FACULTY OF FISHERIES AND PROTECTION OF WATERS
UNIVERSITY OF SOUTH BOHEMIA IN ČESKÉ BUDĚJOVICE

Sperm competition in common carp (*Cyprinus carpio*)

Kompetice spermií u kapra obecného (*Cyprinus carpio*)

Vojtěch Kašpar

Vodňany, Czech Republic, 2010

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CHAPTER 1

GENERAL INTRODUCTION

1.1. SPERM COMPETITION

Sperm competition during the process of fertilization can be considered as part of a nechanism involved in sexual selection that occurs in wide range of animals (Birkhead and Møller, 1998; Birkhead and Pizzari, 2002). Competition led to wide range of behavioral, morphological and physiological adaptations that enhance the success of a male´s sperm relative to the sperm of rival (Parker, 1970). This phenomenon has an important effect on the reproductive behavior in many animals, including fish with external and internal fertilization.

Parker (1970) defined sperm competition as "*competition with a single female between the sperm from two or more males for the fertilization of ova*". More recently Parker (1998) re-defined it as "*competition between the sperm from two or more males for the fertilization of given set of ova*".

According to Parker (1970), males´ behavioural, morphological and physiological adaptations evolved to enhance the success of an individual's sperm against sperm of a rival male. These adaptations involve spermatozoa (through variation in size, number and structure); reproductive organs; behavior (such as aggression, courtship and mating); and social systems.

Parker (1997) defined sperm competition risk and sperm competition intensity. Sperm competition risk is defined as the probability that the ejaculates of the 2 different males will compete for access to a female's eggs whereas sperm competition intensity is number of males competing for the same clutch of eggs.

Sperm competition risk models (Parker et al., 1997) predict that if there is a low probability that the males' ejaculates will compete, individual males should invest less in each mating, whereas if the probability of competition is high, males should invest more (increase ejaculate size) in each mating (Parker et al., 1997; Parker, 1998). Under the sperm competition intensity models males are predicted to have smaller ejaculate sizes as the number of competing males increases beyond one competitor (Parker et al., 1996; Parker, 1998). According to theory of Evolutionary Stable Strategy, the optimal strategy for a male depends on the strategy adopted by other individuals in the population (Parker, 1996; Ball and Parker, 1996). Evolutionanary Stable Strategy predicts concepts of both inter-specific and intra-specific variation in male reproductive characteristics (Petersen and Warner, 1998).

Fertilization success of sperm is determined by the number of sperm, their quality in terms of motility and longevity when sperm of individual males fertilize eggs but competitive fertilizations and patterns affecting competitive fertilization are still not well understood.

Sperm competition in fish with external fertilization is rather rule than exception and sperm competition in fish, especially those with external fertilization, is represented by alternative mating strategies (Taborsky, 1994; 1998). Many descriptive and functional terms are used to describe these strategies (forum by Taborsky, 1997). There have been different concepts to the assessment of sperm competition in a variety of fish species. Methods to evaluate sperm competition include: studies of reproductive behavior and tactics, particulary in species with two life history strategies (Gross, 1985; Fuller, 1998; Evans and Magurran, 1999; Leach and Montgomerie, 2000; Vladic and Jarvi, 2001; Burness et al., 2004; Reichard et al., 2004), comparative studies of sperm competition across species with external fertilization, evaluation of the intensity of competition by the relative investment into gametes using the gonadosomatic index GSI (Stockley et al., 1997; Stoltz et al., 2005) and studies evaluating the reproductive success of spawners using DNA fingerprinting.

These studies have been performed both in natural spawning systems (Colbourne et al., 1996; Foote et al., 1997; Mjolnerod et al., 1998; Hoysak et al., 2004; Reichard et al., 2004) and using in vitro fertilization trials (Withler, 1988; Withler and Beacham, 1994; Gage et al., 2004; Linhart et al., 2005; Yeates et al., 2007). Sperm competition is present even in fish species with internal fertilization (Macias-Garcia and Saborio, 2004; Apsbury, 2007)

1.2 ALTERNATIVE MATING TACTICS AND LIFE STRATEGIES

Amazing diversity of fish (more than 25 thousands of species according to Nelson (1994), more than 27 thousands of species according to Eschemeyer (1998) is linked with very high diversity of mating and reproductive systems. Sperm competition, reproductive behavior and alternative mating tactics were subjects of considerable interest. Alternative mating tactics and different life strategies were reported in behavioral studies. Best studied examples of alternative mating tactics and life strategies are called "sneaker and guardner" which was reported for bluegill sunfish (Colbourne et al., 1996; Fu et al., 2001; Neff et al., 2003; Burness et al., 2004) and "parr and anadromous" reported for salmons (Vladic and Jarvi, 2001; Vladic et al., 2002). In case of "sneaker and guardner" strategy, two forms of males evolved. Sneakers are smaller males having disadvantaged temporal position, lower total volume of ejaculate but higher relative ammount of sperm. Guarders exhibit bigger males having dominant position in proximity of female. Alternative mating tactic named "parr and anadromous" was described for salmons, where dominant anadromous male is accomanying and defending female while younger parr males try to release sperm directly in a time of egg release.

Forum published by Taborsky (1997) offers description of functional and descriptive terms used in connection with alternative mating tactics. Same author named alternative mating tactics as "bourgeious" and "parasitic". Term "bourgeois" was used for males that monopolize resources as spawning sites and females and prevent parasitic males from enhancing reproductive success.

Studies of sperm competition confirmed that different patterns of sperm competition depend on anatomy of broodfish, GSI, sperm morphology (Petersen and Warner, 1998; Birkead and Moller, 1998).

Fact that younger, smaller and subdominat males produce relatively high-quality sperm in terms of sperm motility and velocity was many times reported (Vladic and Jarvi, 2001; Vladic et al., 2002; Evans et al., 2003; Burness et al., 2004; Rudolfsen et al., 2006). High-quality sperm of subdominat males is supposed to compensate disfavorate roles and position of males in competitive fertilization in nature.

Leach and Montogomerie (2001) found sperm of parental males of bluegill sunfish (*Lepomis macrochirus*) to have higher longevity than sperm of sneaking males but difference in this longevity was not significant.

Longer motility period of sperm of parental males in contrast to cuckolder sperm in same species was reported by Neff et al. (2003) and longevity of parental sperm was 40% longer.

Burness et al. (2004) observed traits of spermatozoa in buluegill sunfish and velocity and percentage of motile spermatozoa after activation of motility decreased in differently. Rates of decline differed between males of different tactics. Higher intracellular stocks of ATP in sneaker males were supposed and higher initial velocity of sperm cells was explained by trade-off between sperm velocity and longevity.

Parr males of Atlantic salmon (*Salmo salar*) were found to have higher spermatocrit than anadromous males and significant differences were observed between parr and anadromous males in percentage of motile sperm while no difference was reported in mean longevity and velocity (Vladic and Jarvi, 2001; Vladic et al., 2002).

Sperm competition was studied in European bitterling (*Rhodeus sericeus*) which spawns on the gills of mussels. Smith et al. (2004) reported males of bitterling to incerease inspection rate of mussels containing extract of testes containing sperm. Presence of sperm in mussels did not influence behavior of males leading females to spawning but males increased inspection rate of mussels containg extract of testis. Teritorial males ejaculated into mussels at low rate in the absence of competiting males but increased frequency of ejaculations in competition.

Morphological and behavioral traits in male success in copetition of males of bitterling was studied by Reichard et al. (2005). Altough dominant males monopolized females, subordinate males sired some offspring throug sneaking. Male dominance and intensity of colouration were found to be best predictors of competitive fertilization success.

1.3 INVESTMENT TO GAMETES

Theory of sperm competition sugests that gonadosomatic index (GSI, weight of gonads relative to the weight of individual) is growing with potential for sperm competition (Parker, 1990). Higher investment into gametes in species with higher risk of sperm competition revealed by higher GSI was also reported in amphibians (Gage, 1994), birds (Jennions et al., 1993) or mammals (Harcourt et al., 1995).

Inter-specific studies of sper competition in fish can be influenced by existence different male forms and species with two male forms incline to mask ability of GSI to reflect sperm competition intensity.

Stockley et al. (1997) reported significant positive correlation between the intensity of sperm competition and relative testis size reflected by GSI exhibiting higher investment into gametes in species with higher intensity of sperm competition. This study revealed data available for 89 fish species. Beside increase of GSI, significant positive correlation of sperm risk of sperm competition and number of sperm in ejaculate was reported (Stockley et al., 1997).

Allometric relationship between body mass and gonad mass and utilization of GSI as a parameter to evaluate sperm competition in fish was objective of work of Stoltz et al. (2005) who studied allometry in 23 species from different families and who pointed out that allometric growth is not rule.

According to Tomkins and Simmons (2002) using of GSI to evaluate intensity or risk of sperm competition is not correct because GSI has not isometric relationship between gonad mass and body mass in individual species. Relationship between gonad size and body size was not found to be isometric, negative allometric relationship was reported – larger individuals were reported to have relatively smaller gonads than smaller individuals.

Vladic and Jarvi (2001) observed testes size of Atlantic salmon (*Salmo salar*) with alternative male reproductive strategies – parr and anadromous males. They found parr males to produce gonads with weight reaching only 2% of weight of testes produced by anadromous males but having GSI more than two times higher (11.22 vs. 4.08%).

Investment to gametes was studied in internaly fertilizing sailfin mollies (*Poecilia latipina*) by Aspbury (2007). Males produced more sperm in the high-intensity treatment than in the low-intensity treatment.

Sailfin mollies in the risk present treatments expended more sperm than those males in the risk absent treatment and small males produced more sperm than medium and large males. In next species with internal fertilization – eastern mosquito fish (*Gambusia holbrooki*) males produce more sperm under high risk than under low risk (Evans et al., 2003).

1.4 MORPHOLOGY OR MOTILITY OF SPERMATOZOA AND SPERM COMPETITION

Sperm morphology is very simple in teleost fish (Jamieson, 1991). Spermatozoa of externaly fertilizing fish are released into the water and in case of freshwater teleost fish motility period is activated by decrease of osmotic pressure or by altering the concentration of specific ions such as potassium (e.g. in salmonids). Effect of ions on initiation of sperm motility in teleost fish was studied by Morisawa and Suzuki (1980). Period of motility is very short, typicaly from 30–120 s.

Sperm morphology in contrast to sperm competition was described in mammals (Baker and Bellis, 1988; Moore et al., 1999; Ramm and Stockley, 2010) but data related to fish are quite rare. Comparative analysis of sperm competition in fishes of Stockley et al. (1997) reported that sperm length negatively correlated with intensity of sperm competition and Burness et al. (2004) reported significant positive correlation between length of sperm flagella and velocity of sperm cells movement and negative correlation between sperm length and longevity. Taborsky (1998) summarized possibilities why sperm longevity can be favoured in competitive situations. First argument was that length that sperm travel before reaching the eggs is not fixed and the spatial coordination of mates may be greatly disturbed. As a second argument he used preovipositional shedding of sperm into a nest or spawning pit which may result in a higher fertilization potential (this is case of bitterlings – *Rhodeus sericeus* or *Rhodeus ocellatus*).

Significant difference in longevity was observed between individuals representing different reproductive strategies in bluegill sunfish (Neff et al., 2003). Parental males exhibited significantly longer period of motility than male forms named as sneakers and satellites but difference in sperm longevity reported by Leach and Montgomeri (2000) in two forms of same species of sunfish was not found to be significant.

1.5 REPRODUCTIVE SUCCESS DETERMINED BY DNA FINGERPRINTING

Paternity determination using microsatellite markers revealed that multiple males contributed sperm to most spawnings in cod (*Gadus morhua*) when 1,340 offspring and 102 spawnings distributed across a spawning season were analysed (Bekkevold et al., 2002). Results showed that multiple males contributed sperm to most spawnings but paternity frequencies were highly skewed among males, with larger males on average siring higher proportions of offspring. Reproductive success of males was dependent on the magnitude of the size difference between a female and a male.

Determination of paternity using allozyme markers was done to evaluate sperm competition in internaly fertilizing Amarillo fish (*Girardinichthys multiradiatus*). Females were exposed to two males and half of the females produced progeny sired by 2 males where one male always sired more than 70% or progeny. Difference in number of offspring sired was correlated to number of courtship displays with individual males (Macias-Garcia and Saborio, 2004).

Besides of evaluation of competitive success in natural conditions, determination of parentity or paternity was used for evaluation of fertilization success in experimental conditions or in vitro fertilization. Microsatellite markers were used for evaluation of competitive success in Atlantic salmon (*Salmo salar*) (Gage et al., 2004, Stockley et al., 2007) or silver catfish (*Rhamdia quelen*) (Ribolli and Zaniboni, 2010).

Biochemical markers – allozymes were used for paternity determination in experiments conducted in chinook salmon (*Oncorhynchus tschawytscha*) counting with hatchery induced sperm competition (Withler, 1998; Withler and Beacham, 1994).

1.6 HATCHERY INDUCED SPERM COMPETITION

As mentioned above, superior quality of sperm of subordinate males was reported in species with alternative mating tactics and two life strategies. High-quality of sperm of subordinate males may compensate their role in natural fertilization but these subordinate males can get advatige if used for hatchery propagation. Life strategy and reproductive behaviour has to be taken into account for design and strategy of artificial reproduction.

From the practical viewpoint of a hatchery manager, the extent of sperm competition is very important for the logistics of genetic programmes, because the common practice of pooling sperm from different males may have very detrimental effects if sperm competition levels are high (McKay and McMillan, 1991). Due to the potential for a high level of sperm competition, pooling sperm from different males may result in a much lower number of males being effectively represented in the offspring as numerically present in the competition, with predictable consequences of inbreeding and loss of genetic variability through genetic drift that such type of bottleneck is known to generate. This has to be taken into account – no matter if artificial reproduction is realised for captive breeding of progeny or restocking. The extent of the problem may be very different according to the use of the hatchery products. If the only aim of the hatchery production is to stock ponds or tanks for growing fish to commercial size, a loss of genetic variability is not necessarily a problem, as it will not accumulate over time. However, if the stock produced aims at restocking natural populations or at being the basis of a selective breeding or domestication program (i.e. if the products are to be used as broodstock), then the loss of genetic variability will accumulate over time, together with inbreeding, and quickly produce negative effects on the performance of the population.

Sperm competition under hatchery conditions, when using pooled sperm, was confirmed in salmonids (Gharrett and Shirley, 1985; Withler, 1988; Withler and Beacham, 1994; Gage et al., 2004).

Contributions of males of chinook salmon (*Oncorhynchus tschawytscha*) to progeny was tested when pooled milt was used for fertilization (Withler, 1998). Paternity was determined by biochemical markers when four sets of three males used for fertilization of eggs pooled from two or three females. Potency (as a fertilization rate) of individual males was observed in contrast to fertilization rates of control fertilization and spermatocrit values and was not correlated.

Withler and Beacham (1994) used biochemical markers again to determine paternity of progeny in competitive trials in chinook salmon where equal volumes of sperm of each male were used for fertilization of batch of eggs. Individual males sired from 5 to 83% of progeny in two competitive trials when mixture of sperm was used for fertilization immediately after pooling. Pooled sperm was kept for 15 and 60 minutes and competitive trials were repeated.

There was no difference in relative male contributions in fertilization after 15 minutes of storage but relative contributions of males were more equilibrated when fertization realized after 60 minutes of storage.

Gage et al. (2004) observed sperm competition in Atlantic salmon using 18 experimental trials – 18 males in 9 pairs were used to fertilize eggs of 9 males – firstly using equal volumes of sperm and using volumes in a ratio 2:1 in an attempt to clarify which of spermatozoal traits is responsible for competitive fertilization success (more below in next chapter).

Effect of sequentioal adition of semen was tested (Withler and Beacham, 1994) and position of male in fertilization seqence showed to be determinant of fertilization success in competitive trials. Sequentional adition of milt was studied by Yeates (2007) showed that 2 second delay in application of milt of delayed male caused only very poor representation of delayed male in competitive trials in Atlantic salmon (*Salmo salar*).

It appears that the outcome of sperm competition is not necessarily linked to its fertilization success when used separately in hatching control test, neither to spermatocrit or sperm longevity (Withler, 1988; Gage et al., 2004).

Pooled sperm was used for fertilization in Atlantic halibut (*Hippoglossus hippoglossus*) and sperm competition was studied (Ottesen et al., 2009). Sperm of four males was pooled to have mixtures containg equal number or equal volume of sperm and batches of eggs from two females were fertilized. Number of spermatoza and percentage of motile sperm showed to be correlated with relative fertilization success in all sperm/egg ratios tested.

Wedekind et al. (2007) reviewed outcome of sperm competition under conditions of in vitro fertilization in Alpine whitefish (*Coregonus zugensis*) using parameter of number of effective males. Not controlling for sperm density or milt volume in design of artifical reproduction at a constant population size decreased the number of effective males represented in progeny by around 40–50%.

Above mentioned studies showed that sperm competition is a reality, and can lead to highly disequilibrated progeny numbers between males when using pools of sperm or sequential addition of sperm. But pooling of sperm is rather rule than exception in hatchery practise protocols and pools of sperm are used for fertilization in an attempt to minimize effect of males with low quality on success of hatchery propagation and to minimize work load for hatchery managers.

1.7 MALES' TRAITS – PREDICTORS OF FERTILIZATION SUCCESS

Sperm vary in morphology, behavior (speed, trajectory, and motility) and longevity, but the mechanism for this variation is poorly understood (Cosson et al., 1999). Above mentioned studies of hatchery induced competition of Withler (1998) and Withler and Beacham (1994) included spermatocrit measurement only. The effect of sperm traits on fertilization success during *in vitro* fertilization was also studied (Gage et al., 2002, 2004; Linhart et al., 2005; Liljedal et al., 2008).

Velocity of male's sperm relative to the velocity of spermatozoa of the competiting male was reported as a best predictor of male fertilization success in salmonids (Gage et al., 2004; Liljedal et al., 2008).

Experiment of Gage et al. (2004) was conducted using 18 experimental trials, first 9 with equal volumes of sperm next 9 using volumes in a ratio 2:1 to reveal which of spermatozoal traits is primary determinant of fertilization success. Sperm traits were recorded and paternity of progeny was determinated by microsatellite markers.

Results showed that males with faster sperm sired higher proportion of progeny and sperm velocity owershadowed sperm numbers in competitive trial.

Sperm competition was studied in Arctic charr (*Salvelinus alpinus*) by Liljedal et al. (2008). Male to male competitive trials were performed with ballanced number of sperm cells from competiting males. Sperm motility was videorecorded and evaluated and data showed that velocity of a male´s sperm relative to the velocity of spermatozoa of the competiting male´s sperm was the best predictor of fertilization success. Paternity was determined using microsatellite markers. Genotypes of spawners were compared and fertilization success of males sharing more microsatellite alleles with female was higher.

Linhart et al. (2005) studied sperm competition in common carp using 36 male to male competitive trials. Sperm concentration estimates were used to compute equilibrated doses of spermatozoa. Colour of progeny determined paternity when codominantly inherrited colour markers and control hatching rates were used to check potency of every male.

Spermatozoal traits were recorded and analyzed and sperm quality in terms of sperm motility and velocity of spermatozoa explained only part of the variation in male competitive ability – link between sperm velocity and subsequent hatching rate in a controlled hatching test was not reported, whereas motility did affect hatching rate. Recorded sperm parameters – spermatocrit, motility, velocity of movement, single trial fertilization success had an impact on males´ potency in common carp, but that none of these can be considered as the main explanatory variable. Moreover, even with all parameters combined, a large proportion of the variance among males competitive success in sperm competition remained unexplained (Linhart et al., 2005). This is in contrast with data reported in salmonids, which showed sperm velocity to be the primary determinant of sperm competition success (Gage et al., 2004; Liljedal et al., 2008).

Experiment of Ottesen et al. (2009) studied sperm competition in Atlantic halibut (*Hippoglossus hippoglossus*) when pooled sperm was used for fertilization. Male having highest percentage of motile sperm cells with highest velocity sired majority of progeny in competition trials.

1.8 MALES' SECONDARY SEX TRAITS AND COMPETITION

Expression of secondary sex traits was linked to competitive ability of sperm (Liljedal et al., 2008) and association between sperm quality and male ornamentation was reported (Kortet et al., 2004).

Immnocompetence handicap hypothesis of sexual selection of Folstad and Karter (1992) suppose that male sexual ornamentation is produced and maintained by elevated levels of immunosuppresive androgens and signal of sperm quality. Males with more pronounced sex ornamentation are suppoused to produce sperm of better quality but may be more susceptible according to this theory. This was studied in roach (*Rutilus rutilius*) where males with more pronounced sexual ornamentation produced sperm with better longevity but sperm concentrations and and velocity of movement were not significantly different (Kortet et al., 2004).

Sperm potency in individual fertilization tests in Alpine whitefish (*Coregonus zugensis*) was not found to be significantly correlated to occurrence of breeding tubercles or condition factor of males (Wedekind et al., 2007).

Coloration of males of Arctic charr (*Salvelinus alpinus*) was tested as a factor that might influence competitive success in paired tests but velocity of male´s sperm relative to the velocity of the competing male´s sperm showed to be best predictor of male fertilization success.

But brightly colored males from pair had the lowest fertilization probability and paler males were suppposed to produce more competitive sperm than more colored males (Liljedal et al., 2008).

The extent of of red iris colouration in bitterling (*Rhodeus sericeus*) had not significant effect on male dominance or reproductive success and parasite load has no effect on colouration of males (Reichard et al., 2005).

1.9 OVARIAN FLUID INFLUENCE ON SPERM TRAITS

Sperm traits of externally fertilizing fish species are typically measured in fresh or salt water depending on the typical spawning environment. Released ova contain ovarian fluid. In several species of externally fertilizing fishes, ovarian fluid has been documented to influence both sperm velocity and longevity (Litvak and Trippel, 1998; Turner and Montgomerie, 2002; Elofsonn et al., 2003; Woolsey et al., 2006; Hatef et al., 2009).

Enhanced sperm longevity, motility and swimming speed in Arctic charr (*Salvelinus alpinus*) was reported when 50% of ovarian fluid was present in activating medium (Turner and Montgomerie, 2002). Percentage of motile sperm and the duration of sperm motility in rainbow trout (*Oncorhynchus mykiss*) were both higher when sperm were activated in solution of 50% ovarian fluid compared to freshwater (Woolsey et al., 2006).

Sperm of Atlantic cod (*Gadus morhua*) activated in ovarian fluid swam faster and higher percentage of sperm cells was motile for longer period compared to standard activation by sea water (Litvak and Tripell, 1998).

Rosengrave et al. (2009) measured sperm traits of chinook salmon (*Oncorhynchus tshawytscha*) in both fresh water and diluted ovarian fluid and reported that spermatozoa swam faster, had both higher percent of motility and a straighter path trajectory for a longer period of forward motility when activated in ovarian fluid compared with fresh water. Relevance of data on sperm motility measured in fresh water as an index of male´s sperm quality is discussed by Rosengrave et al. (2009): previous reports of swimming speeds for fish spermatozoa activated in water underestimates sperm swimming speeds under natural conditions and overall use of sperm motility, swimming speed and linearity measured in freshwater is inaccurate index of male´s sperm quality.

Ovarian fluid was reported to influence velocity and longevity of sperm in Arctic charr (Turner and Montgomerie, 2002; Urbach et al., 2005) but effect of ovarian fluid on sperm velocity vas dependent on male-female combination. This finding is sugesting a possibility of cryptic female choice or interaction between sperm and ovarian fluid based on compatibility (Urbach et al., 2005).

Rosengrave et al. (2009) found that sperm traits were variable and dependent on individual male/female combinations when sperm activation was realised using solution of ovarian fluid and suggested that ovarian fluid could be a mechanism of cryptic female choice.

In rainbow trout (*Oncorhynchus mykiss*) Dietrich et al. (2007) reported that ovarian fluid enhanced sperm motility in terms of sperm velocity and trajectory of movement, not percentage of motile cells. Females producing ovarian fluid with motility enhancement capacity were reported and authors presumed existence of cryptic female choice in this species.

Exceptional influence of ovarian fluid presence in activating medium was reported for sperm of three-spined stickleback (*Gasterosteus aculeatus*). Sperm cells are usually motilie for very short period but sperm of stickleback activated by medium containing 25% of ovarian fluid was motile after 7 h (Elofsson et al., 2003).

Similar experiment with sticklebacks revealed even very low concetrations as 0.75 or 1.56% prolonged sperm motility, concentration of 6.25% prolonged duration of motility of sperm cells to more than one hour (Elofsson et al., 2006). Effect of artificial ovarian fluid was explored in this experiment and sperm showed same response to artificial ovarian fluid, manitol solution of osmolarity similar to ovarian fluid prolonged to more than 30 minutes.

THE AIM OF THIS THESIS WAS:

- *To evaluate effect of equalization of sperm numbers in pooled sperm for practical reproduction in common carp. Pooled sperm is used on hatcheries for many reasons but consequences of this common practice on males´representation in progeny were not described. Fertilization with equal volume and equal number of sperm in pool of sperm were realised and males´ representation in progeny was determined thanks to paternity assessment based on genotyping of progeny by microsatellite markers. Propability of explanatory models of males´ competitive success based on spermatozoal traits of competiting males was evaluated.*

- *Study sperm competition and its conseqences in different conditions of in vitro fertilization. Fertilization success of individual males in competitive situation concerning different conditions in terms of sperm/egg ratio was evaluated and explanatory models of competitive success of males based on spermatozoal traits were studied. Similairly to first aim mentioned above, propability of explanatory models based on spermatozoal traits of competiting males was evaluated.*

- *To evaluate consequences of hatchery induced sperm competition in common carp using parameter of number of effective males N_{em}. Hatchery induced sperm competition cause unequal representation of males in progeny when pool of sperm of different males is used for fertilization of batch of eggs which results in reduction of the effective size of the populations and a loss of genetic variability.*

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CHAPTER 2

EQUALIZING SPERM CONCENTRATIONS IN A COMMON CARP (*CYPRINUS CARPIO*) SPERM POOL DOES NOT AFFECT VARIANCE IN PROPORTIONS OF LARVAE SIRED IN COMPETITION

Kaspar, V., Kohlmann, K., Vandeputte, M., Rodina, M., Gela, D., Kocour, M., Alavi, S.M.H., Hulak, M., Linhart, O., 2007. Equalizing sperm concentrations in a common carp (*Cyprinus carpio*) sperm pool does not affect variance in proportions of larvae sired in competition. Aquaculture 272 S1, S204–S209.

RUNNING HEAD: COMPETITION IN CARP SPERM

EQUALIZING SPERM CONCENTRATIONS IN A COMMON CARP (*CYPRINUS CARPIO*) SPERM POOL DOES NOT AFFECT VARIANCE IN PROPORTIONS OF LARVAE SIRED IN COMPETITION

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ABSTRACT

Proportions of offspring from five common carp contributing to a sperm pool composed of equalized sperm concentrations (N-progeny) or equal sperm volumes (V-progeny) were each compared to a uniform distribution. Four microsatellite markers (*MFW1*, *MFW6*, *MFW7*, *MFW28*) were used to determine the paternity of the progeny. The homogeneity of offspring numbers from each male for the two types of progeny, were tested using an exact test for the likelihood-ratio chi-square. Numbers of offspring in the progeny groups were highly variable (0.4–50% in V-progeny, 2.4–41.2% in N-progeny) and highly significantly different as shown by Pearson chi-square statistics (χ^2 = 189, 4 d.f., $p < 0.0001$ in V-progeny and χ^2 = 139, 4 d.f., $p < 0.0001$ in N-progeny). Significant heterogeneity between treatments ($p < 0.05$) together with reduction of χ^2 value from 189 to 139 showed that equalization of sperm concentration reduced heterogeneity in numbers of offspring. Number of sperm per male, sperm motility (71–98%), sperm velocity (97–155 μm s^{-1}) and control hatching rates (81–91%) all affected the observed number of offspring sired by each of the males, but the high variability in proportion of progeny among the 5 males remained unexplained.

Keywords: fish, common carp, Cyprinus carpio*, sperm, competition, males, microsatellite markers, aquaculture*

1. INTRODUCTION

Sperm competition occurs when sperm from different males compete for fertilization (Parker, 1970). This form of post-copulatory sexual selection is recognized as a significant and widespread force in the evolution of male reproductive biology and as a key determinant of differential male reproductive success (Birkhead and Moller, 1998).

Mating systems among fish are very diverse. Fish show one of the widest ranges of sperm competition intensity of any animal group (Petersen and Warner, 1998). Sperm competition in fish, especially those with external fertilization, can occur by alternative mating strategies (Taborsky, 1994, 1998). These have been described as "bourgeois" males and parasitic spawning (Taborsky, 1994) and sexual parasitism (Kortet et al., 2004). Alternative mating strategies and mating behavior have been studied in several fish species (Reichard et al., 2004a, 2004b; Kortet et al., 2004; Burnes et al., 2004; Gage et al., 1995). Theory suggests that increased sperm competition is achieved by higher [relative] investment into gamete production (Parker, 1990). Comparative study of fishes with external fertilization revealed a positive correlation between gonadosomatic index I_g and intensity of sperm competition (Stockley et al., 1997).

Sperm vary in morphology, behavior (speed, trajectory, and motility) and longevity, but the mechanism for this variation is poorly understood (Cosson et al., 1999). Paternity analysis based on microsatellite DNA fingerprinting revealed that, for Atlantic salmon, relative sperm velocity was the primary determinant of fertilization success and that sperm longevity correlated negatively with competition success (Gage et al., 2004).

The effect of sperm competition during *in vitro* fertilization, has been studied (Gage et al., 2002, 2004; Linhart et al., 2005). Linhart et al. (2005) identified progenies in the common carp (*Cyprinus carpio* L.) by dominant wild-green and recessive gold markers (Katasonov, 1973, 1978), 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 males used ($P < 0.001$, $R^2 = 0.91$). The best male sired an average of 88% of the offspring in its competition, while the worst male sired only 5%. Sperm quality explained only part of the variation in male competitive ability. There was no link between sperm velocity and subsequent hatching rate in a controlled hatching test, whereas motility did affect hatching rate in the same experiment (Linhart et al., 2005).

The objective of this study was to determine if equalizing individual sperm concentrations (N-progeny) in a sperm pool derived from five common carp males would reduce the variance in number of progeny compared to a pool comprising equal volumes (V-progeny) of semen from the same 5 males. Offspring were assigned to their sires *a posteriori* based on microsatellite markers.

2. MATERIALS AND METHODS

BROODSTOCK HANDLING AND COLLECTION OF GAMETES

The first part of the experiment was done at the experimental station of the Department of Fish Genetics and Breeding, Research Institute of Fish Culture and Hydrobiology University of South Bohemia, Vodnany, Czech Republic. Experiments were performed in accordance with EU and Czech Republic legal requirements.

Preliminary genotyping of [available] females and males was performed with four microsatellite markers (for methods see below). Candidate males were selected so that each would express discriminatory alleles on at least one locus. One female and five males were chosen based on their genotypes (Table 1).

Fish were maintained in a pond before the experiment, then they were stocked separately in $4m^3$ hatchery tanks with a water flow rate of 0.2 l s^{-1}, a temperature of 18–22 °C and 6–7 mg l^{-1} O_2.

Fish were anesthetized before handling with a 1:1,000 aqueous solution of 2-phenoxyethanol. Males were injected with carp pituitary extract at 1 mg kg^{-1}, 24h before stripping. The female was injected with the same extract at 0.4 mg kg^{-1} 24h before stripping and then again with 2.1 mg kg^{-1} 12h before stripping. Semen was collected from each male and the samples stored in a thin layer in cell culture vessels under aerobic conditions at 0 °C. Sperm concentrations of each of 5 males were estimated microscopically (Olympus BX 41) at 400x using a Burker cell hemocytometer. The female was stripped, and the ova stored under aerobic conditions at 17–19 °C during the time that sperm were being counted.

Table 1. *Genotypes of five males, strain 435 and one female, strain Amur.*

Spawners		Genotypes			
No.	**Sex**	**MFW 1**	**MFW 6**	**MFW 7**	**MFW 28**
M1	Male	211–211	150–158	199–199	306–306
M2	Male	169–219	128–158	197–203	294–298
M3	Male	211–225	150–154	195–271	292–296
M4	Male	169–219	158–158	197–203	306–306
M5	Male	217–217	150–154	195–261	294–294
F 1	Female	217–223	136–162	191–253	–

FERTILIZATION BY EQUAL VOLUME OF SPERM (V-PROGENY)

Two hundred g of ova (800 ova g^{-1}) were placed into a one liter dish and an 'equal volume' sperm pool was added. This sperm pool comprised 1.4 ml containing $32x10^9$ sperm prepared from 280 µl of sperm from each of the five males (V-progeny), giving a final concentration of 200,000 sperm per ova for fertilization. Hatchery water (200 ml) was added to activate the gametes.

FERTILIZATION BY EQUAL NUMBER OF SPERM (N-PROGENY)

Two hundred g of ova (800 ova g^{-1}) were placed into a one liter dish and an 'equal number' sperm pool was added. This sperm pool comprised 1.4 ml containing $32x10^9$ sperm prepared from equal numbers of sperm ($6.4x10^9$ $male^{-1}$) from each of the same five males (N-progeny), based on the estimated sperm concentrations. This produced 200,000 sperm per ova for fertilization. Hatchery water (200 ml) was added to activate the gametes.

INCUBATION

After 2 min of gamete activation with hatchery water, egg stickiness was eliminated with a milk solution, containing 40 g l^{-1} milk powder, over a period of 60 min. The ova from each of the two trials were incubated to hatching in Zug jars for 4.5 days at 22 °C.

HATCHING CONTROL OVA

Samples of 5 g of ova were fertilized with sperm from each of five males using equal sperm volumes (7 µl male^{-1}, V-progeny) and equal sperm numbers (0.16x10^{9}, N-progeny). This was repeated in quadruplicate for each male. The dishes were placed on an orbital agitator (200 rpm, 10 mm deflection). Gametes were activated with 5 ml of hatchery water, and after 2 min samples of approximately 100 ova were transferred to Petri dishes and placed in an incubator supplied with UV-sterilized and dechlorinated tap water (22 °C, 9 mg l^{-1} O_2).

Live and dead eggs were counted in each incubator during incubation and dead eggs were removed. Hatched fry were counted after 5 days of incubation at 22 °C. The control hatching rate was calculated as the ratio of hatched larvae to the initial number of eggs incubated.

SPERM MOTILITY AND VELOCITY RECORDING

Sperm activity was videorecorded using dark-field microscopy (Olympus BX 50; stroboscopic lamp Strobex 9630, Chadvick-Helmut, USA) to evaluate motility and velocity. Using a CCD video camera (Sony, SSC-DC50AP), the microscopic field was transferred to a video monitor and recorded with a S-VHS system (Sony, SVO-9500 MDP). The strobe frequency was set to automatic register with video frames (50 Hz) for sperm velocity measurement. Motility and velocity were examined at 20x objective magnification immediately after mixing 1 µl of sperm (prediluted sperm with immobilizing solution 1:200; Linhart et al., 2005) with 49 µl of distilled water + 0.1% BSA, on a glass slide pre-positioned on the microscope stage. The final dilution was 1:10,000. The BSA was added to prevent sperm heads from sticking to the glass slide. Within 10 s after mixing, a video recording was made for 2 min for the evaluation of sperm swimming activity. The focal plane was positioned near the glass slide surface.

SPERM MOTILITY AND VELOCITY EVALUATION

Velocity and motility were assessed 15 s after activation. The successive positions of the videorecorded sperm heads were analyzed from video frames by means of Olympus MicroImage software (Version 4.0.1. for Windows with a special macro by Olympus C & S). Velocity and percentage motility were calculated from sperm head positions on five successive frames with three different colors (frame 1 red, frames 2–4 green and frame 5 blue). Twenty to 40 sperm were counted for each frame. Sperm that moved were visible in three colors, while non-moving sperm were white. The percentage of motile sperm was calculated from the number of white and red cells. Sperm velocity was calculated as µm s^{-1} based on length traces of sperm from blue to green and red heads, calibrated for magnification.

CARP LARVAE SAMPLING

We took 250 individuals from each fertilization group (N-progeny and V-progeny) after hatching and placed them in 1.5 ml Eppendorf tubes filled with 96% ethanol. Samples were stored in a refrigerator at 4 °C to prevent evaporation of ethanol prior to DNA extraction.

PATERNITY ANALYSIS

Paternity analysis was carried out at the Leibnitz-Institute of Freshwater Ecology and Inland Fisheries, Dept. of Inland Fisheries, Berlin. Genomic DNA from whole fry was extracted using the E.Z.N.A. tissue DNA Kit (Peqlab, Germany). Microsatellite DNA fingerprinting of four microsatellite loci *MFW1*, *MFW6*, *MFW7* and *MFW28* (Crooijmans et al., 1997) was used for paternity analysis. Conditions for PCR amplification of the four loci did not differ from those described by Crooijmans et al. (1997). PCR was carried out on total volumes of 15 µl. Products were checked on an agarose gel and then used for fragment analysis with an automated sequencing device (CEQ 8000; Beckman Coulter). Because two different colors of primer labeling were used and lengths of amplified products differed, PCR products could be combined into two duplexes. Finally, due to the pre-selection of males according to their genotypes, segregation of alleles at these four loci enabled reliable paternity determination of the 500 progeny.

STATISTICAL ANALYSIS

During this experiment, data from concentration estimates and control hatching rates were collected. From the record of sperm activity, data describing sperm motility and velocity were obtained.

The proportions of offspring from each of the five males in the N- and V-progeny trials were compared to a uniform distribution. As some cell counts were lower than 5, we used a Monte-Carlo estimate (100,000 samples) of the exact test for the Pearson chi-square statistic. Homogeneity of offspring number between the N- and V-progeny was tested using an exact test for the likelihood-ratio chi-square. These analyses were performed with SAS-Freq®.

Several models were also used to predict expected values of percentage offspring sired per male. For the V-progeny, the following four models explain the proportional contribution of males to number of progeny:

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

Model 2, includes number of sperm ($p_{2i} = C_i / \sum_i C_i$) where C_i is the initial sperm concentration of each male i.

With this model the offspring sired were expected to be proportional to the number of sperm per male.

Model 3, includes number of motile sperm ($p_{3i} = C_i M_i / \sum_i C_i M_i$) with M_i the motility of sperm i in the first 15 s.

With this model the offspring sired were expected to be proportional to the number of motile sperm per male.

Model 4, includes velocity of motile sperm ($p_{4i} = C_i M_i V_i / \sum_i C_i M_i V_i$) with V_i the velocity of sperm from male i in the first 15 s.

With this model the offspring sired were expected to be proportional to the cumulative distance covered in the first 15 s by the motile sperm of each male.

In N-progeny, initial numbers of sperm were equalized, so C_i=constant and therefore *Model 1* and *Model 2* described for V-progeny were equivalent. The other two models describing [contribution] of males in progenies were the same.

The models were compared using log-likelihood ratios. The log-likelihood ratio between models A and B was calculated as:

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

with X the observed data set, N_i the observed number of offspring from male i, and p_{ai} and p_{bi} the expected proportions of offspring sired in models A and B, respectively.

Motility and sperm velocity parameters used for log-likelihood estimates were also analyzed by ANOVA (SAS Proc GLM, Student Newman-Keuls test for comparison of means).

3. RESULTS

Hatching rates for the control demonstrated the high quality of sperm and eggs used for the competition experiment (Table 2), which corresponded with 90% hatching in N- and V-progeny experiments. Initial sperm concentrations (17.97–34.53x10^9 sperm ml^{-1}), percentage of sperm motility (71.3–98.4%), sperm velocity (96.8–154.9 μm s^{-1}) and control hatching rates (80.89–90.8%) were evaluated for five males (Table 2). The control hatching rate was not significantly different among the five males, but both motility and sperm velocity differed, with male 4 showing the lowest values. This may be connected with the very low levels of progeny sired by male 4, which contributed only 0.4% of V-progeny and 2.4% of N-progeny competitions.

Table 2. *Initial sperm concentration, sperm motility, velocity and control hatching rates for 5 common carp. Groups with a common superscript do not differ significantly (P > 0.05).*

Male No.	Initial sperm concentration x 109 ml^{-1}	Motility %	Velocity μm s^{-1}	Control hatching rate %
M1	34.53	94.97 ab	135.58 ab	85.89 a
M2	22.19	98.34 a	154.95 a	90.79 a
M3	24.06	98.44 a	152.60 a	80.93 a
M4	23.75	71.29 b	96.79 c	87.55 a
M5	17.97	88.37 ab	140.44 bc	87.95 a

Segregation of alleles in four loci allowed determination of paternity in all 500 individuals sampled. Individual males achieved a contribution of 0.4–50% in V-progeny and 2.4–32% in N-progeny competitions (Figure 1). In both N- and V-progeny competitions the contribution of the five males expressed by Pearson chi-square statistic were not uniform (χ^2 = 189, 4 d.f., P < 0.0001 for V-progeny and χ^2 = 139, 4 d.f., P < 0.0001 for N-progeny). The contributions of the males were not homogeneous across progeny types (P < 0.05), showing an effect of the equalization of numbers of sperm per male used for fertilization of ova in N-progeny.

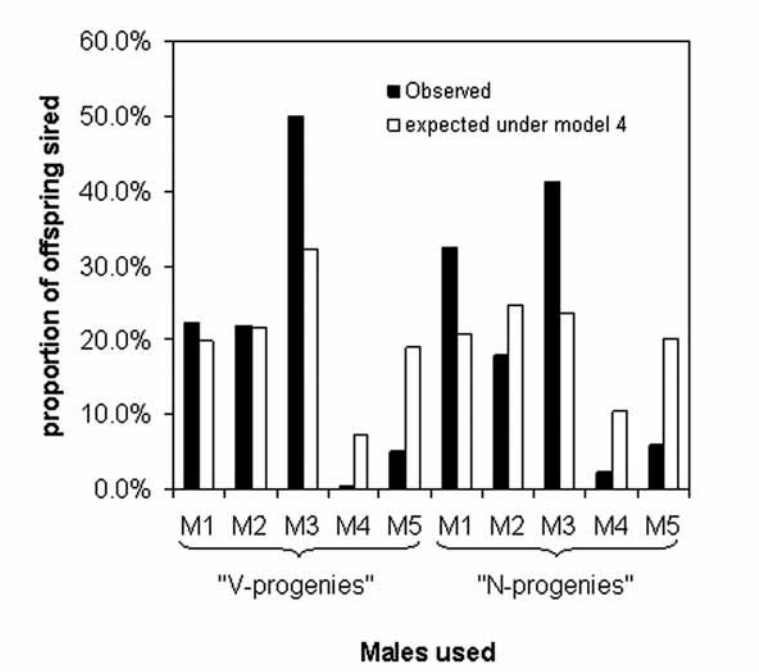

Figure 1. *Proportions of offspring sired by 5 competing male common carp when sperm quantities were equalized by volume (V-progeny) or by number of sperm per male (N- progeny). The observed proportions were compared to the expected proportions using Model 4, where the males were expected to sire offspring in proportion to the cumulative distance covered by motile sperm (taking account of number of sperm, and their motility and velocity).*

Comparison of the explanatory models including sperm concentration, motility and velocity showed that all models were different (Table 3), as the minimum log-likelihood ratio was greater than 5, meaning that *Model 4* was at least 100,000 times more probable than *Model 3*, from the observed data. The best model to describe the data was *Model 4* including numbers of sperm, their motility and velocity.

Chi-square goodness of fit analysis comparing observed numbers of offspring per male and expected values using *Model 4* was highly significant ($I < 0.0001$, 4 d.f., $\chi^2 = 66$ in V progenies, $\chi^2 = 93$ in N progenies), showing a large unexplained residual variability in males contributions.

Contributions of males were different in V- and N-progeny competition. Together with the decrease in χ^2 values (189 in V, 139 in N), this showed that the equalization procedure reduced the heterogeneity in male representation, which nevertheless remained far from homogeneous ($P < 0.0001$). Numbers of sperm, their motility and velocity contributed to the observed number of offspring per male, but a very high unexplained variability remained.

Table 3. *Log-likelihood ratios for the comparison of explanatory models for fertilization success of sperm from 5 competing male common carp (Model 1 = equal fertilization from each male, Model 2 = fertilization proportional to number of sperm, Model 3 = fertilization proportional to number of motile sperm, Model 4 = fertilization proportional to distance covered by motile sperm), in trials where initial sperm quantities were equalized by volume (V-progeny) or by number of sperm per male (N-progeny).*

		Model A				
		V-progenies			*N-progenies*	
		Model 2	Model 3	Model 4	Model3	Model4
	Model 1	15.5	21.6	26.9	6.6	11.7
Model B	Model 2		5.9	11.3	*	*
	Model 3			5.4		5.2

**Model 2 not relevant for N-progeny, as number of sperm per male was equalized*

4. DISCUSSION

Oviparous fish species such as common carp have a reproductive strategy in which a very short period of sperm reaction is needed to achieve fertilization. Here, when compared to other competitive experiments on fish with external *in vitro* fertilization, we attempted to use not only the same quantity of sperm from rival males, but also sperm with relatively similar (good) motilities. When the effect of sperm number is suppressed, then according to the literature on fish with external *in vitro* fertilization, fertilization success reflects sperm motility (Lahnsteiner et al., 1998) and sperm velocity (Gage et al., 2002, 2004). Surprisingly, the variation in number of sired offspring was very high and cannot be explained only by number of sperm, motility and velocity. On the other hand in male 4 the sperm velocity and motility clearly seem connected with the very low proportion of offspring sired (0.4% in V-progeny and 2.4% in N-progeny). Results from this male confirm that low velocity and motility correspond to low numbers of sired offspring. Other studies have not always shown a positive effect of velocity, e.g. the highest sperm velocities were associated with reduction of fertilization rate in rainbow trout (Lahnsteiner et al., 1998).

The application of an adequate sperm:egg ratio for high fertilization and hatching rates was an important parameter in our experimental design. In classical fertilization studies, an optimal sperm: egg ratio is used for discrimination of fertilization effect (Billard and Cosson, 1992), and where the fertilization and hatching success is used as a potential predictor of sperm quality. In the present study, the control hatching rates ranged from 81 to 91%, motility was more than 71% (71 to 98%) and velocity was higher than 97 μm s^{-1} (97 to 155 μm s^{-1}; Table 2), proving the good quality of sperm from individual males. Numbers of fresh sperm ranging from 9,000 to 30,000 per ovum are required for minimal *in vitro* fertilization in common carp, but the practical quantity of sperm recommended for artificial propagation is 100,000–200,000 (Billard et al., 1995; Linhart et al., 2003). We used 200,000 sperm per ovum for the N- and V-progeny experiment. The rates of sperm and ovum dilution with activating medium (water, activation solution) during the process of fertilization are other variables that must be well-adjusted. A sperm dilution ratio of 1:143 (sperm:water) was used with an ovum dilution ratio of 1:1. Both rates were suitable for artificial propagation of common carp in previous studies (Linhart et al., 2003, 2005). The present results showed that equalization of sperm by volume or by sperm numbers does not allow the production of equal numbers of progeny per male in a competitive situation, and leaves a wide range of unexplained variability in competition success.

In the Atlantic salmon, sea urchins and fowl, the same is observed, and in these cases, sperm containing faster sperm are the most efficient (Gage et al., 2004; Levitan, 2000; Birkhead et al., 1999). In Atlantic salmon, sperm number is not the primary determinant of sperm competition success (Gage et al., 2004), in contradiction to the theory of competition (Parker, 1982) and comparative (Stockley et al., 1997) and experimental (Martin et al., 1974) findings in other species (Gage et al., 2004). In a common carp competition experiment the male effects alone explained 91.4% of the observed variance, consisting of 17.1% explained by sperm motility and 32.5% by control hatching rates in single fertilizations. Undetermined male effects explained 41.8%. The velocity of sperm had no effect on the outcome of sperm competition in this study (Linhart et al., 2005).

Finally, having sperm with low velocity and motility, as in male 4 (Table 2), may explain the low level of sired offspring in V- and N-progeny trials and consequently its low ability in competition with other males, although all males used here had different but good motilities. When different sperm parameters were analyzed by comparing the likelihood ratios of different models, it appeared that the variance in larvae sired in competition could be explained partly by differences in sperm motility, initial sperm concentration and sperm velocity.

There remains a large unexplained component of the variance in proportions of larvae sired in competition. Such phenomena can be explained by male to male competition but, as Birkhead and Møller (1998) have pointed out, such simple mechanisms potentially belie much more complex male-female interactions. Such phenomena can also be explained by mechanisms which result in the differential utilization of sperm, depending on the recognition of gametes according to their immunological competition (Wedekind et al., 1995; Zeh and Zeh, 1997).

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CHAPTER 3

IN VITRO STUDY ON SPERM COMPETITION IN COMMON CARP (*CYPRINUS CARPIO* L.)

Kaspar, V., Hulak, M., Kohlmann, K., Vandeputte, M., Rodina, M., Gela, D., Linhart, O., 2008. In vitro study on sperm competition in common carp (*Cyprinus carpio* L.). Cybium 32 (2), 303–306.

IN VITRO STUDY ON SPERM COMPETITION IN COMMON CARP (*CYPRINUS CARPIO* L.)

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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 competitive success of five males using heterosperm with equal number of sperm of each male and four different sperm/egg ratios 5,000, 10,000, 20,000 and100,000 spermatozoa per egg to understand competitive fertilization succes of different males. The role of percentage of motile sperm and sperm velocity were studied.

Fertilization and hatching rates of control tests of individual males with 100,000 spermatozoa per egg were measured in a range 23.7–94.8% and 23.7–92.2%, respectively. Sperm velocity and percentage of motile sperm were measured in range of 85.0 to 137.6 μm s^{-1} and 2.0 to 93.5% at 15 sec post sperm activation, respectively. Contribution of individual males explored by DNA fingerprinting was very diverse in progenies. Males with very low level of sperm motility (M3 4.45%, M4 1.95%) were represented by low contribution in all groups of progenies. Significant differences in the contribution of males were also found among individuals of similar percentage of motile spermatozoa. Probability of several models to explain contribution of males in progenies was tested.

Keywords: aquaculture, artificial reproduction, paternity assignment

1. INTRODUCTION

Sperm competition occurs when sperm from different males compete for fertilization (Parker, 1970). Sperm competition was explored in many behavioral studies in several fish while works aimed to the sperm competition in controlled conditions of in vitro fertilization in aquaculture important species are quite a rare (Gage et al., 2004, Linhart et al., 2005). Theory of sperm competition suggests that increased intensity of sperm competition leads to the higher relative investment of competitive males into gamete production (Parker, 1990). In a case of 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, explained only part of the variation in male competitive success.

The aim of presented study was to evaluate competitive success and effect of spermatozoal parameters of five males in a competitive trial in contrast with studies observing sperm competition mentioned above (Gage et al., 2004; Linhart et al., 2005). Experiment was designed to include different sperm/egg ratios for an in vitro fertilization (5,000; 10,000; 20,000 and 100,000 spermatozoa per egg) and equal number of spermatozoa from each of the males entering the competition trial.

2. 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. Whole the combination of the female and five males was selected to enable paternity assignment using four microsatellite loci. Males and female were artificially reproduced according Linhart et al. (2005).

Sperm was collected individually and stored under aerobic conditions at 0 °C. Sperm concentrations of each of 5 males were estimated using a Burker cell hemocytometer. The female was stripped and the ova were stored under aerobic conditions at 17–19 °C. After the sperm concentration estimates, part of sperm was stored individually for fertilization and hatching control tests and additional part was used for preparation of heterosperm composed from 20% number of spermatozoa of each of five males.

FERTILIZATION

Five grams of ova (800 ova.g^{-1}) were placed into a four dish on an orbital agitator volume of a heterosperm adequate to the ratios of 5,000 (G1), 10,000 (G2), 20,000 (G3) and 100,000 (G4) spermatozoa per egg was added. Gametes were activated on an orbital agitator (200 rpm, 10 mm deflection) by adding a 5 ml of hatchery water. After 2 min of mixing approximately 100 ova were transferred to Petri dishes and placed in an experimental incubator supplied with UV-sterilized and dechlorinated tap water (22 °C, 9 mg l^{-1} O_2).

FERTILIZATION AND HATCHING CONTROL FOR INDIVIDUAL MALES

Samples of 5 g of ova were fertilized with sperm from each of five males using equal sperm (100,000 spermatozoa per egg). This was repeated in duplicate for each of males. The dishes were placed on an orbital agitator, activated and incubated as described in the text above. Live and dead eggs were counted in each incubator during the time of incubation and dead eggs were removed and hatched fry were counted after incubation.

SPERM MOTILITY AND VELOCITY RECORDING AND EVALUATION FOR INDIVIDUAL MALES

Sperm activity was videorecorded using dark-field microscopy (Olympus; stroboscopic lamp, a S-VHS system Sony; see Linhart et al., 2005) and examined at 20x objective magnification immediately after mixing 1 µl of 1:200 prediluted sperm (imobilizing solution) with 49 µl of distilled water + 0.1% BSA, on a glass slide pre-positioned on the microscope stage. Velocity and motility were assessed 15 s after activation. Velocity at µm s^{-1} and percentage of motility were analysed (Olympus software) and calculated from sperm head positions on five successive frames of videorecord.

SAMPLING AND PATERNITY ANALYSIS

The fry was individually sampled into Eppendorf tubes filled by 96% ethanol soon after the hatching. Genomic DNA was extracted using the E.Z.N.A. tissue DNA Kit (Peqlab GmbH). Microsatellite DNA fingerprinting of four microsatellite loci MFW1, MFW6, MFW7 and MFW28 (Crooijmans et al., 1997) was used for paternity analysis. Conditions for PCR amplification of the four loci did not differ from those described by Crooijmans (1997). Based on two different colors of PCR primer labeling and different lengths of amplified products, PCR products were combined into two duplexes for a fragment analysis with an automated sequencing device (CEQ 8000; Beckman Coulter).

3. STATISTICAL ANALYSIS

Descriptive statistics and analysis of variance (ANOVA) of the data from fertilizing and hatching control rates, sperm motility and velocity measurement was performed by Statistica 6.0 (StatSoft).

The effect of the different sperm/eggs ratio was evaluated by a likelihood-ratio chi-square test for homogeneity, where we evaluated if the contributions of each male in each of the conditions were consistent with the global contribution of each male in 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 males representation. The following four models were assumed:

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

Model 2, that includes number of motile spermatozoa ($p_{2i} = M_i / \sum_i M_i$) with M_i the motility of sperm i in the first 15 s.

With model 3, 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 ($p_{3i} = M_i V_i / \sum_i M_i V_i$) 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 cumulated 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 ($p_{4i} = H_i / \sum_i H_i$) 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:

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

with X the observed data set, Ni the observed number of offspring from male i, log10 the decimal logarithm and pai and pbi the expected proportions of offspring sired in models *A* and *B*, respectively. The differences between models were appreciated 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 a strong evidence that Model *A* is more likely than model *B*. Conversely, a value lower than – 2 was considered a strong evidence that model *B* was more likely than model *A*.

4. RESULTS AND DISCUSSION

Because of the nuisance to use well selected combination of males, the males for the competition trials differed in parameters of sperm finally. 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 (ά = 0.05) did not show significant differences in this parameters for three of the spawners – M1, M2 and M5 (Figure 1). Differences were reported in the sperm motility, sperm of two males showed very low percentage of motile spermatozoa – M3 4.44%, M4 1.99%. Spermatozoa velocity ranged from 85.03 to 137.56 $\mu m.s^{-1}$ and the analysis of variance did not reported significant differences in four of males – M1, M2, M4 and M5 (Figure 2). Contribution of males in four groups of progenies is shown by figure (Figure 3). Fertilization and hatching rates of heterosperm in a sperm/egg ratios of experimental in vitro fertilization are showni in figure. (Figure 4).

Males contributions were highly disequilibrated in all fertilization conditions ($P < 0.0001$, Table 1). 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 5 males.

Likelihood ratios for the comparison of the tested explanatory models for male representation after competitive fertilization are shown in Table 2. The results show, not surprisingly, that all models (motility, motility and velocity, hatchability) are better than the uniform representation model, which, we saw before, does not fit the data at all. Among all models, the best fitting model is Model 2, where motility is the explanatory variable. Models 3 and 4, where motility*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.

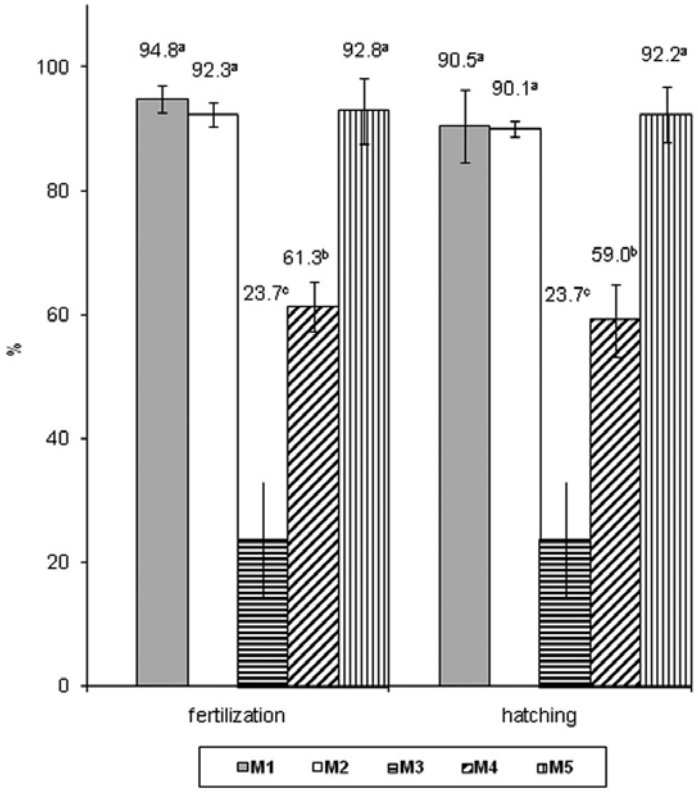

Figure 1. *Individual fertilization and hatching control rates of males M1–M5 when the rate of 100,000 spermatozoa per egg was used for fertilization. Groups of values with a same superscript do not differ significantly (P > 0.05).*

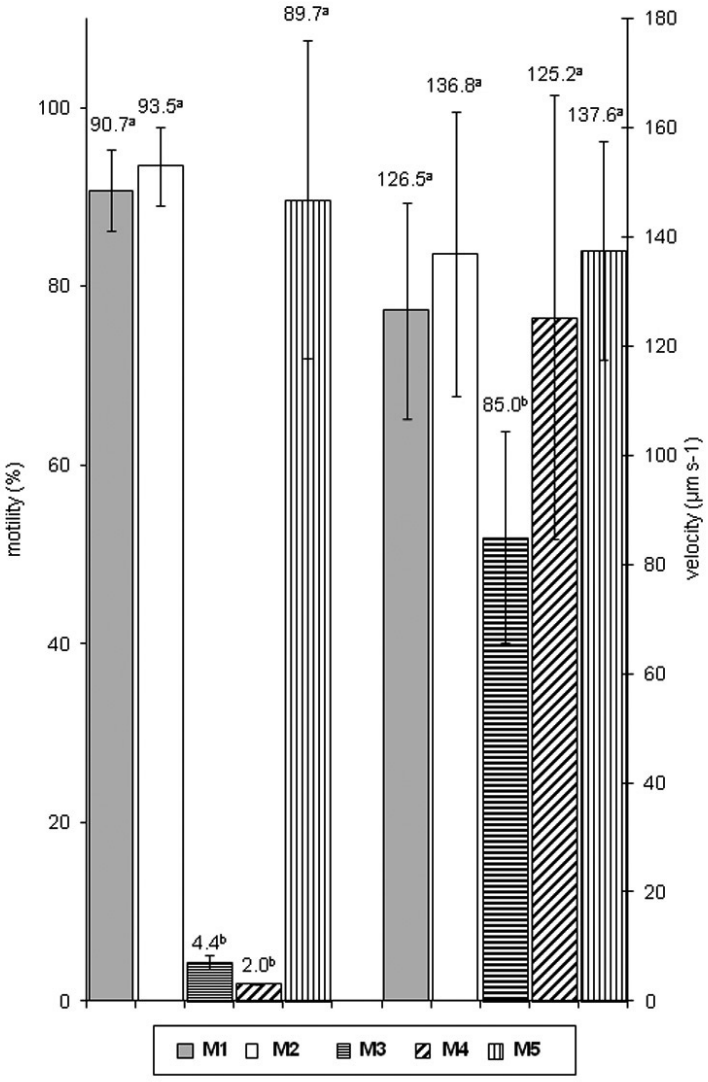

Figure 2. *Spermmotility and velocity of the individual males M1–M5 Groups of values with a same superscript do not differ significantly (P > 0.05).*

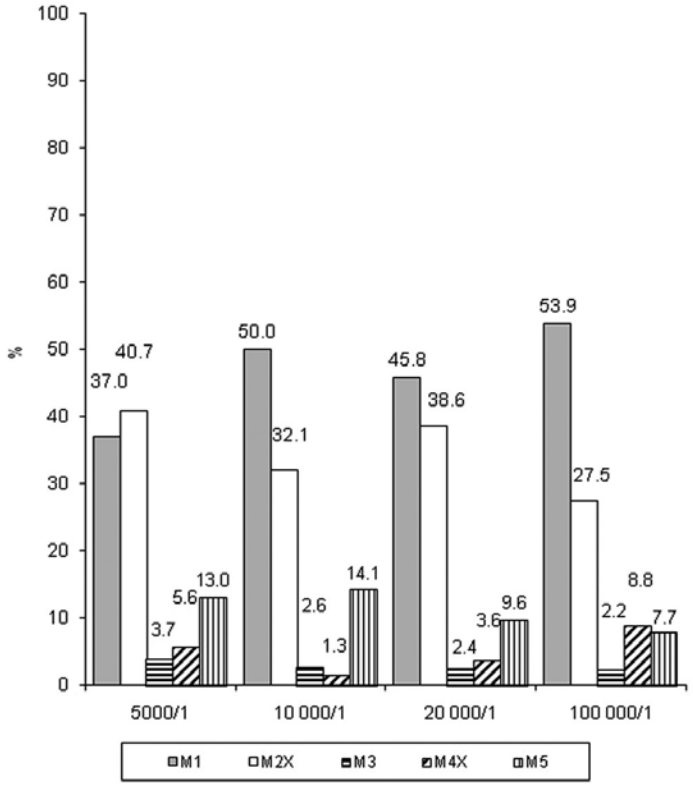

Figure 3. *Contribution of males in four groups of progenies constructed using four different heterosperm/egg ratios (5,000; 10,000; 20,000 and 100,000). Heterosperm was composed from 20% number of spermatozoa of each of five males, M1–M5.*

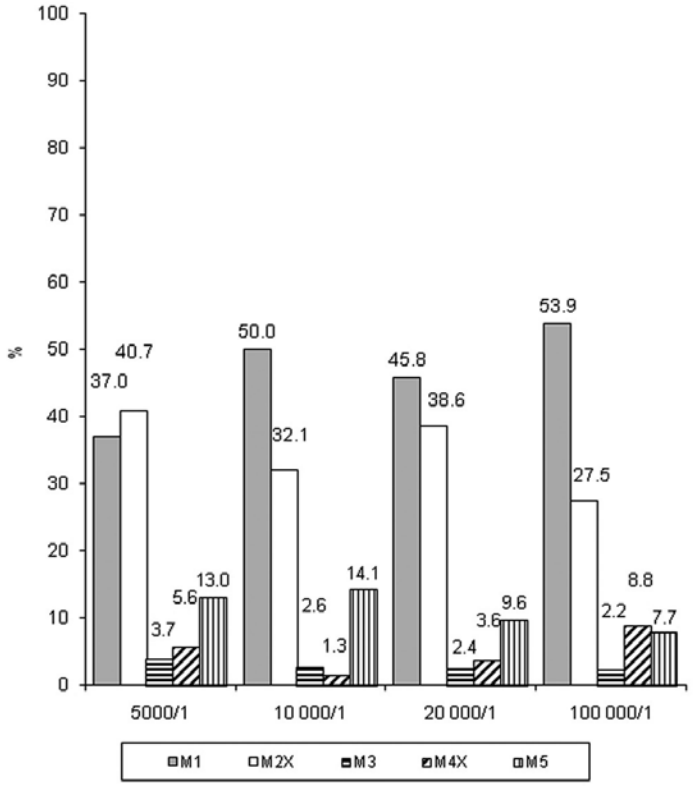

Figure 4. *Fertilization and hatching control rates for a heterosperm containing 20%number of spermatozoa of each of males M1–M5.*

Table 1. *Chi-square tests for equi-representation of the 5 males in the four heterosperm/eggs ratios tested.*

	N spermatozoa/egg				
	5,000	10,000	20,000	100,000	all
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 2. *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

5. CONCLUSIONS

Contribution of individual males explored by DNA fingerprinting was very diverse in progenies. Males with very low level of motility were represented by low number of sired individuals, but striking differences were found in a contribution of males with similar level of all the observed factors.

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CHAPTER 4

A PROPOSAL AND CASE STUDY TOWARDS A CONCEPTUAL APPROACH OF VALIDATING SPERM COMPETITION IN COMMON CARP (*CYPRINUS CARPIO* L.), WITH PRACTICAL IMPLICATIONS FOR HATCHERY PROCEDURES

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A PROPOSAL AND CASE STUDY TOWARDS A CONCEPTUAL APPROACH OF VALIDATING SPERM COMPETITION IN COMMON CARP (*CYPRINUS CARPIO* L.), WITH PRACTICAL IMPLICATIONS FOR HATCHERY PROCEDURES

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SUMMARY

Pooled sperms are used in hatcheries to reduce the impact of sperm of low quality and in an attempt to maintain genetic variability. This paper reviews sperm competition experiments that were conducted in common carp (*Cyprinus carpio* L.), and evaluates the genetic consequences of sperm competition under conditions of *in vitro* fertilization by variance in the effective number of males N_{em}. Whereas fertilization using equal volumes of sperm from each of five males resulted in 42.4% reduction in N_{em}, using pool of sperm with equal number of spermatozoa reduced the loss in N_{em} to 34.9%. Other two experiments implied only competition tests with equal numbers of spermatozoa per male - mean reduction in N_{em} of 28.4% was reported within 36 male-to-male competion challenges and four fertilizations by different sperm:egg ratios generated mean reduction in N_{em} of 42.2%. Lower number of males effectively represented in the hatchery progeny result in inbreeding and loss of genetic variability so that management procedures able to reduce this effects are discussed.

1. INTRODUCTION

It has been postulated that sperm competition during the process of fertilization can be considered as part of a nechanism involved in sexual selection that occurs in wide range of animals (Birkhead and Møller, 1998; Birkhead and Pizzari, 2002). This common phenomenon has an important effect on the reproductive behavior in many animals, including fish with external and internal fertilization.

Parker (1970) defined sperm competition as "*competition with a single female between the sperm from two or more males for the fertilization of ova*". More recently he re-defined it as "*competition between the sperm from two or more males for the fertilization of given set of ova*" (Parker, 1998). Furthermore, Parker et al. (1996) also distinguished between the risk and the intensity of sperm competition. *The risk of sperm competition* refers to the probability that a male will spawn with at least one other male competing for a batch of eggs, whereas *the intensity of sperm competition* refers to the number of males whose sperm are competing for a batch of eggs.

Sperm competition in fish, especially those with external fertilization, is represented by alternative mating strategies (Taborsky, 1994; 1998). Many descriptive and functional terms are used to describe these strategies (forum by Taborsky, 1997). There have been different concepts to the assessment of sperm competition in a variety of fish species. Methods to evaluate sperm competition include: studies of reproductive behavior and tactics, particulary in species with two life history strategies (Gross, 1985; Fuller, 1998; Evans and Magurran, 1999; Leach and Montgomerie, 2000; Vladic and Jarvi, 2001; Burness et al., 2004; Reichard et al., 2004), comparative studies of sperm competition across species with external fertilization, evaluation of the intensity of competition by the relative investment into gametes via the gonadosomatic index GSI (Stockley et al., 1997; Stolz et al., 2005) and studies evaluating the reproductive success of spawners using DNA fingerprinting. These studies have been performed both in natural spawning systems (Colbourne et al., 1996; Foote et al., 1997; Mjolnerod et al., 1998; Hoysak et al., 2004; Reichard et al., 2004) and using *in vitro* fertilization trials (Withler, 1988; Withler and Beacham, 1994; Gage et al., 2004; Linhart et al., 2005; Kaspar et al., 2007; Yeates et al., 2007; Kaspar et al., submitted;).

From the practical viewpoint of a hatchery manager, the extent of sperm competition is very important for the logistics of genetic programmes, because the common practice of pooling sperm from different males may have very detrimental effects if sperm competition levels are high (McKay and McMillan, 1991). Due to the potential for a high level of sperm competition, pooling sperm from different males may result in a much lower number of males being effectively represented in the offspring as numerically present in the competition, with predictable consequences of inbreeding and loss of genetic variability through genetic drift that such type of bottleneck is known to generate. Still, the extent of the problem may be very different according to the use of the hatchery products. If the only aim of the hatchery production is to stock ponds or tanks for growing fish to commercial size, a loss of genetic variability is not necessarily a problem, as it will not accumulate over time. Still, some advocate that a higher genetic variability helps to reduce the spread of diseases in a population (Springbett et al., 2003). However, if the stock produced aims at restocking natural populations or at being the basis of a selective breeding or domestication program (i.e. if the products are to be used as broodstock), then the loss of genetic variability will accumulate over time, together with inbreeding, and quickly produce negative effects on the performance of the population. This effect may have quite strong consequences, like hatchery strains which perform less in a farm environment than wild strains, as seen in tilapia in Asia (Eknath et al., 1993).

This paper is not an original contribution but conceptually reviews sperm competition experiments that were conducted with common carp (*Cyprinus carpio* L.), and evaluates the genetic consequences of sperm competition in hatchery practice. Management procedures able to reduce these effects are discussed.

2. THE DETERMINANTS OF SPERM COMPETITION IN COMMON CARP

Sperm competition under hatchery conditions, when using pooled sperm, was confirmed in salmonids (Gharrett and Shirley, 1985; Withler, 1988; Withler and Beacham, 1994; Gage et al., 2004). These studies showed that sperm competition is a reality, and can lead to highly disequilibrated progeny numbers between males when using pools of sperm. It appears that the outcome of sperm competition is not necessarily linked to its fertilization success when used alone in fertilization control tests, neither to spermatocrit or sperm longevity (Withler, 1988; Gage et al., 2004).

However, sperm velocity is correlated with competition success in Atlantic salmon (Gage et al., 2004), and is considered to be a good predictor of sperm potency when equal numbers of spermatozoa per male are used in a pool (Wedekind et al., 2007).

In common carp it seems to be different. In a first experiment involving a series of male-to-male sperm competition trials, we showed that sperm competition was high, but that sperm velocity had no impact on the outcome of sperm competition (Linhart et al., 2005). On the contrary, both sperm motility (percentage of motile spermatozoa) and fertilization success in single trials could explain part of the differences between male potencies. Sperm motility accounted for 17.1% of the variance between males, and fertilization success in single trials for 32.5%, still leaving 41.8% of the variance between males unexplained.

In a second experiment conducted with sperm pooled from five males, spermatocrit values, sperm motility, sperm velocity and hatching rate in separate tests all had an impact on the male potency. However, this still left a high proportion of the variation between the males unexplained (Kaspar et al., 2007).

A third experiment, also with sperm pooled from five males, showed that the sperm motility seems to be the main determinant of male potency (Kaspar et al., submitted). However, in this particular study, sperm motility was very low (< 5%) in two of the males used. This is expected to magnify the effect of motility on potency, when compared to other trials where all males had a high motility (> 70%). In the latter experiment, by fertilizing different egg batches with 5,000; 10,000; 20,000 and 100,000 spermatozoa per egg, using the same pool of 5 males, we also tested possible impact of the total number of spermatozoa per egg in the sperm pool on male potency. Undoubtedly it was demonstrated that total sperm concentration had no impact on male potency.

From the above it can be concluded that all recorded sperm parameters (spermatocrit, motility, velocity, single trial fertilization success) could have an impact on male potency in common carp, but that none of these can be considered as the main explanatory variable. This is in contrast to salmonids, where velocity seems to be the primary determinant of sperm competition success (Gage et al., 2004). Moreover, even with all parameters combined, a large proportion of the variance among males competitive success in sperm competition remains unexplained (Linhart et al., 2005; Kaspar et al., 2007).

3. GENETIC CONSEQUENCES OF SPERM COMPETITION

One of the main practical problems caused by sperm competition is unequal representation of males in hatchery progeny when pooled sperms are used for fertilization. This results in reduction of the effective size of the populations and a loss of genetic variability (e.g. McKay and McMillan, 1991; Wedekind et al., 2007). Pooled sperm are frequently used in hatcheries to reduce the impact of males with low sperm quality on the overall fertilization rate, allowing the use of several males in a single fertilization in an attempt to maintain genetic variability.

The basic parameter that is modified by unequal male representation, linked to the amount of genetic variance lost at the next generation, is the effective number of males, N_{em}. This can be calculated as:

$$N_{em} = (N_m k_m - 1)/(k_m - 1 + V_m/k_m) \qquad (1)$$

Where Nm is the number of males used, km the mean number of progenies per male, and Vm the variance of the number of progeny per male (Kimura and Crow, 1963). If all males have the same potency, any offspring has an equal chance to originate from each of the males used, and the number of progeny per male is expected to follow a Poisson distribution. In a Poisson distribution, the variance and mean are equal, then $k_m = V_m$. This simplifies equation 1 to:

$$N_{em}=N_m-1/k_m \quad (2)$$

The latter will give the expected effective number of males if all males had the same reproductive success.

The above equations were used to estimate the expected and true values of N_{em} in the mentioned 3 experiments on sperm competition in carp:

- *In the first experiment (Linhart et al., 2005) 36 competition challenges between 2 males were performed with a total of 14,720 offspring recorded. The theoretical N_{em} in the absence of competition was 2.0 on average, and the true N_{em} was 1.43 on average (1.02–1.98). This resulted in a mean reduction in N_{em} of 28.4%.*
- *In the second experiment (Kaspar et al., 2007), where equal volumes of sperm from 5 males were mixed and 250 offspring were evaluated, the theoretical N_{em} was 4.98, and the true N_{em} was 2.87, representing a 42.4% reduction.*
- *In Kaspar et al. (2007) four trials were conducted with the same pool from 5 males, using different sperm:egg ratios. The expected N_{em} was 4.93 on average, and the true N_{em} ranged from 2.68 to 3.21 (mean 2.85), showing a mean reduction of 42.2%.*

The above show that using pooled sperm leads to a severe reduction of the effective number of males used. This can generate important reductions in genetic variability, if this technique is used in hatchery propagation of common carp broodstock. Similar calculations, using data published by Withler (1988) and Withler and Beacham (1994) in chinook salmon, give a mean reduction in N_{em} equal to 31.4% (13–52%), which is quite comparable to our results found for common carp.

4. ALLEVIATING THE GENETIC CONSEQUENCES OF SPERM COMPETITION

We now know that sperm competition arising from fertilization using pooled sperm can have severe consequences on the genetic variability in populations of common carp, the question arises of how to reduce these negative effects. With current hatchery practices in mind, the first recommendation would be to equalize the volume of sperm used from each male. In salmonids ignoring of this practice is expected to generate losses in N_{em} in the range of 40–50% (Wedekind et al., 2007). Use of equal volumes of sperm from each male was tested in Kaspar et al. (2007), and generated a 42.4% reduction in N_{em}. In the same experiment, we also tried to set up a pool of sperm with equal number of spermatozoa per male, compensating spermatocrit differences by using unequal volumes of sperm from each male. Although this reduced the loss in N_{em} (-34.9% instead of -42.4%), the value remains high. Moreover, the other two experiments (Linhart et al., 2005; Kaspar et al., 2007) both involved only competition tests with equal numbers of spermatozoa per male, and we previously found this to still generate high reductions in N_{em}. In chinook salmon, Withler and Beacham (1994) showed that holding a pool of sperm for 60 minutes prior to use allowed a good, if not total re-equilibration of male contributions.

Using the N_{em} calculation approach with the data of Withler and Beacham (1994) the initial reduction in N_{em} when the pool was used immediately was 32.8% on average, whereas it was only 7.6% when the pool was held for 60 minutes prior to fertilization. This approach was not tested in common carp, but could be interesting to assess in future, using more males than were previously tested in salmon (only 3 males were used, which is lower than the number practically used in hatcheries, which is 5 at least for a batch of eggs by *in vitro* fertilization in common carp).

Another possibility to reduce the consequences of sperm competition in common carp would be to divide eggs into batches equal to the number of males used and to fertilize them separately before combining them for incubation. This was tested in common carp in two experiments, where the males were identified with microsatellite markers. In the first experiment 24 males were crossed with 10 females in a full-factorial design (see Vandeputte et al., 2004 for details). The number of offspring assigned to parents was 524, the theoretical N_{em} was 24.0, and the true N_{em} was 23.0, resulting in a 4.2% reduction. In a second experiment, where 147 males were crossed with 8 females in a full factorial design (more details in Kocour et al., 2007), 615 offspring were assigned to parents. The theoretical N_{em} was 146.7, and the true N_{em} of 124.9 represents a reduction of 14.9%. Thus, by using separate fertilizations of egg batches, it is possible to obtain male representations in progeny that are close to the optimum. For the time being, in the absence of a good predictor of a male's potency, the above approach could be recommended for the propagation of carp broodstocks in hatcheries.

The above is in line with theoretical studies which showed that factorial designs can reduce the variance in family sizes (Busack and Knudsen, 2007). Moreover, apart from the reduction of the variance in family sizes, factorial mating designs are also known to improve the conservation of genetic variance, either with or without selection (Dupont-Nivet et al., 2006), making their use even more beneficial. With a good predictor of a male's individual potency available, the use of factorial designs produced with pools of sperm could be an option. However, in the absence of such predictors, the use of separate fertilizations to produce factorial designs is highly recommended for the propagation of carp broodstocks in hatcheries. However, as outlined before, these recommendations are essential for hatchery batches that will generate the future broodstock for the next generation, but not necessarily for the mass production of fingerlings for stocking grow-out ponds, which should be much less affected by a reduction in genetic variability.

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CHAPTER 5

GENERAL DISCUSSION ◆ ENGLISH SUMMARY ◆ CZECH SUMMARY ◆ ACKNOWLEDGEMENTS ◆ LIST OF PUBLICATIONS ◆ TRAINING AND SUPERVISION PLAN DURING STUDY ◆ CURRICULUM VITAE

GENERAL DISCUSSION

Hatchery induced sperm competition, extent of sperm competition and consequences of design of artificial propagation are very important for management of genetic programmes. Knowledge of extent of sperm competition and its effect on genetic variability of progeny resulting from different designs of artificial reproduction and hatchery practice is very important for management of genetic resources or captive broodstocks, fish breeding programmes or production of stocking fish. Pooling of sperm i.e. induction of sperm competition is common practice on hatcheries and this is sometimes paradoxicaly done in an attempt to enhance genetic variability of progeny. Sperm from several males is applied simultaneusly or sequentialy to prevent low fertilization rate which may result in loss of eggs in case of bad quality of sperm or male infertility.

In general, relative production of gametes is very high in fish with external fertilization, especially in species of high commercial interest. Limited potential of hatchery for keeping of broodfish may result in low number of individuals used for practical reproduction. In adition, sperm volume obtained from one idividual is very high and sperm can be stripped repeatedly from same males in many comercially important species. Protocols for artificial reproduction of species of commercial interest are well established which allows effective use of obtainded gametes. If the number of male individuals is limited and sperm quality in terms of sperm motility is unknown, pooled sperm is used for fertilization in an attempt to reduce effect of sperm of bad quality and to enhance high fertilization and hatching rate.

Low number of individuals creating next generation may reduce the genetic variability of progeny itself and pooling of sperm from different males is additional factor affecting genetic variability of progeny. From the practical viewpoint of a hatchery manager, the knowledge of extent of sperm competition is very important for management of breeding programmes and maintanace of genetic resources, because the common practice of pooling sperm from different males may have very detrimental effects if sperm competition levels are high (McKay and McMillan, 1991).

Common carp (*Cyprinus carpio*) is leading species of aquaculture in Czech Republic with total annual production reaching 18,000 tons according to statistics of Czech Fish Farmers Association. Numerous local breeds of common carp have been developed and several common carp breeds have been introduced in the past. Audit of common carp breeds revealed 12 original local breeds to lost or crossbred and nine local breeds considered to be pure (Pokorny et al., 1995). These are kept in Live Gene Bank under National Programme of Conservation of Genetic Resources. Artificial propagation of common carp is well established (Billard et al., 1995; Linhart et al., 2003).

The objective of first study (Kaspar et al., 2007; Chapter 2) was to determine if equalizing individual sperm numbers in a sperm pool of five common carp males would reduce the variance in number of progeny compared to a pool comprising equal volumes of semen from the same 5 males. Second study (Kaspar et al., 2008; Chapter 3) was done in an effort to compare competitive success of five males using heterosperm with equal number of sperm of each male and four different sperm/egg ratios 5,000; 10,000; 20,000 and 100,000 spermatozoa per egg to understand competitive fertilization success of different males. Sperm concentrations were evaluated in both experiments to enable equalization of sperm contributions of all males in fertilization trials.

Sperm/egg ratio 200,000 spz/egg was applied for fertilization with equalized number of spermatozoa. Sperm/egg ratio from 9,000 to 30,000 is required for minimal in vitro fertilization in common carp and sperm/egg ratios from 100,000 to 200 000 are recommended for practical propagation (Billard et al., 1995; Linhart et al., 2003). Optimal sperm/egg ratio is used for discrimination of fertilization effect (Billard and Cosson, 1992) and control fertilization and hatching rates are then used as a potential predictor of sperm quality.

Altough our aim was to use only male individuals producing sperm with comparable motility, sperm egg/egg ratio reaching higher limit of the range recommended for practical reproduction of common carp was used in an attempt to mask minimal differences in sperm motility and velocity. Low sperm/egg ratios in second study were designed to reveal consequences of sperm competition in a stituation of sperm limitation but highest sperm/egg ratio in fertilization trials was in a range which is supposed to be optimal for artificial reproduction. Hatching control rates ranged from 81 to 91% in first study and from 23% to 92% in second study respectively. Control hatching rates reported in first study demonstrated the high quality of sperm and eggs used for the competition experiment corresponding with 90% hatching in both experimental fertilizations Significant differences were not reported in hatching in first study, two males from second study had significantly lower hatching than three others whose hatching was higher than 90%. Low hatching rates reported in second experiment were explained by low motility of spermatozoa as described below.

Fertilization trials of first study were similar to the conditions of hatchery practise when 2 batches of 200 g of eggs were fertilized whereas design of second study was more experimental with fertilization of 5 g portions of eggs. A sperm dilution ratio of 1:143 (sperm/water) was used in first experiment with an egg/activating water dilution ratio of 1:1. In experimental fertilization of second study, gametes were activated by adition of 10 ml of water to the beaker placed on orbital agitator and dilution ratio was 1:2. Both rates were suitable for artificial propagation of common carp according to previous studies (Linhart et al., 2003; 2005).

Percentage of sperm motility in a range 71.3% to 98.4% with sperm velocity from 96.8 to 154.9 $\mu m.s^{-1}$ were reported for experiment evaluating effect of equalization of sperm numbers. Both motility and sperm velocity significantly differed, showing very low value for one of males. Very low values of sperm motility and velocity resulted in very low contribution of this male to progeny, reaching 0.4% of progeny from fertilization trial using same volumes and 2.4% of progeny in fertilization trial using same number of spermatozoa. In a second experiment testing different conditions of fertilization, motility ranged from 2 to 93% with velocity of spermatozoal motility from 85.03 to 137.56 $\mu m.s^{-1}$. Altough initial aim was to use sperm of high and comparable quaility, two males in experiment produced sperm with significantly lower motility having 2 and 4.4% of motile spermatozoa. Velocity of sperm cells was not significantly different in 4 males in these fertilization trials. Very low motility of spermatozoa of two males significantly influenced percentage of males sired by these males in all fertilization trials.

In both fertilization trials of first experiment, the contributions of the five males expressed by chi-square statistic were not uniform. The contributions of the males were not homogeneous across progeny types showing an effect of the equalization of sperm numbers for fertilization of eggs. In second experiment where competition in conditions of different sperm/egg ratios was tested, contributions of males were highly disequilibrated in all fertilization conditions. The homogeneity test for contribution of males was not significant, showing that the different number of spermatozoa per egg had no effect on the representation of males.

Results of equalization of sperm number showed an effect on representation of males, but striking differences were reported in representation of males whose sperm traits were not significantly different. This equalization of sperm by numbers does not enable equal representation of males in progeny in competitive situation.

Comparison of the explanatory models in experiment evaluating effect of equalization of sperm numbers showed minimum log-likelihood ratio in model including numbers of sperm, their motility and velocity. But chi-square goodness of fit analysis comparing observed numbers of offspring per male and expected values based on this model was highly significant, showing a large unexplained residual variability in males contributions. In experiment with different sperm/eggs ratios tested results showed that all models tested were better than prediction of uniform representation of all males. Best fitting model was model including motility as explanatory variable and other models where motility and velocity or hatchability were found to be less probable. However, sperm motility was very low (< 5%) in two of the males used. This is expected to magnify the effect of motility on fertilization success, when compared to other trials where all males had a high motility. In this experiment, by fertilizing different egg batches with 5,000; 10,000; 20,000 and 100,000 spermatozoa per egg (using the same pool of 5 males) was demonstrated that total sperm concentration had no impact on male fertilization success.

Sperm competition in common carp was studied (Linhart et al., 2005) and fertilization success of males in paired design of experiment was compared with evaluated sperm traits. The mean percentage of offspring sired was strongly influenced by the males used. The best male sired an average of 88% of the offspring in its competition, while the worst male sired only 5%. Sperm quality explained only part of the variation in male competitive ability. No link between sperm velocity and subsequent hatching rate in a control hatching test was reported, whereas motility affected hatching rates in the same experiment (Linhart et al., 2005).

Hatchery induced sperm copetition was widely studied in salmonid species but first studies did not evaluate spermatozoal parameters as sperm motility and velocity. Sperm competition was confirmed in chinook salmon (*Oncorhynchus tschawytscha*) by using pooled milt for fertilization and examined paternity by biochemical markers (Withler, 1998). Potency (fertilization success) of individual males was evaluated in contrast to fertilization rates of control fertilization trials and spermatocrit but these parameters were not correlated with fertilization success of individual males when pooled sperm used.

Equal volumes of sperm of each male used for fertilization of batch of eggs (Withler and Beacham, 1994). Individual males sired from 5 to 83% of progeny in two competitive trials when mixture of sperm was used for fertilization immediately after pooling.

In Atlantic salmon (*Salmo salar*), sperm competition and factors affecting relative success of males in fertilization trials were studied (Gage et al., 2004) when equal volumes of sperm and then volumes in a ratio 2:1 were used for fertilization in an effort to clarify which of spermatozoal traits is responsible for competitive fertilization success. Sperm velocity, not sperm number, was found to be primary determinant of success in copetition. This is in contradiction to theory of sperm competition (Parker, 1982) which suppose that increased level of sperm competition result in production of more sperm and in contradiction to the comparative studies of sperm competition in fish with external fertilization based on evaluation of risk of sperm competition according to production of gametes if velocity, not number, is primary determinant of sperm competition. This evaluation of risk of sperm competition by production of gametes pronounced by gonadosomatic index was doubted (Stoltz et al., 2005) because allometric growth in fish is not common.

GSI is not correct parameter to evaluate intensity or risk of sperm competition (Tomkins and Simmons, 2002) because GSI has not isometric relationship between gonad mass and body mass in individual species.

Nevertheless, findings that sperm velocity is primary determinant success in competition as reported in Atlantic salmon (Gage et al., 2004) are in contradiction to study of sperm copetition in Atlantic halibut (*Hippoglossus hippoglossus*) (Ottesen et al., 2009) where sperm of four males was pooled to have mixtures containg equal number or equal volume of sperm and batches of eggs from two females were fertilized. Number of spermatozoa and percentage of motile spermatozoa showed to be correlated with relative fertilization success in all sperm/egg ratios tested.

Some studies showed that the outcome of sperm competition is not necessarily linked to its fertilization success when used separately in hatching control test (Withler, 1988) neither to spermatocrit (Withler, 1998; Gage et al., 2004) or sperm longevity (Gage et al., 2004). Gage (2004) found sperm velocity to be primary determinat of success of fertilization in Atlantic salmon and our study evaluating effect of equalization of sperm numbers (Chapter 2, Kaspar et al., 2007) showed that all parameters as hatching rates, sperm numbers, motility and velocity positively influenced probability of theoretical explanatory model, altouhgh large part of variability of males´ representation in both types of progeny remained unexplained. Second study of sperm competition under condition of *in vitro* fertilization in common carp (Chapter 3, Kaspar et al., 2008) revealed that contributions of males were highly disequilibrated in all fertilization conditions and homogeneity test for contribution of males was not significant, showing that the different number of spermatozoa per egg had no effect on the representation of males. Explanatory models in this study showed sperm motility to be most important factor which correlates data reported for Atlantic halibut (Ottesen et al., 2009) where number of spermatoza and percentage of motile sperm showed to be premises of competitive success in all sperm/egg ratios tested in study of hatchery induced sperm competition when different sperm/eggs ratios tested.

Study of *in vitro* fertilization where different sperm/eggs ratios tested, very low sperm motility in two of the males was expected to magnify the effect of motility on fertilization success (Kaspar et al., 2008; Chapter 3). In study of hatchery induced sperm competition in halibut (Ottesen et al., 2009), computer-assisted sperm analysis of spermatozoa from 4 males used in experiment revealed high differences in percentage of motile cells – motily 30–36% vs. 88% in one of males. Fertilization trials were performed using different sperm/egg ratios and contributions of males were highly disequilibrated in all fertilization conditions. In all conditions of in vitro fertilization, striking differences in relative contribution of three males of comparable spermatozoal parameters were found which means that difference in sperm motility between those three males of bad quality and resting one male with good quality in terms of sperm motility overestimated influence of sperm motility as explanatory variable.

Besides of fertilization trials described above, effect of sequentioal adition of semen was tested (Withler and Beacham, 1994) when sperm of three males was applied in 45 s intervals and then activated by adition of water. Position of male in sequence was linked to fertilization success in competitive trials. Altough the first male was favoured in progeny he never fertilized all of the eggs and males in second or third position still sired part of progeny and differences were reported in crosses when pooled sperm was applied for fertilization immediately after pooling of sperm. This showed effect of simultaneous or sequential addition of sperm of multiple males to stripped eggs prior activation of gametes by hatchery water as a common practice on hatcheries.

Gamete association after release into the environment seems to be very fast. Sequentional adition of sperm was then studied (Yeates et al., 2007) and delay in application of sperm of second male caused only very poor representation of delayed male in competitive trials in Atlantic salmon (*Salmo salar*). In this case, sperm of second male was added 2 seconds after application of first male into the turbulent water.

In our first study (Kaspar et al., 2007, Chapter 2), sperm concentrations were evaluated to enable equalization of sperm numbers. Fertilization with ballanced number of spermatozoa from each of five males showed only limited effect on representation of males in progeny of common carp. Effect of storage of pooled sperm before fertilization was studied (Withler and Beacham, 1994). Pooled sperm was kept for 15 and 60 minutes and egg batches were fertilized. There was no difference in relative males´contributions when fertilization realised after 15 minutes of storage if compared with direct fertilization but males´contributions were more equilibrated when fertization was done after 60 minutes of storage but fertilizatin success in competitive trials was not related to the fertilization rate in control test.

Sperm traits are typically evaluated in activating medium which is fresh or salt water depending on the natural spawning environment. Micro-environment of fertilization contains ova released together with ovarian fluid. This ovarian fluid has been documented to influence both sperm velocity and longevity of motile spermatozoa in several species of externally fertilizing fishes (Litvak and Trippel, 1998; Turner and Montgomerie, 2002; Elofsonn et al., 2003; Woolsey et al., 2006; Hatef et al., 2009). Relevance of data on sperm motility measured in activating medium as index of male´s sperm quality was discussed (Rosengrave et al., 2009) because previous reports of swimming speeds for fish spermatozoa activated in water underestimates sperm swimming speeds under natural conditions and overall use of sperm motility, swimming speed and linearity measured inf freshwater is inaccurate index of male´s sperm quality.

Examples of different sperm selection have been identified in different organisms (Birkhead and Møller, 1998). According to the cryptic female choice hypothesis, females may mate with several males but they are able to control which male sires the offspring by actively affecting by which sperm her eggs will be fertilized after copulation (Eberhard, 1996; Zeh and Zeh; 1996).

Ovarian fluids influence on sperm motility parameters is one of supposed forces of cryptic female choice in fish (Wedekind, 2004). Influence of ovarian fluids on sperm motility was mostly studied in salmonids where stripped eggs contain 10–15% of ovarian fluid. Presence of 50% of ovarian fluid in activating medium enhanced sperm longevity, motility and swimming speed in Arctic charr (*Salvelinus alpinus*) according to Turner and Montgomerie (2002). Ovarian fluid presence in activating medium prolonged duration of sperm motility and increased percengate of motile cells in rainbow trout (*Oncorhynchus mykiss*) as reported by Woolsey (2006). Sperm of Atlantic cod (*Gadus morhua*) activated in ovarian fluid was reported to swam faster, percentage of motile sperm cells was reported to be higher and cells were motile for longer period compared to standard activation by sea water (Litvak and Tripell, 1998). Ovarian fluid presence in activating medium influenced both velocity of sperm cells and its longevity in Arctic charr (Turner and Montgomerie, 2002; Urbach et al., 2005). But this effect of ovarian fluid on sperm velocity was dependent on individual male-female combination. This finding of male-female combination dependent effect of ovarian fluid on sperm motility suppose an interaction between sperm and ovarian fluid based on compatibility (Urbach et al., 2005).

Sperm of chinook salmon (*Oncorhynchus tshawytscha*) in activating medium containing diluted ovarian fluid swam faster, have higher percent of motility and a straighter path trajectory for a longer period of motility (Rosengrave et al., 2009). These changese in sperm motility traits were variable and dependent on individual male/female combinations when sperm activation was realised using solution of ovarian fluid and Rosengrave et al. (2009) suggested that ovarian fluid could be a mechanism of cryptic female choice. This supposition is in harmony with reference of Liljedal (2008) that sperm competition among males and sperm selection by females may operate simultanously, potentially imposing different selection pressure on sperm cells. Examples of different sperm selection have been identified in different organisms (Birkhead, 1998). According to the cryptic felmale choice hypothesis, females may mate with several males but they are able to control which male sires the offspring by actively affecting by which sperm her eggs will be fertilized after copulation (Eberhard, 1996; Zeh and Zeh, 1996). Wedekind (2004) reviewed three potential possibilities for cryptic female choice in fish resulting in non-random gamete fusion: sperm signaling their haploid specifity at certain loci and eggs accepting sperms selectively, selection occuring in the phase of formation of second polar body, ovarian fluids having a selective effect on sperm.

Examples of female differential sperm selection were identified in a number of reproductive systems (Pizazzari and Birkhead, 2000; Pilastro et al., 2004). Eggs of tunicates favour sperm carrying different alleles at a so called "fusibility" locus and sperm with identical alleles are less compatible for egg penetration (Scofield et al., 1982) whereas opposite mechanism was reported in sea urchins where external fertilization is mediated by protein binding and eggs select sperm with a binding genotype similar to their own (Palumbi, 1999).

The major histocompatibility complex (MHC) is the most polymorphic region in the vertebrate genome and the gene products are under Darwinian positive selection. High variability of the MHC genes results in the presence of numerous alleles and, as a consequence, numerous haplotypes within a population (Rakus et al., 2003). MHC is an important part of the immune system of vertebrates and is supposed to have an important role in mate complementarity (Penn and Potts, 1999; Landry et al., 2001; Reusch et al., 2001) because several studies reflected that mate choice was at least partially influenced by complementarity of their mates´ MHC alleles.

The vertebrate major histocompatibility complex is large cluster of genes involved in the acquired immune response and immunological self/non-self recognition. MHC genes encode cell surface glycoproteins responsible for the recognition and presentation of foreign antigens to the immune effector cells (Potts and Wakeland, 1990). The presence of multiple independently segregating histocompatibility loci in fish has already been postulated by Hildeman (1970) and Kalmann (1970) who studied skin transplantation possibilities in several fish species. Genomic organisation of the MHC genes in teleosts is different from tetrapods and cartilagous fish – class I and class II genes are not linked together and they are localized on different chromosomes (Sato et al., 2000). Besides the main functions of MHC, role of MHC in mate choice was reported by many studies in different species. Major histocompatibility complex is best-studied example of genes that are important in both, host-pathogen co-evolution and sexual selection (Wedekind et al., 2004).

Mate choice optimizes MHC variation in the offspring of mammals, birds and fishes (Penn, 2002). MHC mediated mating preferences were described in stickebacks and salmonids. MHC locus is duplicated in sticklebacks and individuals with intermediate number of alleles have higher resistance to pathogence and higher reproductive success than individuals with high or low number of alleles.

Females of sticklebacks (*Gasterosteus aculeatus*) were reported to select males with optimal number of MHC alleles to enhance viability and resistance of progeny (Reusch et al., 2001). Evidence for MHC-disassortative mating preferences was reported in salmon (*Salmo salar*) where parentage studies of naturaly spawning population showed higher level of offspring heterozygosity at MHC genes than predicted for random mating (Landry and Bernatchez, 2001; Landry et al., 2001). Mate choice in salmonids was supposed to be driven by tendency to maximize MHC heterozygosity of progeny. But brown trout *(Salmo trutta*) females have been shown to prefer males with intermediate MHC amino acid divergence whereas males with higher MHC divergence were supposed to have lower adaptation on local pathoges (Forsberg et al., 2007). *In vitro* fertilization trials in whitefish (*Coregonus* sp.) showed that MHC clas II has an effect on disease resistance but no MHC influence on relative fertilization success was reported (Wedekind et al., 2004). Higher fertilization success of males of certain MHC class II genotype in condition of in vitro fertilization was reported in arctic charr (*Salvelinus alpinus*) by Skarstein et al. (2005) but effect of male-female interactions in fish can not be accurately reflected by fertilization and hatching tests for individual male-female combinations in factorial design but cryptic female choice or non-random gamete fusion can occur in terms of sperm limitation or sperm competition.

Influence of variation in MHC class I genotype on fertilization success in competition was studied in Atlantic salmon (*Salmo salar*) by Yeates et al. (2008). Males geneticaly simillar and males genticaly different in MHC class I gene were crossed in paired design and spermatozoal traits were assessed. This design of *in vitro* fertilization competition trials showed that males competiting for fertilization of eggs of female with similar MHC class I genotype had about 15% higher fertilization success than males geneticaly distant (Yeates et al., 2008). Altough MHC influence on sperm competition was studied in salmonids, no data related to cyprinids is available. Study of MHC influence on fertilization success in competitive fertilization and non-random gamete fusion in common carp is engagement for future.

Hatchery induced sperm competition may influence broodstock management or conservation efforts. Transfer of as many genes as possible to following generation is most important issue in broodstock management and conservation. In this case, high stress has been given to the number of spawners used (Ryman et al., 1995; Wang and Ryman, 2001). Spawning procedures practiced in hatcheries are supposed to affect the genetic variation o f the ensuing generation and can be chosen to minimize the stochastic genetic changees that occur as a result of founder effects and genetic drift (Withler and Beacham, 1994). One of the main practical problems caused by hatchery induced sperm competition is unequal representation of males in hatchery progeny when pooled sperm is used for fertilization. This results in reduction of the effective size of the populations and a loss of genetic variability (McKay and McMillan, 1991; Wedekind et al., 2007).

Loss of genetic variability at the next generation can be evaluated by investigation of genetic variability of progeny but if paternity determined, basic parameter effective number of males, N_{em}., can be evaluated. Comparision of theoretical and reported values of N_{em} illustrates unequal male representation and this is linked to the amount of genetic variance lost.

In our experimental fertilization trials we observed sperm competition during *in vitro* fertilization events and evaluated spermatozoal as variables of competitive success of males. Using of qual volumes of sperm from each male (Chapter 2, Kaspar et al., 2007) was reported to generate 42.4% reduction in N_{em} and fertilization with pool of sperm containing equal number of spermatozoa per male generated reduction of N_{em} about 34.9%.

In the second experiment (Chapter 3, Kaspar et al., 2007) where pool of sperm containing equal volumes of sperm from 5 males used for fertilization and different sperm/egg ratios tested, reduction of males representation in progeny remained very high about 42.2%. Altough comparison of number of effective males showed effect of equalization of sperm numbers in first study, fertilization using pool of sperm with ballanced number of spermatozoa in second study was reported to generate high reduction of mumber of effective males. This reduction of number of effective males was comparable with value reported for fertilization using same volumes of sperm in first study. However, sperm motility was very low (< 5%) in two of the males used in all fertilization trials in this study and this was expected to magnify the effect of motility on fertilization success when effect of spermatozoal traits was evaluated. Representation of males with very low level of motility was very low and this significantly influenced reduction of number of effective males similairly to evaluation of explanatory variables in theoretical models.

On the other hand, data from male to male competitive trials performed with ballanced number of spermatozoa of competiting males based on concentration estimates showed mean reduction in N_{em} of 28.4% (Linhart et al., 2005). This reduction of number of effective males is similar to our first study and showing effect of equalization of sperm numbers based on concentration estimates. Evaluation of sperm concentrations or spermatocrit and equalization of sperm mumbers together with estimates of sperm motility is highly recomended then.

Altough comparison of propability of the explanatory models in experiment evaluating effect of equalization of sperm numbers showed minimum log-likelihood ratio in model including numbers of sperm, their motility and velocity. But chi-square goodness of fit analysis comparing observed numbers of offspring per male and expected values based on this model was highly significant, showing a large unexplained residual variability in males´ contributions. In experiment with different sperm/eggs ratios tested results showed that all models tested were better than prediction of uniform representation of all ales. Best fitting model was model including motility as explanatory variable and other models where motility, velocity and hatchability were found to be less probable. However, sperm motility was very low (< 5%) in two of the males used. This is expected to magnify the effect of motility on fertilization success when compared to other trials where all males had a high motility. In this experiment, by fertilizing different egg batches with 5,000; 10,000; 20,000 and 100,000 spermatozoa per egg, using the same pool of 5 males, was demonstrated that total sperm concentration had no impact on male fertilization ability.

Storage a pool of sperm for 60 minutes prior to fertilization enabled significant equilibration of male contributions (Withler and Beacham, 1994). Using the N_{em} calculation approach for data obtained in this experiment showed the initial reduction in N_{em} when the pool was used immediately was 32.8% on average, whereas it was only 7.6% when the pool of sperm containing same volumes of spermatozoa was held for 60 minutes prior to fertilization. Fertilization trial was performed with three males only and no data about spermatozoal traits is available, equilibrated contributions of males to progeny are promising. This practice and its influence on representation of males in progeny was not tested in cyprinids.

Possibility how to reduce the consequences of sperm competition in common carp is to divide eggs into number of batches equal to the number of males and to fertilize them separately by sperm of individual males and finally combine them for incubation. Data of Vandeputte et al. (2004) from paternity determination were used for calculation of number of effective males.

When 24 males were crossed with 10 females in a full-factorial design described above, theoretical number of effective males N_{em} was 24.0 and true N_{em} computed for this data was 23.0 which means 4.2% reduction. In a second experiment, where 147 males were crossed with 8 females in a full factorial design (Kocour et al., 2007), 615 offspring were assigned to parents. The theoretical N_{em} was 146.7,and the true N_{em} of 124.9 represents a reduction of 14.9%. Separate fertilizations of egg batches is then possible way how to obtain equal representations of all males in progeny. The above approach could be then recommended for the propagation of carp broodstocks in hatcheries.

Hatchery induced sperm competition in common carp and its influence on genetic variability of progeny was evaluated by comparison of semi-polyspermatic design of reproduction and full-factorial design (Kaspar et al., unpublished data). Two broodstocks as a parental generation and progeny resulting from two designs of reproduction were genotyped in four microsatellite markers. Semi-polyspermatic approach was done by dividing of pooled eggs into five aliquots and each of those was fertilized with sperm of 5 males. Factorial design of fertilization was done by dividing pooled eggs into 25 aliquots and fertilizing with individual male. Significant differences in allelic and genotypic frequencies between spawners and their progeny were not reported in any of the fertilization scheme and breed. However, in most cases distribution of alleles and genotypes within each locus was more similar with parents in progeny established by factorial design.

Calculations of number of effective males based on data of Kocour et al. (2007) and Vandeputte et al. (2004) showed that factorial design of reproduction is more sutiable for broodstock management and this is in line with theoretical studies that showed factorial design to reduce the variance in family sizes (Busack and Knudsen, 2007). Factorial design showed to improve the conservation of genetic variance (Dupont-Nivet et al., 2006) and our study of hatchery induced sperm competition in common carp showed that high differences in males´ representations were reported among males with comparable spermatozoal parameters. Observation of sperm motility for the needs of artificial reproduction is not guarantee of fertilization success in competitive situation and factorial design of reproduction is recomended then. Data displaying extent of hatchery induced sperm competition and spermatozoal traits´ influence of competitive success in fertilization has alredy been published but all factors affecting competitive fertilization success are still not unovered.

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ENGLISH SUMMARY

Sperm competition in common carp (*Cyprinus carpio*)

Vojtěch Kašpar

Sperm competition in fish with external fertilization is rather rule than exception. Fertilization success of sperm is determined by the number of sperm, their quality in terms of motility and longevity when sperm of individual males fertilize eggs but competitive fertilizations and patterns affecting competitive fertilization are still not well understood.

Pooled sperm is widely used in hatchery practise, which means that hatchery induced competition occurrs. Knowledge of hatchery induced sperm competition, extent of sperm competition and influence of design of reproduction are very important for management of breeding programmes or genetic resources. Pooling of sperm is common practice on hatcheries, sometimes paradoxicaly done in an attempt of hatchery manager to enhance genetic variability of progeny. Sperm from several males is applied simultaneusly to prevent low fertilization rate if quality of sperm in terms of sperm motility and velocity is unknown.

The objective of first study was to determine if equalizing individual sperm numbers in a sperm pool of five common carp males would reduce the variance in number of progeny compared to a pool comprising equal volumes of sperm from the same 5 males. Spawners were selected based on their genotype in four microsatellite markers to enable easy and undoubted paternity identification. Males´traits as sperm motility and velocity of spermatozoa were recorded. Numbers of offspring sired by individual males were highly variable in both types of progeny – 0.4–50% in progeny constructed using same volume of sperm and 2.4–41.2% in progeny constructed using same number of sperm of individual males. Males´contributions were significantly different as shown by Pearson chi-square statistics ($\chi^2 = 189$, 4 d.f., $p < 0.0001$ in first and $\chi^2 = 139$, 4 d.f., $p < 0.0001$ in second type of progeny) showing effect of equalization of sperm numbers for fertilization. Number of sperm per male, sperm motility (71–98%), sperm velocity (97–155 μm s^{-1}) affected the observed number of offspring sired by each of the males, but the high variability in proportion of progeny among the 5 males remained unexplained.

Second study compared competitive success of five males when pool of sperm with equal number of sperm of each male was applied to fertilize eggs in four different sperm/egg ratios 5,000; 10,000; 20,000 and 100,000 spermatozoa per egg to understand competitive fertilization succes.

Perctentage of motile cells ranged from 2 to 93% with velocity of from 85.03 to 137.56 μm.s^{-1}. Altough initial aim was to use sperm of high and comparable quaility, two males in experiment produced sperm with significantly lower motility – with 2 and 4.4% of motile spermatozoa. Velocity of sperm cells was not significantly different in 4 males in these fertilization trials. Males' contributions ranged from 1.3 to 53.9% and were highly disequilibrated in all fertilization conditions ($P < 0.0001$). 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 5 males. Very low motility of spermatozoa of two males significantly influenced percentage of males sired by these males in all fertilization trials.

Loss of genetic variability at the next generation can be evaluated by investigation of genetic variability of progeny but if paternity determined, basic parameter effective number of males, N_{em}., can be evaluated. Comparision of theoretical and reported values of N_{em} illustrates unequal male representation and this is linked to the amount of genetic variance lost.

According to calculation, using equal volumes of sperm from each male was reported to generate 42.4% reduction in N_{em} whereas fertilization using equalized sperm numbers generated reduction of N_{em} about 34.9%. When pool of sperm containing equal volumes of sperm from five males was used for fertilization in different sperm/egg ratios, reduction of males representation in progeny remained very high about 42.2%. This was influenced by very low motility of sperm of two males contained in pooled sperm. Altough reduction of N_{em} was reported to be very high, equalization of sperm numbers showed to have effect in first experiment and is recomended for alleviating consequences of hatchery induced sperm competition.

As showed by calculations based on data from experiments of diferent authors, most effective way how to reduce conseqences of sperm competition in common carp is to divide divide eggs into batches equal to the number of males used and to fertilize them separately in full-factorial design before combining them for incubation. Data displaying extent of hatchery induced sperm competition during *in vitro* fertilization and spermatozoal traits´ influence of competitive success in fertilization has alredy been published but all factors affecting competitive fertilization success are still not unovered.

CZECH SUMMARY – SOUHRN

Kompetice spermií u kapra obecného (*Cyprinus carpio*)

Vojtěch Kašpar

Kompetice spermií je u ryb s externím oplozením spíše pravidlem nežli výjimkou. Obecně je úspěšnost oplození určena množstvím spermií, kvalitou spermatu, co se týče motility nebo doby pohyblivosti spermií, ale úspěch oplození v kompetici a zákonitosti ovlivňující úspěšnost oplození v kompetici nejsou ještě úplně objasněny.

Směs spermií několika samců je využívána v umělé reprodukci ryb, a je tak navozována situace, kdy dochází ke kompetici spermií. Poznatky o kompetici spermií, rozsahu kompetice spermí a vlivu designu umělé reprodukce jsou velmi důležité pro management chovných programů nebo genetických zdrojů. Sperma několika samců je pro potřeby umělé reprodukce používáno současně, někdy paradoxně se záměrem zachovat genetickou variabilitu potomstva. Sperma několika samců je používáno při umělé reprodukci ryb ve snaze předejít nízké oplozenosti, a tím pádem nízké efektivitě umělé reprodukce.

Cílem první práce bylo určit, jaký efekt bude mít vyrovnání počtu spermií jednotlivých samců ve směsi, tj. jakým způsobem ovlivní vyrovnání počtu spermií rozdíly v zastoupení samců v potomstvu ve srovnání s potomstvem získaným oplozením stejnými objemy spermatu jednotlivých samců. Generační ryby byly vybrány na základě genotypu ve čtyřech mikrosatelitních lokusech s cílem maximálně usnadnit určení paternity. Motilita a rychlost pohybu spermií byly analyzovány. Podíl potomků jednotlivých samců byl velmi variabilní v obou skupinách potomstva – 0.4–50 % pro oplození stejným objemem spermatu a 2.4–41.2 % pro oplození stejným počtem spermií jednotlivých samců. Zastoupení jednotlivých samců bylo velmi rozdílné podle Pearsonovy chi-square statistiky ($\chi^2 = 189$, 4 d.f., $p < 0.0001$ pr první a $\chi^2 = 139$, 4 d.f., $p < 0.0001$ pro druhý typ potomstva), což ukazuje efekt vyrovnání počtu spermií pro oplození. Počet spermií jednotlivých samců, motilita spermií (71–98 %) a rychlost pohybu spermií (97–155 μm s^{-1}) ovlivnily zastoupení jednotlivých samců v potomstvu, ale vysoký podíl variability zastoupení v potomstvu pěti samců zůstává nevysvětlen.

Druhá práce porovnávala úspěšnost oplození v kompetici pěti samců, když směs spermií obsahovala stejný počet spermií jednotlivých samců a byla použita k oplození při různých poměrech spermií na jikru – 5 000, 10 000, 20 000 a 100 000. Analyzovaná motilita spermií byla od 3 do 93 % a rychlost pohybu spermií od 85.03 do 137.56 μm.s^{-1}. Ačkoliv původním záměrem bylo použít sperma samců s porovnatelnými hodnotami motility a rychlostí pohybu, sperma dvou samců vykazovalo pouze velmi nízkou motilitu – 2 a 4.4 % motilních spermií. Určení paternity pomocí mikrosatelitních markerů odhalilo zastoupení jednotlivých samců v potomstvu od 1.3 do 53.9 %, přičemž zastoupení samců v potomstvu bylo značně nevyrovnané v rámci všech oplození ($P < 0.0001$). Test homotenity zastoupení jednotlivých samců nebyl signifikantní ($\chi^2 = 12.5$, d.f. = 12, $P > 0.4$), různý poměr spermií na jikru tak neměl efekt na zastoupení jednotlivých samců v potomstvu. Velmi nízká motilita spermií dvou samců znatelně ovlivnila podíl potomstva těchto samců v rámci všech oplození.

Ztráta genetické variability v další generaci může být vyhodnocována podle genetické variability potomstva. Jestliže je však vyhodnocována paternita, nabízí se také vyhodnocení parametru počet efektivních samců N_{em}. Porovnání teoretických a skutečných hodnot parametru pečet efektivních samců N_{em} zobrazuje nerovnoměrné zastoupení samců v potomstvu a je spjato se ztrátou genetické variability.

Oplození směsí spermatu obsahující stejný objem spermatu každého z pěti samců mělo za následek snížení tohoto parametru N_{em} o 42.4 %, zatímco oplození směsí spermatu obsahující stejný počet spermií každého ze samců vykazovalo snížení N_{em} o 34.9 %.

Když byla směs spermií obsahující stejný počet spermií jednotlivých samců použita k oplození jiker v různých poměrech spermií na jikru, snížení počtu efektivních samců v rámci všech oplození dosáhlo 42.2 %. Toto bylo ovlivněno velmi nízkou motilitou spermií použitých k oplození u dvou ze samců. Ačkoliv snížení parametru N_{em} bylo velmi výrazné oproti teoretické hodnotě N_{em}, vyrovnání počtu spermií jednotlivých samců pro oplození efektivně ovlivnilo zastoupení samců v potomstvu v prvním experimentu a je tedy doporučeno pro snížení následků kompetice spermií.

Na základě výpočtů založených na datech z experimentů jiných autorů, nejefektivnějším způsobem, jak se vyhnout vlivu kompetice spermií v rámci umělé reprodukce kapra a jak zamezit snížení genetické variability potomstva, je faktoriální desing reprodukce, tj. rozdělit jikry k oplození do dávek a ty pak oplodnit spermatem jednotlivých samců a poté společně inkubovat. Rozsah kompetice spermií během umělé reprodukce kapra obecného a vliv kvality spermií byl popsán, ale všechny faktory ovlivňující úspěšnost oplození v kopetitivní situaci dosud popsány nebyly.

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LIST OF PUBLICATIONS

PEER-REVIEWED JOURNALS

Butts, I.A.E, Litvak, M.K., **Kaspar, V.**, Trippel, E., 2010. Cryopreservation of Atlantic cod *Gadus morhua* L. spermatozoa: effects of extender composition and freezing rate on sperm motility, velocity and morphology. Cryobiology. Accepted.

Henrotte, E., **Kaspar, V.**, Rodina, M., Psenicka, M., Linhart, O., Kestemont, P., 2009. Effects of dietary n-3/n-6 ratio on the biochemical composition of *Perca fluviatilis* semen and indicators of sperm quality. Aquaculture Research. In press.

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ABSTRACTS AND CONFERENCE PROCEEDINGS

Henrotte, E., **Kaspar, V.**, Rodina, M., Psenicka, M., Linhart, O., Kestemont, P., 2009. Effects of dietary n-3/n-6 ratio on the biochemical composition of *Perca fluviatilis* semen and indicators of sperm quality. Larvi 2009, 5th fish & shellfish larviculture symposium, Ghent University, Belgium, September 7–10, 2009.

Kaspar, V., Kocour, M., Hulak, M., Rodina, M., Gela, D., Linhart, O., 2009. An effect of fertilization practise on distribution of alleles in common carp. Genetics in Aquaculture 2009, June 22–26, Bangkok, Thailand, (Poster presentation).

Hulak, M., **Kaspar, V.**, Kohlmann, K., Tesitel, J., Rodina, M., Gela, D., Kocour, M., Linhart., O., 2009. Genetic diversity and differentiation of foreign common carp breeds farmed in the Czech Republic based on microsatellite DNA variation. Genetics in Aquaculture 2009, June 22–26, Bangkok, Thailand, (Poster presentation).

Kaspar, V., Hulak, M., Rodina, M., Kocour, M., Gela, D., Linhart, O., 2008. PCR-RFLP analysis of the mitochondrial ND-3/4 and ND-5/6 region polymorphism in the common carp (*Cyprinus carpio* L.). Aquaculture Europe 08, Krakow, September 15–18, 2008. (Poster presentation).

Hulak, M., **Kaspar, V.**, Kocour, M., Gela, D., Rodina, M and Linhart O. Spatial genetic structure of local European common carp breeds: implication for conservation and aquaculture. Aquaculture Europe 08, Krakow, September 15–18, 2008. (Oral presentation).

Hulak, M., **Kaspar, V.**, Gela, D. and Linhart, O. Effects of 17 alpha-methyltestosterone and estrogen receptor antagonist (tamoxifen) on effectiveness of sex reversal in gynogenetic tench (*Tinca tinca* L.) juveniles. In: Proceeding of the Vth International Workshop on Biology and Culture of the Tench, September 29 – October 3, 2008, Ceresole d'Alba, Italy. (Poster presentation).

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Kaspar, V., Kohlmann, K., Vandeputte, M., Rodina, M., Gela, D., Kocour, M., Alavi, S.M.H., Hulak, M., Linhart, O., 2006. Equalization of sperm concentrations in a pool of sperm does not prevent large variance in males contribution in common carp progeny. International Symposium of Genetics in Aquaculture, Montpellier, 25–30 June, 2006, France (Poster presentation).

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Kaspar, V., Hulak, M., Kohlmann, K., Vandeputte, M., Rodina, M., Gela, D., Linhart, O., 2007. Sperm competition during in vitro fertilization in common carp (*Cyprinus carpio* L.). 8th Syposium of Fish Physiology and Reproduction, Saint Malo, 3–9 June, France (Oral presentation).	2007
Kaspar, V., Hulak, M., Kohlmann, K., Vandeputte, M., Rodina, M., Gela, D., Linhart, O., 2007. A case study on sperm competition in common carp (*Cyprinus carpio* L.). In: Book of Abstracts (ed. O. Linhart), 1st International Workshop on the Biology of Fish Sperm. August 29–31, 2007, Vodnany. (Poster presentation).	2007

International conferences	**Year**
Kaspar, V., Hulak, M., Rodina, M., Kocour, M., Gela, D., Linhart, O., 2008. PCR- RFLP analysis of the mitochondrial ND-3/4 and ND-5/6 region polymorphism in the common carp (*Cyprinus carpio* L.). Aquaculture Europe 08, Krakow, September 15–18, 2008. (Poster presentation).	2008
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