Spermatozoal competition in common carp (*Cyprinus carpio*): what is the primary determinant of competition success?

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Abstract

The percentage of sperm motility (92–100%), spermatozoan velocity (112–163 $\mu\text{m}\cdot\text{s}^{-1}$) and control hatching rates (83–96%) were evaluated for each of six gold and five green male common carp (*Cyprinus carpio*). In all 30 possible paired combinations of sperm-competition tests, hatching rates of 90–97% were achieved. The mean percentage of offspring sired was strongly influenced by the male used ($P < 0.001$, $R^2 = 0.91$). The best male sired an average of 88% of the offspring in its competition tests, and the worst male sired only 5%. Spermatozoan-quality parameters could explain only part of the variation in male competitive ability. The male effects alone explained 91.4% of the observed variance, consisting of 17.1% explained by spermatozoan motility and 32.5% by control hatching rates in single fertilizations. Undetermined male effects explained 41.8%. The velocity of spermatozoa had no effect on the outcome of sperm competition. Neither was there any link between spermatozoan velocity and hatching rate in a control hatching test, whereas there was an effect of motility on hatching rate in this same test.

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Introduction

Sperm competition occurs when the spermatozoa of several males compete for fertilization. Competition is a major force for spermatozoan selection, but its implications for mating systems and on life-history evolution are only just beginning to be understood (Parker 1998, Gage *et al.* 2004). Trade-offs between gamete characteristics and relative gametic investment has been modelled by evolutionary stable strategy (ESS) approaches, when the optimal strategy for a male depends on the strategy adopted by other individuals in the population (Ball & Parker 1996, Parker *et al.* 1996, Parker 1998, Petersen & Warner 1998). All of the models assume that males obtain more fertilization success by releasing more sperm, this being done at a cost to investment in other fitness-enhancing functions such as growth, finding additional males, investment in female function, mate defence, territoriality or releasing more sperm in other spawnings. As applied to fish, sperm-competition trials with paternity analysis have been conducted in several salmonid species (Gage *et al.* 1995, 2002, 2004, Gile & Ferguson 1995, Liley *et al.* 2002) and recently also in cyprinids (Kortet *et al.* 2004, Reichard *et al.* 2004a, 2004b) or other species such as bluegill sunfish (*Lepomis macrochirus*; Burness *et al.* 2004). In rainbow trout

(*Oncorhynchus mykiss*) male potency, defined as the ability of males to sire progeny when their sperm is pooled with that of other males, was not affected by the female gametes used, and decreased significantly when sperm concentrations were equalized (Gile & Ferguson 1995).

Sperm vary in morphology, behaviour (speed, trajectory, percentage of sperm motility, etc.) and longevity in fishes. Currently, the mechanism of this variation is poorly explained (Cosson *et al.* 1999). There appear to be intraspecific differences that may correlate with male-mating type in Atlantic salmon (*Salmo salar*; Gage *et al.* 1995). Fishes show one of the widest ranges of sperm-competition intensity of any animal group (Petersen & Warner 1998). A comparative study of fishes with external fertilization found that both the gonadosomatic index (I_G) and sperm numbers increase with intensity of sperm competition across species, but that sperm length decreases (Stockley *et al.* 1997). Specific models for continuous fertilization suggest that if length increases a sperm's speed but decreases its longevity, sperm length should increase with sperm-competition intensity, whereas models for instant fertilization suggest that sperm length should remain constant. The negative relationship found between sperm competition and sperm length was not predicted (Stockley *et al.* 1997). The competitive fitness effects of

Atlantic salmon sperm during *in vitro* fertilization were studied recently (Gage *et al.* 2002, 2004). Microsatellite DNA fingerprinting revealed that a male's relative sperm velocity was the primary determinant of sperm-competition success, whereas there was no significant relationship between fertilization success and either relative sperm number or total length of sperm. Sperm longevity showed an inverse relationship with competition success. Atlantic salmon sperm were considered to enter a competition analogous to a race in which the fastest sperm had the highest probability of success (Gage *et al.* 2002, 2004). Positive association between spermatocrit and energy charge was also detected in Atlantic salmon. Sperm-length parameters correlated positively with ATP, energy charge and fertilization success, whereas no evidence for an effect of sperm morphology on longevity was found. Long sperm tail and midpiece may be selected to optimize energetic demands under conditions of increased sperm competition intensity (Vladic & Jarvi 2001, Vladic *et al.* 2002).

Studying competition, different models are proposed according to the reproductive strategies of various species. However, most models are not well-enough designed to simply explain sperm competition during *in vitro* fertilization, because they involve other variables that are irrelevant in this simple case (trade-offs between physiological and/or behavioural fitness-related traits, for example).

For this study of sperm competition in common carp (*Cyprinus carpio*), we used typical *in vitro* fertilization conditions that closely shared many fundamental characteristics of natural fertilization (Linhart *et al.* 2003). The objective of this study was to find out the extent of competition between sperm from two male carp, identified by green and gold genetic markers, in a factorial crossing design testing all possible sperm combinations of six gold males and five wild-green males. We used experimentally regulated identical numbers of spermatozoa in all doses, with sperms chosen to be highly motile.

Materials and Methods

Colour markers for offspring identification

The offspring in each competition test were identified by wild-green (dominant) and gold (recessive) genetic markers (Katasonov 1973, 1978) in a factorial design testing all possible combinations (30) of five wild-green males (nos 7–11) and six gold Japanese fancy (Koi) males (nos 1–6), all crossed with a single gold female. For each paired competition test, one wild-green male and one gold male were used. The percentages of gold and green offspring gave the result of the competition test, according to Mendelian inheritance (Mendel 1865): wild-green males gave 100% green offspring and gold males gave 100% gold offspring. There was no evidence of codominance in crosses involving various colour varieties (Kirpichnikov 1979).

Broodstock handling and collection of gametes

The reproduction and culture of common carp was carried out at the Department of Fish Genetics and Breeding, RIFCH, University of South Bohemia at Vodnany in the Czech Republic in 2004. Experiments with fish were performed in accordance with UK legal requirements. 4-year-old wild-green males, 7-year-old gold Koi males and 7-year-old female broodstock were maintained in separate ponds until used in the experiments. Broodfish suitable for stripping were selected in May and stocked separately in the hatchery in 4 m³ tanks with a water flow rate of 0.2 l·s⁻¹, temperature of 18–22 °C and 6–7 mg·l⁻¹ O₂.

For the experiment, all fish were first anaesthetized in a solution of 2-phenoxyethanol (1:1000). 16 males (eight green and eight gold) were injected with carp pituitary at 1 mg·kg⁻¹, 24 h before stripping at 21 °C. Sperm was collected individually from each male and kept in a thin layer in cell-culture vessels under aerobic conditions at 0 °C. Prior to fertilization, the motility of sperm was checked by microscopy and only 11 males (six gold and five green) with sperm motility of better than 90% were used for the sperm-competition experiment.

Two gold females were used for the experiment and injected with carp pituitary at 0.4 and then 2.1 mg·kg⁻¹, 24 and 12 h before stripping, respectively (Linhart *et al.* 2003). The females were stripped into separate dishes. Ova were stored under aerobic conditions at 17–19 °C for 2–4 h prior to artificial insemination (Rothbard *et al.* 1996). For the experiment, only the female exhibiting ova without whitish colour (which mostly occurs in over-mature ova) and without pollution (i.e. by urine or excrement) was used.

Prior to the fertilization experiments with different males, the initial sperm concentration of all 11 males was estimated using a Burkner cell haemocytometer (16 squares counted for each sperm of each male) under Olympus microscope BX 41 (400 ×) and expressed in billions per ml (Table 1). From this concentration the volume of sperm from each male was calculated as containing 4×10^8 spermatozoa, so that there would be 100 000 spermatozoa from each male per egg in the competition tests. Three batches of approximately 0.3 g (around 200 eggs) of unfertilized eggs were weighed to the nearest 0.0001 g, and then fixed in 4% formaldehyde for later counting and determination of non-fixed mean egg weight. The number of eggs was then calculated in each experiment from the weight of eggs and expressed as number per g eggs.

Competition of two males during *in vitro* fertilization

Eggs (5 g; 800 eggs per g) were placed into a 50 ml dish and sperm from one green and one gold male were simultaneously added in volumes corresponding to 40×10^7 spermatozoa per male. The dish was then placed on an orbital agitator (200 r.p.m., 10 mm deflection). Water (5 ml) was added for activation. 2 min later, 300–600 fertilized eggs were placed with three replicates into special incubation cages of 200 ml supplied with UV-sterilized

Table 1 Mean characteristics of the 11 male common carp in sperm-competition tests, including covariates describing sperm quality: sperm motility (%), spermatozoan velocity, initial sperm concentration and hatched larvae (%) in a control fertilization test.

Sire	Genetic marker	Male weight (kg)	% Sired larvae in competition	Motility (%)	Velocity ($\mu\text{m}\cdot\text{s}^{-1}$)	Initial concentration (spz/ml $\times 10^9$)	% Hatched larvae in control
1	Go	3.1	66.0	98.9	127	25.5	91.4
2	Go	2.7	5.5	96.6	148	22.3	83.4
3	Go	2.1	42.5	99.1	138	29.8	90.2
4	Go	1.8	69.6	100.0	163	26.7	94.7
5	Go	2.1	74.1	99.7	151	21.7	96.0
6	Go	1.7	86.3	100.0	140	25.3	86.8
7	Gr	1.1	27.6	100.0	145	30.0	86.1
8	Gr	1.3	40.9	94.1	111	26.6	90.8
9	Gr	1.0	55.8	98.5	129	20.2	92.2
10	Gr	1.0	34.9	93.0	149	22.8	93.3
11	Gr	1.3	28.2	94.9	125	19.8	89.0

Go, gold; Gr, green; spz, spermatozoa.

recirculated dechlorinated tap water at 22 °C and $9\text{ mg}\cdot\text{l}^{-1}$ O_2 . For each male, a sperm fertilization and hatching control test was also performed, using the appropriate volume to fertilize 5 g eggs with 8×10^8 spermatozoa.

Calculation of fertilization and hatching

The eggs were counted in each cage and during incubation dead eggs were counted and removed, and then hatched fry (green and gold) were counted, usually up to 4.5 days of incubation at 22 °C. The fertilization rate was calculated as the ratio of live eggs at 24 h post-fertilization to the initial number of eggs per cage.

The hatching rate was calculated as the ratio of hatched larvae (green and gold, *H*) to the initial number of eggs per cage. The total number of offspring scored was 12 239 with 282–501 per individual cross.

Observation of sperm motility and velocity

Directly after *in vitro* fertilization, sperms were video-recorded for exact evaluation of the percentage of motility and velocity from three individual records per male. Dark-field microscopy and a Sony camera were used for measurements as described by Linhart *et al.* (2002). Percentage of motility and velocity were examined under $20\times$ objective magnification immediately after mixing $1\mu\text{l}$ (prediluted sperm with immobilizing solution, 1:200; Linhart *et al.* 2002) sperm with $49\mu\text{l}$ distilled water + 0.1% BSA, on a glass slide positioned previously on the microscope stage. The final dilution was 1:10 000. The BSA was added only to prevent sperm heads from sticking to the glass slide. Within 10 s of mixing, a video recording was made for 2 min to be used in the evaluation of spermatozoan swimming activity. The focal plane was always positioned near the glass slide's surface. The movements of spermatozoa were recorded using a S-VHS (Sony; SVO-9500 MDP) video recorder at 50 half frames $\cdot\text{s}^{-1}$ using a CCD video camera (Sony; SSC-DC50AP) mounted on a dark-field microscope (Olympus BX 50) and visualized on a video monitor illuminated with a stroboscopic lamp of

Strobex (model no. 9630; Chadwick-Helmut, USA). The adjustable frequency stroboscopic flash illumination was set in automatic register with video frames (50 Hz) for sperm velocity measurement.

Evaluation of the velocity and percentage of motility

Velocity and motility were assessed at 15 s after activation: the successive positions of the video-recorded sperm heads were analysed from video frames by means of Olympus MicroImage software (version 4.0.1. for Windows with a special macro by Olympus C&S). The velocity and percentage of motility were measured by evaluating spermatozoan head positions on five successive frames with three different colours (frame 1, red; frames 2–4, green; frame 5, blue). 20–40 spermatozoa were evaluated from each frame. Spermatozoa that moved were visible in three colours, while non-moving spermatozoa were white. The percentage of motile spermatozoa was easily calculated from white versus red cells. The velocity of spermatozoa was calculated as $\mu\text{m}\cdot\text{s}^{-1}$ based on length traces of spermatozoa from blue to green and red heads, calibrated for magnification.

Statistical analysis

Prior to analysis, all percentages (variables and covariates) were arcsine-transformed ($Y_2 = \arcsine(\sqrt{Y_1})$) for variance stabilization. To evaluate the effect of sires on competition results, the percentages of green larvae in each competition cross (one green male against one gold male) were analysed with a two-way analysis of variance (ANOVA), with gold male ID and wild-green male ID as independent factors. The analysis was performed on the percentage of green larvae, but as the sum of green and gold larvae was 100%, it gave equivalent information on the percentage of gold larvae (for example, a gold male having a negative effect on the percentage of green larvae also has a positive effect on the percentage of gold larvae). Differences between means of males were assessed with Tukey's

studentized range test. Normality of residuals was assessed with the Shapiro–Wilk test.

Analysis of covariance (ANCOVA) was conducted on the mean (arcsine-transformed) proportion of offspring sired by each male in all the crosses where it had been used, to assess the impact of male origin (gold or green strain) and covariates on the mean competitive value of a male. The covariates tested were sperm-quality indices: arcsine-transformed percentage motility, arcsine-transformed percentage hatching in sperm control tests, spermatozoan velocity and initial sperm concentration (number of spermatozoa per ml). The weight of males at the time of stripping was also used as a covariate. Covariates were tested for homogeneity of slopes among gold and green males. All analyses were performed with SAS-Glm.

Results

Fertilization and hatching were not significantly different; therefore, only hatching results are presented. Hatching results in the control experiments show the good quality of the sperm and egg samples used for the competition experiment (Table 1). Percentage of sperm motility (93–100%), spermatozoan velocity ($111\text{--}163\ \mu\text{m}\cdot\text{s}^{-1}$) and control hatching rates (83–96%) were evaluated for each gold and green male. In paired competition tests, hatching rates of 90–97% were achieved. The mean percentages of offspring sired, given for each male (green offspring for green males and gold offspring for gold males) were significantly the best for gold male nos 1, 4, 5 and 6, with 67–89% gold offspring, and for green male no. 9 with 56% green offspring. By contrast, gold male no. 2 sired only 5% of the offspring on average (Table 2). Both gold and green males had a highly significant effect on the proportion of green offspring ($P < 0.001$), and their crossed effects explained more than 91% of the observed variance, showing that male effects on sperm competitive ability are both strong and essentially additive (although interaction could not be tested, most of the variance is explained by the main effects). In the ANCOVA analysis, there was an effect of male type (green or gold) on the

percentage of offspring sired ($P < 0.05$). The total percentage ratio of gold:green offspring was 58:42.

Regression slopes were homogeneous among types of males for the covariates percentage hatching in sperm control tests ($P > 0.8$), initial sperm concentration ($P > 0.4$), spermatozoan velocity ($P > 0.8$) and weight of males at the time of stripping ($P > 0.6$). When percentage motility was used as a covariate, the regression slopes were heterogeneous ($P < 0.05$), with a strong positive association between percentage motility and percentage of offspring sired in gold males (slope $a = 4.24$, $P < 0.05$) and no association ($a' = -0.08$, $P > 0.9$) in green males (Fig. 1). However, the difference between the two colour strains came mainly from one green male (male 7), which had 100% motility but only 27% of the offspring sired. Without this male, the slopes would have been homogeneous ($P > 0.1$), with a positive association between the percentage of offspring sired and percentage motility ($R^2 = 0.57$, $a = 2.13$, $P < 0.02$).

Regression of the percentage of offspring sired on the percentage of hatching in sperm control tests was relatively close to significance, with a positive (although not significant) association ($a = 1.89$, $P = 0.09$). When weight of males at the time of stripping was used as a covariate, the regression was again close to significance ($P = 0.10$), this time with a negative association with percentage of offspring sired ($a = -0.28$). There was no significant regression on percentage of offspring sired when initial sperm concentration ($P > 0.6$) or spermatozoan velocity ($P > 0.9$) were used as covariates.

When differences between motilities and hatching rates were introduced as covariates in the initial two-way ANOVA, they were both significant ($P < 0.01$), but the effects of green and gold males also remained significant ($P < 0.0001$), showing that these sperm quality parameters explain only part of the variation in males competitive ability. In the initial analysis, the percentage of variance explained by the effects of males was $R^2 = 91.4\%$. When the covariates were added, difference in sperm motilities explained 17.1% of the variance, difference in control

Table 2 Sperm competition between two male common carp identified by green (Gr) and gold (Go) genetic markers, in a factorial crossing design testing all possible combinations of six gold males (nos 1–6) and five green males (nos 7–11). For each cell, percentages of gold:green offspring are given. Mean percentages of offspring sired are given for each male (offspring for green males, and offspring for gold males). Means with different superscripts are significantly different (Tukey's test, $P < 0.05$). The total number of offspring scored was 12.239 (282–501 per individual cross). Hatching rates were 90% or higher for each cross.

Gold male	Gold:green offspring (%)					Mean % of gold offspring	
	Green male...	7	8	9	10		11
1		70.6:29.4	62.1:37.9	24.1:75.9	87.7:12.3	85.4:14.6	66.0 ^{ab}
2		10.4:89.6	6.4:93.6	1.2:98.8	2.8:97.2	6.5:93.5	5.5 ^c
3		68.4:31.6	35.8:64.2	7.9:92.1	37.2:62.8	63.0:37.0	42.5 ^b
4		82.8:17.2	77.8:22.2	32.8:67.2	65.0:35.0	89.8:10.2	69.6 ^a
5		83.8:16.2	92.8:7.2	55.1:44.9	52.4:47.6	86.2:13.8	74.1 ^a
6		97.4:2.6	78.5:21.5	65.6:34.4	94.5:5.5	95.6:4.4	86.3 ^a
Mean % of green offspring		27.6 ^b	40.9 ^b	55.8 ^a	34.9 ^b	28.2 ^b

Figure 1 Mean arcsine-transformed proportion of offspring sired in sperm competition conditions by 11 gold (Go) or green (Gr) male common carp, compared with arcsine-transformed sperm motility (AMOT). Partial regressions for gold and green males, as well as for all males except male 7, are plotted; a = slope of the regression.

hatching rates explained 32.5% and the combined male effects explained 41.8% of the variance.

Discussion

Oviparous fish species such as common carp have a reproduction strategy in which a very short period of sperm reaction is needed to achieve their goal. The sperm exhibit a hypermotile behaviour remarkably similar to the so-called hypermotility of mammalian sperm in the vicinity of eggs before fertilization (Cosson *et al.* 1999). The hypermotility of fish sperm is fully triggered when released in a new ionic/osmotic environment and is expressed by an initial high beat frequency, high velocity and consequently fast energy consumption. It is possible that such behaviour is dictated by an evolutionary constraint, that is, the short period in which an egg can be fertilized due to the limited time (a few seconds in many fish species) of micropyle permeability (Cosson *et al.* 1999).

Here, when compared with other competitive experiments with external fertilization in fish, the attempt was made to use not only the same quantity of sperm from rival males, but also sperm with similar, good motility. When the effect of sperm number is suppressed, then, according to published results in fishes with external *in vitro* fertilization, fertilization success reflects sperm motility (Lahnsteiner *et al.* 1998) and velocity of spermatozoa (Gage *et al.* 2002, 2004). Surprisingly, the variation of sired offspring was very high with gold:green ratios ranging from 1:99 to 98:2, but these proportions were not significantly influenced by the large variation in velocity (from 112 to 163 $\mu\text{m}\cdot\text{s}^{-1}$) of competitive spermatozoa. However, published results do not always show a positive effect of velocity; for example, the highest sperm velocities were associated with a reduction of fertilization rate in rainbow trout (Lahnsteiner *et al.* 1998).

The application of an adequate sperm:egg ratio for high fertilization/hatching rate was an important parameter in

the experimental design. In classical fertilization studies, optimal sperm:egg ratio is used for discrimination of fertilization effect (Billard & Cosson 1992), when the fertilization/hatching success is used as a potential predictor of sperm quality. In the present study, the control hatching rates ranged from 83 to 96% (Table 1), motility was more than 93% (with variation from 93 to 100%) and velocity was higher than 111 $\mu\text{m}\cdot\text{s}^{-1}$ (with variation from 111 to 163 $\mu\text{m}\cdot\text{s}^{-1}$), proving the very good quality of sperm from individual males. Sperm with high motilities and velocities were selected to avoid or minimize evident effects on the competitive ability of males (e.g. non-motile versus highly motile sperm would evidently give a positive linkage between motility and competitive ability). Numbers of fresh spermatozoa ranging from 9000 to 30000 per egg are required for minimal *in vitro* fertilization in common carp, but the practical quantity of sperm recommended for artificial propagation is 100000–200000 (Billard *et al.* 1995, Linhart *et al.* 2003). 200000 spermatozoa per egg were used in the present experiments. The rates of sperm and egg dilution with activating medium (water, activation solution, etc.) during the process of fertilization are other parameters that must be well adjusted. A sperm-dilution ratio of 1:25 (sperm:water) and an egg-dilution ratio of 1:1 were used. Both rates were suitable for artificial propagation of common carp in previous studies (Linhart *et al.* 2003). Present results show that some carp males reproductively win a greater proportion of the paternity in a competitive situation. In the Atlantic salmon, sea urchins and fowl, the same is observed, and in these cases the sperm milt containing the faster spermatozoa are the most efficient (Birkhead *et al.* 1999, Levitan 2000, Gage *et al.* 2004). These results in Atlantic salmon and sea urchins are logical, because increased velocity will enable sperm to search the spawning microenvironment at a faster rate per time unit, and to penetrate earlier than rival sperm (Gage *et al.* 2004). Interestingly, in Atlantic salmon, sperm

number is not the primary determinant of sperm competition success (Gage *et al.* 2004), in contradiction with 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 the present results, there were no links between sperm velocity and percentage of offspring sired in competition or between sperm velocity and percentage of hatching in the sperm control test ($N = 11$, $R^2 = 0.03$, $P > 0.6$; Table 1). On the other hand, there was an effect of motility on hatching in the sperm control test ($N = 11$, $R^2 = 0.38$, $P < 0.05$; Table 1), and a strong association between sperm motility and percentage of offspring sired in gold males but not in green males. When one green male (male no. 7), with a low level of sired offspring (27.6%; Table 1), was excluded, then there was a global association, for all males, between motility and percentage of offspring sired in competition.

Finally, sperm motility seems to explain part of the strong male effect on competitive ability, although all males used here had different but excellent motilities (from 93 to 100%). Percentage sperm motility has been recognized as an important spermatozoal trait for male fertility in rainbow trout (Moccia & Munkittrick 1987, Ciereszko & Dabrowski 1994, Lahnsteiner *et al.* 1998), human (Donnelly *et al.* 1998) and poultry (Donoghue 1999). The present study indicates that 91% of the variance in sperm competition of common carp with different percentages of sired larvae in competition can be explained by differences between the sperm of the competent males (different sperm motility, different hatching, different colour, etc). When different parameters of sperm were analysed, then 17.1% of the variance in larvae sired in competition can be explained by differences in sperm motilities and 33% can be explained by differences in control hatching rates. But still 42% of that variance cannot be explained. Unfortunately when a large quantity of sperm was used for evaluation of sired larvae in competition, differences of spermatozoan velocity were not significant with larvae sired in competition. Given only this information, there was no link between velocity of sperm and percentage of larvae sired in competition.

The 32% variation in velocity of spermatozoa was very high, from 111 to 163 $\mu\text{m}\cdot\text{s}^{-1}$. It could be that such variation of 32% was not important or discriminative for such a quantity of sperm (200 000 spermatozoa per egg) and that such a quantity of sperm oversaturated and masked any effect of a difference in velocity. So, it can be concluded that differences due to variation in the velocity of spermatozoa can be masked by the quantity of sperm, but the effects of sperm motility are masked less by the quantity of sperm. But still there is no explanation for 42% of the variance of larvae sired in competition. Such phenomena can be explained by male–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 & Zeh 1997).

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