model 3	-	-	-1.9

the best fitting model is Model 2, where motility is the explanatory variable. Models 3 and 4, where motility and velocity or hatchability are the explanatory variable, are very strongly less likely than Model 2 (log-likelihood ratio < -2). Model 4 is only moderately to strongly less likely than Model 3.

Conclusions

Contribution of individual males explored by DNA fingerprinting was very diverse in progenies, but was not influenced by the sperm/egg ratio, although variations in this ratio led to very different fertilization and hatching rates. Males with very low level of motility were represented by a low number of sired individuals, but striking differences were still found in the contribution of males with similar values for the sperm quality traits recorded.

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