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IN VITRO INCUBATION AND MATURATION OF CARP *CYPRINUS CARPIO* SPERMATOZOA

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J. Cosson, R. Billard, C. Redondo-Muller and M. P. Cosson (1991) *In vitro* incubation and maturation of carp *Cyprinus carpio* spermatozoa. *Bull. Inst. Zool., Academia Sinica, Monograph* **16**: 249-261. After hormonal stimulation of different males, the collected carp semen was highly variable in terms of volume, osmotic pressure of the seminal plasma and capacity of spermatozoa to move: this capacity was unstable when the spermatozoa were kept within their seminal plasma. We present data which allow us to understand and possibly to correct these characteristics.

Potentiality to movement was measured as the percentage of motile spermatozoa upon dilution in a medium of low osmotic pressure into which sperm movement was known to occur. Occasionally we also measured flagellar beat frequencies and other parameters of the native spermatozoa as well as those of the demembranated-reactivated axonemal movement. The obtained results are: 1) sperm potentiality to movement was preserved upon dilution into a 200 mM KCl medium: semen of initally "poor" qualities or spermatozoa having lost their capacities to move during storage in the semen recovered gradually their potentiality to movement during incubation at 2°C in this medium. 2) a minimal requirement for 50 mM KCl (which could be replaced by NaCl but not by divalent cations) in media of high osmotic pressure was determined for the conservation and/or the regeneration of sperm capacity to move. 3) the K^+ activation was pH-independent from pH 9.03 to pH 6.04. 4) demembranation-reactivation of the axonemes occurred upon ATP-Mg addition, whatever be the initial potentiality of native spermatozoa to move.

It is concluded that, providing appropriate *in vitro* conditions, carp sperm saves or acquires the potentiality to movement which allows to get motile spermatozoa available all the year around. In

addition, the obtaining of potentially non-motile sperm and its reversion is a usefull model to understand the mechanisms of control of the maturation of spermatozoa *in vitro*.

Key words: *Cyprinus carpio*, Spermatozoa, Incubation, Maturation.

The common carp (*Cyprinus carpio*) is one of the most widely cultivated species in the world and one of the few fully domesticated fish. Reproduction was obtained by natural means in large ponds or in spawning ponds until the middle of this century when the first trial of induced spawning and artificial insemination were attempted. Because of this, there has not been much researches on the biology of the semen and the first works were due to Clemens and Grant (1964, 1965). One major problem is to obtain milt in a sufficient quantity: carp in contrast to trout does not usually have large volume of milt available by stripping except during the process of spawning. In the nature it appears that spermiation is strongly stimulated only at the time of spawning when males are exposed to females undertaking the process of oocyte maturation/ovulation (Billard *et al.*, 1989). This was interpreted as a way to stimulate the male for spawning only when the environmental conditions are optimum for spawning which are first perceived by the female (temperature around or above 20°C, spawning substrate...). These environmental factors trigger the process of oocyte maturation and stimulate spermiation in males *via* pheromones. Ovulation and spermiation are quite dependent of these environmental conditions and are relatively fugacious so that it is difficult to obtain continuously carp gametes under natural conditions; therefore researchers as well as fish farmers used currently hormonal injections to induce ovulation and spermiation (so-called technique of hypophysation) and to carry out artificial insemination (Voynarowich, 1961).

The method to obtain a safely semen *via* hormonal treatment was standardized by Courtois *et al.* (1986) and Saad and Billand (1987), using carp pituitary extracts or LHRH-A +pimozide (Billard *et al.*, 1983). The response in terms of volume of milt is directly proportional to the injected dose. The maximum milt production occurs 12 h post injection and declines beyond 24 h, a slight response is often maintained during

a few days or one week. The response is low at 6°C and is considerably increased at 15°C and above (between 5 to 10 ml of milt/kg body weight). Males kept at 15-18°C can produce milt under such stimulation between November and April. This shows that carp spermatozoa are available for research for long periods of time all over the year, much longer than the natural "spermiation" period but they are obtained under rather "artificial" conditions and the problem of their "quality" must be taken into account. The quality of sperm usually refers to the motility which is a prerequisite condition for fertilization; sperm motility refers to the agitation of spermatozoa under the microscope stage after direct mixing of semen and diluent. A deeper analysis of motility is based on an evaluation of the percentage of motile spermatozoa during motion and the beat frequency of the flagellum measured by stroboscopy (Cosson *et al.*, 1985). The concentration of spermatozoa in the semen is usually high in fish (10^{10} spermatozoa/ml) and a dilution of 1/2,000 is necessary to discriminate the individual spermatozoa under the microscope stage; a 2 step dilution procedure is necessary and is currently used for trout sperm in our laboratories (Cosson *et al.*, 1989): a predilution (1/100) is first made in a medium which prevents the initiation of motility and a final dilution (1/20) made directly and quickly on the microscope slide where motility initiates. These technique has clearly shown that carp spermatozoa was highly variable in "quality" with possible interference with factors in the seminal fluid. In addition some improvements were observed after incubation of a few hours in the preincubation medium (Redondo-Muller, 1990; Redondo-Muller *et al.*, 1991). Some of these results are reported in the present publication.

MATERIALS AND METHODS

The experiments were carried out at Villefranche/Mer. Male carp (body weight 1 to 1.5 kg), tagged with coloured string attached to the dorsal fin were kept in captivity in 1 m^3 indoor tanks with partly recycled tap water at ambiant temperature (16°C) and natural photoperiod. Two separate tanks were used, one with routinely fed animals (trout pellet at a daily rate of 0.5% body weight) and the other with males starved for a few days before hormonal injection. The latter consisted of dry carp pituitary (Argent)

injected at a dose of 2 mg/kg body weight in anesthetized males (in phenoxyethanol 0.25 ml/l). Twenty-four hrs later semen was collected by stripping; contamination by feces was prevented by placement of tissue paper in the rectum during sampling. Semen was stored on ice immediately after stripping. Motility was controlled within 10 min following sampling after a 2 step procedure: predilution of 10 μl of semen in 1.5 ml of a 200 mM KCl solution Tris HCl 30 mM pH 8.0 at 2°C (osmotic pressure—o. p.-380 mosmols/kg) and second dilution by mixing on the microscope slide 1 μl of prediluted sperm into 20 μl of distilled water buffered at pH 8 with 30 mM Tris-HCl (35-40 mosmols/kg). This low osmotic pressure in the final dilution medium is necessary to significantly decrease the o. p. of the predilution medium and allows the initiation of motility (Morisawa *et al.*, 1983). This occurs at o. p. less than 150 mosmols/kg and the o. p. at the final dilution step is around 56 mosmols/kg. After mixing directly on a glass slide motility is initiated instantaneously and can be evaluated within a few seconds by direct observation with dark field microscopy of the cell with a forward movement and evaluation of the percentage of motile cells. The precision of this estimation is in the range of $\pm 10\%$. In some experiments, the beat frequency of the flagellum was measured as described in Cosson *et al.* (1985).

Sperm reactivation after demembranation and addition of increasing amount of Mg-ATP was carried out according to the procedure described by Cosson and Gagnon (1988). Demembranation medium: 0.15 mMK+ acetate, 0.5 mM $CaCl_2$, 0.5 mM EDTA, 1 mM DTT, 20 mM Tris HCl, pH 8.2 and 0.04% Triton $\times$100. Reactivating medium: 0.15 mMK+ acetate, 1 mM DTT, 0.1 mM EGTA, 20 mM Tris HCl pH 8.2 and 1 mM Mg-ATP. After 30 sec on ice the demembranated spermatozoa suspension was diluted 20-fold in the reactivation medium.

RESULTS

The percentage of motile carp spermatozoas observed after dilution (initial motility) depends on the pH of the dilution medium. In the experiments reported in Fig. 1, semen was diluted according to the standard procedure described in Materials and Methods in distilled water buffered with Tris HCl 30 mM at pH from 6 to 9; in these condition the maximum percentage of motile cells occurred

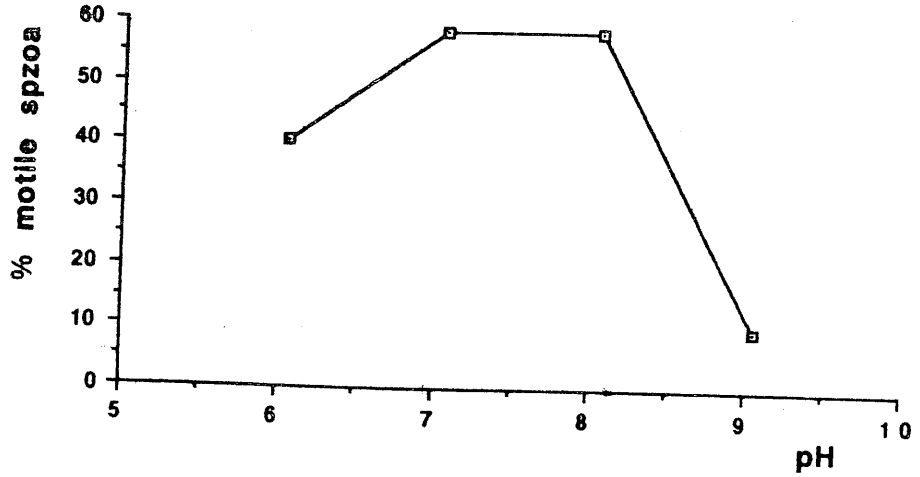

Fig. 1. Change in the percentage of motile spermatozoa after dilution of semen in freshwater buffered at pH from 6 to 9 with Tris 30 mM final dilution 1/3,000.

at pH 7 and pH 8. In the subsequent experiments, pH 8.0 was retained.

The evolution of the percentage of motile cells diluted in the media of an increasing o. p. is shown in Fig. 2. In the distilled water buffered at pH 8 with 30 mM Tris HCl, 80% of the spermatozoas were motile. When osmotic pressure was increased by adding NaCl, this value was maintained up to o. p. 100 mosmols/kg and then decreased regularly as osmotic pressure increased. When KCl was added instead of NaCl to rise the osmotic pressure there was a regular increase of the percentage

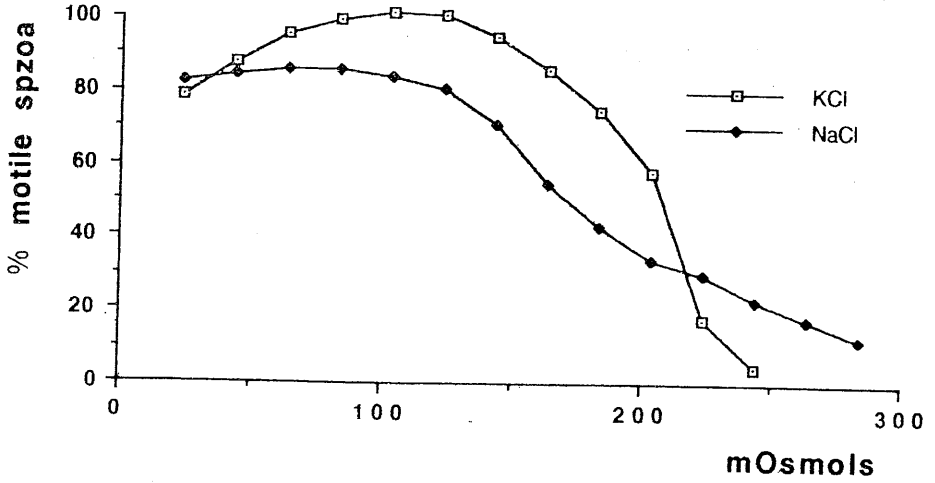

Fig. 2. Changes in the percentage of motile spermatozoa after dilution of semen in KCl and NaCl solutions of increasing osmotic pressure buffered at pH 8. Predilution of semen in KCl 200 mM pH 8 and final dilution 1/3000.

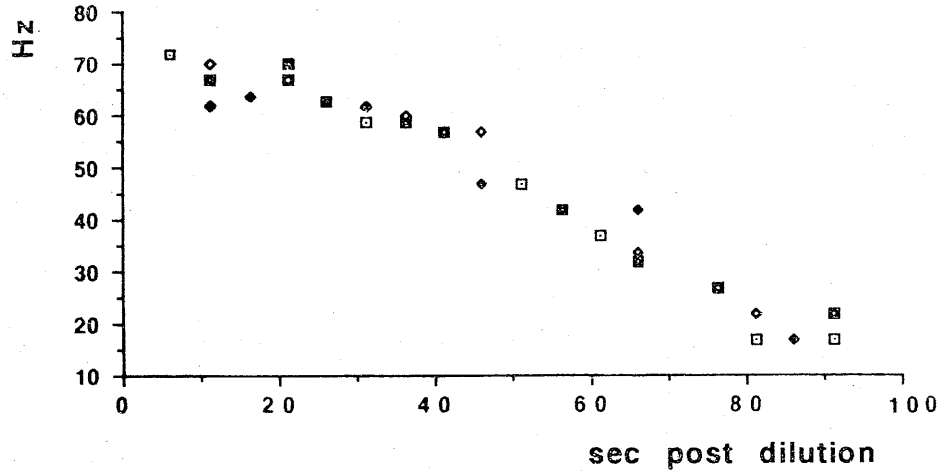

Fig. 3. Changes in the beat frequency of the flagellum of live carp spermatozoa after dilution. The different dots correspond to different males.

of motile spermatozoa which reached 100% at osmotic pressure of 90-110 mosmols/kg and decreased above.

The duration of motility (forward movement) after predilution (1/100) in a 200 mM KCl solution and a final dilution (1/3,000) into the distilled water buffered at pH 8 (Tris-HCl 30 mM osmotic pressure 36 mosmols/kg) is around 1.5 min (Fig. 3). The beat frequency declines from 70 Hz few seconds after dilution to 20-25 Hz, 90 sec later. A plateau is then observed during 2-3 min post-dilution.

The percentage of motile spermatozoas recorded immediately after dilution shows a great variability

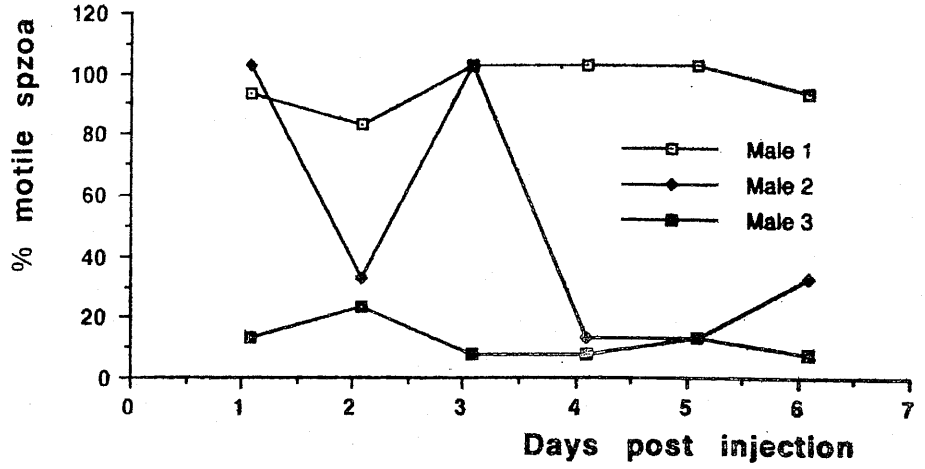

Fig. 4. Changes in the semen quality in 3 males during 6 days after one injection of carp pituitary extract to stimulate spermiation.

which could not be related to any known predicted parameters. In some cases 100% of the spermatozoas were motile, in other samples less 30-40% and sometimes no initiation of motility could be seen. In some males the "quality of semen" assessed by the percentage of motile spermatozoa was consistently high or low but sometimes it shows high variations from one day to another (Fig. 4). When semen with no motile spermatozoa were incubated at 2°C (on ice) in a 200 mM KCl solution (pH 8) a progressive recovery of motility was observed and reached 100% in some cases. An example is given in Fig. 5 showing three extreme situations; one male with 100% of motile spermatozoas after dilution (male 1): two others with 50% (male 2) and 0% (male 3) of motile spermatozoa respectively but with a progressive recovery of motility (100% within 30 min for male 2 and 80% within 70 min for male 3). The recovery of the potency for motility of poor quality semen is possible a few hrs after sampling (8 hrs in the example shown in Fig. 6). There is some indications that a factor in the seminal fluid is involved in the inhibition of motility as shown in Fig. 7 where spermatozoas of "good quality" having a motility rate of 100% (male 1) are exposed to seminal

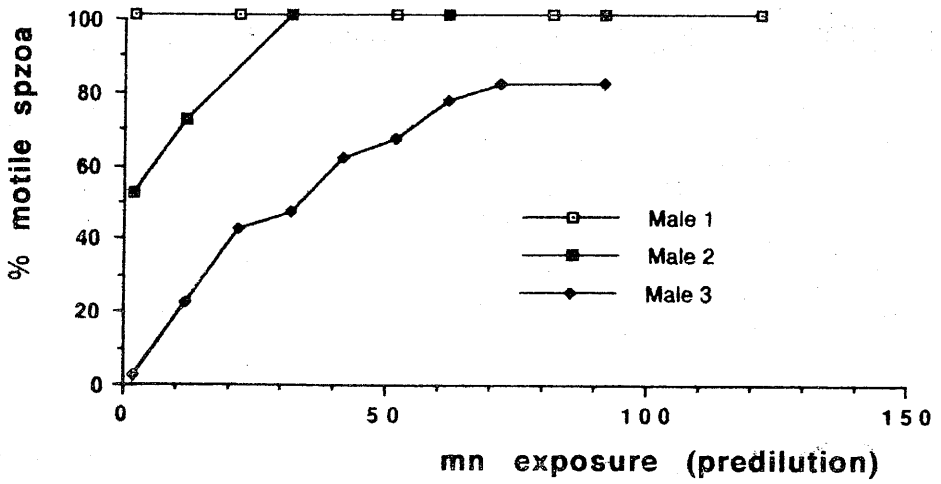

Fig. 5. Example showing semen of different "quality" taken from three different males. 1) good quality sperm showing 100% motility after dilution and keeping this potency during few hours when prediluted in a 200 mM KCl immobilizing solution and stored at 2°C. 2) average quality semen with only 50% initial motility which is however recovered at 100% after 30 min exposure to the predilution medium. 3) semen of very poor quality, partialy recovered during predilution.

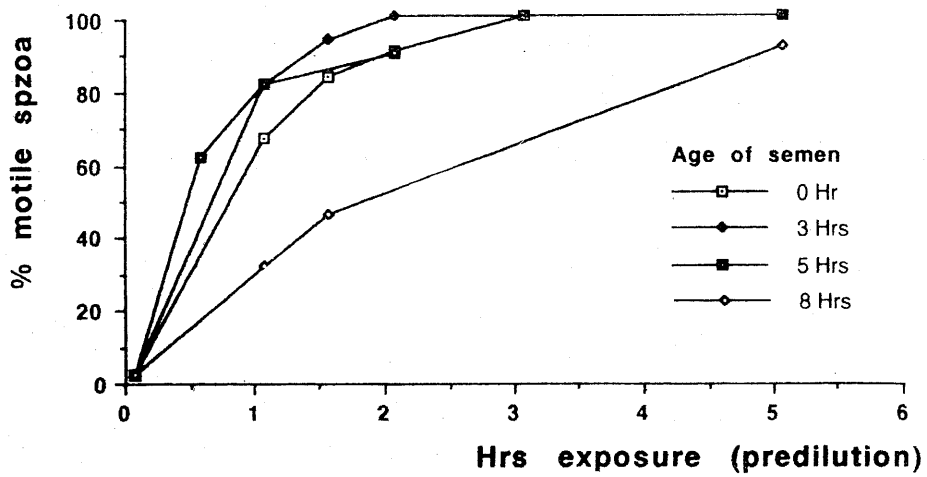

Fig. 6. Recovery of the potency for motiliy of a very poor quality semen immediately after sampling (0 hr) and after 3-5 and 8 hrs of *in vitro* storage of the intact semen at 4°C.

fluid of a semen of poor quality (0% initial motility) (male 2); after 20 min incubation, there is no motile spermatozoa. When those spermatozoa with inhibited motility were washed and exposed to a 200 mM KCl solution as described above the potency for motility was progressively re-established.

Further analysis of this

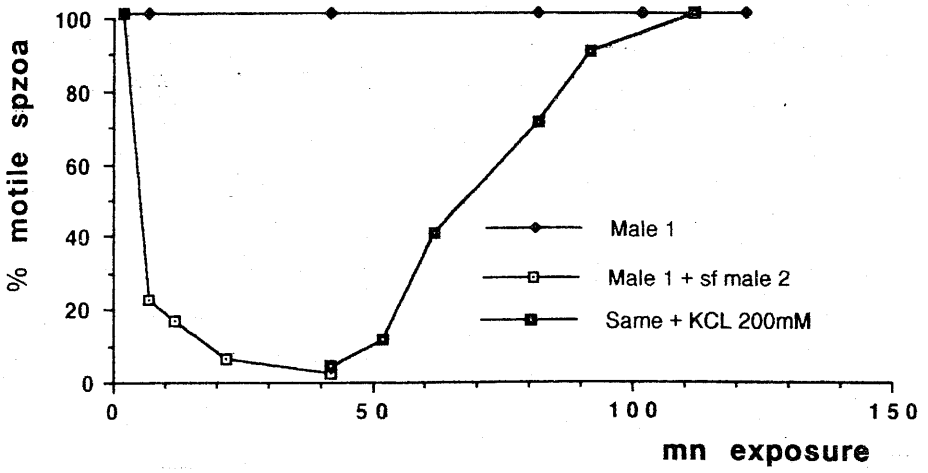

Fig. 7. Example of the change of the motility potential of carp sperm incubated in various conditions: the seminal fluid of a male of poor quality sperm (male 2, 0% motile sperm after predilution in KCl 200 mM and dilution in the activating solution) inhibits the spermatozoa motility of male having good quality sperm. Redilution (1/200) in KCl 200 mM removes this inhibition activity.

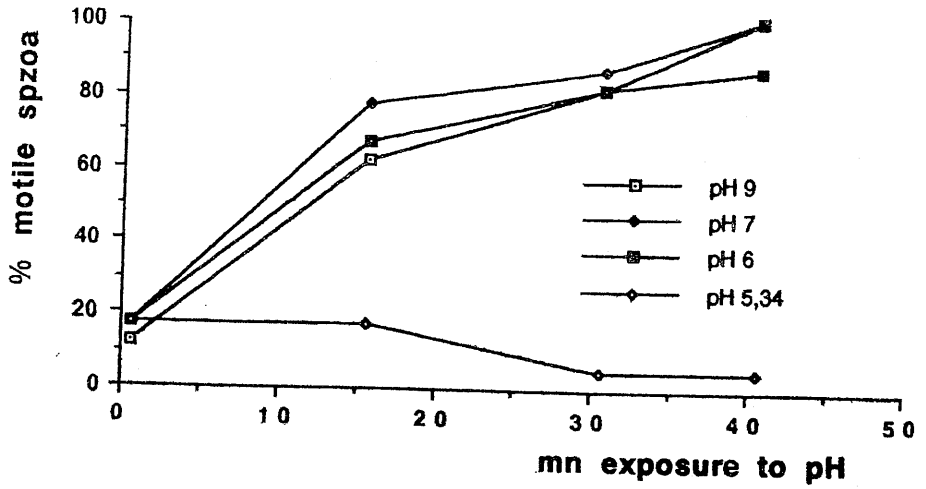

Fig. 8. Effect of pH of the 200 mM KCl solution on the recovery of the potenty for motility of a poor quality semen showing an initial motility of 15-20% of motile spermatozoa.

phenomenon of reversible inhibition of motility have shown that the recovery of potency for movement requires a minimum concentration of 50 mM KCl (or NaCl) and does not occur when divalent cations are substituted to KCl or NaCl (data not shown). The benefic effect of K^+ occurs in a range of pH from 6 to 9 but not at pH 5.34 or below (Fig. 8). Demembranated spermatozoas can be reactivated *in vitro* upon Mg-ATP addition as shown in Fig. 9, such a pattern is observed whatever is the initial potency for movement of the spermatozoas.

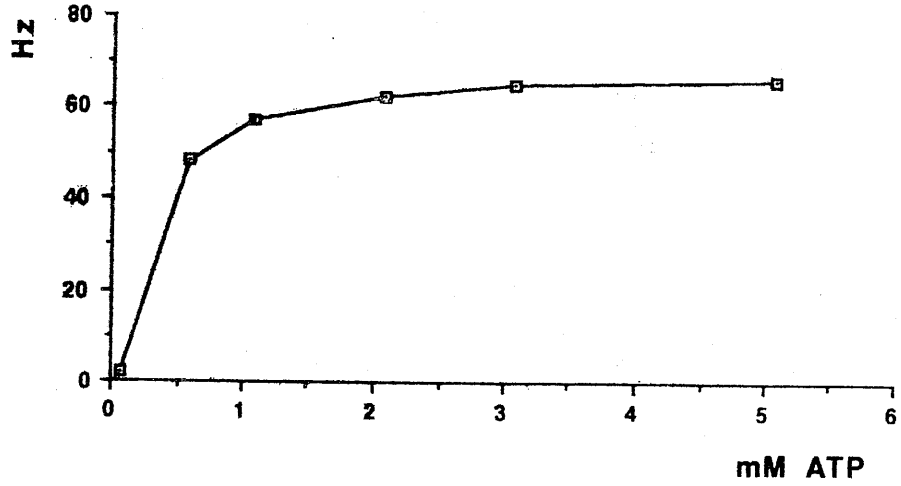

Fig. 9. Pattern of reactivation of demembranated carp spermatozoa with the addition of increasing amount of Mg-ATP.

DISCUSSION

Through the present results, our standard procedure allows the carp spermatozoa obtained after hormonal stimulation to initiate beating in an *homogeneous* manner under appropriate dilution as listed in Materials and Methods: this way, the quality of carp semen can be defined by the percentage of swimming spermatozoa and measured after incubation into various osmotic and ionic environments directly after sampling and after various periods of incubation at 2°C either in seminal fluids or in an artificial medium allowing in some cases the aquisition of the potentiality to movement. It appears that 1) the optimal percentage of motility occurred at pH of the medium between 7.0 and 8.0 with a sharp decrease above and below these values; 2) the percentage of motile spermatozoa was optimally reached for values around 100 mOsm/kg when either NaCl or KCl were added to adjust the osmolarity of the medium; 3) the duration of the motile phase of spermatozoas diluted in the above defined medium was mostly up to one and a half minute during which a progressive decrease of the beat frequency of the sperm population from 70 Hz to 15-20 Hz was homogeneously observed. Nevertheless these observations which define "good quality" sperm were obtained for a small number of males. The sperm qualities vary between fishes and, for a single fish, according to the time delay between the hormonal injection and the successive samplings. Moreover, the sperm potentiality for movement degraded rather quickly when the spermatozoa were kept within their own seminal fluid. However such spermatozoa of variable capabilities for live movement were all capable of ATP-Mg reactivation without noticeable differences which indicated that the axonemes were still functional, excluding the occurrence of an intraflagellar proteolysis during storage in the seminal plasma. Reactivation of the flagellar beating was obtained with no requirement for cAMP as already observed for carp spermatozoa (Cosson and Gagnon, 1988) and for other cyprinids such as the goldfish *Carassius auratus* (Morisawa *et al.*, 1983).

Surprisingly, in all cases where the spermatozoa presented initially a lack of (or a low) potentiality for movement, they were able to recover a good potential movement by incubation either into 200 mM KCl or in 50 mM KCl (with adjustment of osmotic pressure to 300-400 mOsm/

kg). This "activation" process was not pH dependent (between 6.0 to 9.0) and did not occur in media devoid of ions whatever be their osmotic pressure. No clear correlation could be drawn with the semen characteristics: whatever be their initial qualities, all semen degraded with the time when kept at 2°C in contact with air. The variabilities in the quality of carp semen have been sometime noticed (see Stoss, 1983), but the low dilution rate used by the authors might have perturbated the estimation of motile cells.

The present results gave values of the osmotic pressure of the seminal fluid from 200 to 400 mOsm/kg (lower than previously reported by Plouidy and Billard, 1982, or by Morisawa *et al.*, 1983). This might be partially explained by the fact that semen from several males studied in the present work were measured independently and might reflect independent variations. In addition, the observed low osmotic pressures might also be due to the hormone induced spermiation out of the natural reproductive season. Perturbations of semen characteristics have also been reported upon hormonal stimulation, such as an increase of fluid production (Clemens and Grant, 1965; Weil *et al.*, 1986). Morever, the capacities of carp semen for potential movement decreased with the time of incubation in their own semen at 2°C or in non ionic artificial media with balanced osmotic pressure. This phenomenon appeared clearly related to the seminal fluid properties as spermatozoa of initially good performances lost quickly their capacities to move by transfer in seminal fluids from initially "poor" semen. Such unstabilities of carp semen were not observed during the natural period of spermiation as higher percentages of spermatozoas were still capable of movement up to 6 days after sampling (Saad *et al.*, 1988).

On one hand, one cause of instabilities might be related to the process of carp spermatogenesis in which spermatozoa from several generations might be present in the testis at the same time. On the other hand, the present observations might be related to the low doses of pituitary extracts injected for the stimulation of spermiation and to the low temperature. The study of the biochemical composition of such seminal fluid is under progress.

Our observations on carp sperm with potential live movement successively reactivated might reflect some disfunction of the plasma membrane

in relation with modified properties of the seminal fluids or of the state of maturation of the spermatozoa. The importance of the membrane potential has been already demonstrated for trout spermatozoa (Gatty *et al.*, 1990). However, it is not clear of what maintaining sperm immotile in the semen where the osmotic pressure was as low as 212 mOsm/kg, such condition the sperm motility should be observed. A possibility would be the presence of a protein-inhibitor of movement as described in the mammalian sperms (Usselmann and Cone, 1983; Turner and Reich, 1987).

The present results show that the variabilities and unstabilities of carp sperm potential to move could be restored to satisfactory levels indicating that the axonemal machinery was not damaged. As a consequence, this provides the condition in which carp semen movement can be studied all year round and used for fertilization studies in more satisfactory conditions. These data suggest that the carp semen obtained under hormonal stimulation contain spermatozoas with modified membrane properties and seminal fluids which were unable to induce or maintain sperm potential motility. This shows the occurrence of a "maturation" step in carp spermatozoa. The present work establishes the conditions in which an "*in vitro* maturation" can compensate for unability of sperm to initiate flagellar beating and thus provides a model to study the control of such processes.

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