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The effect of ions and cryoprotectants upon sperm motility and fertilization success in the loach *Misgurnus anguillicaudatus*

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Abstract

The solutions commonly used to dilute or cryopreserve sperm are commonly composed of salts, buffers and cryoprotectants, which may affect gametes and subsequent fertilization success. Here, we have evaluated the effects of several cryoprotectants (methanol; MeOH, dimethyl sulfoxide; DMSO and dimethyl acetamide; DMA at concentrations of 0.25, 0.5 and 1%) and different ions (potassium, calcium and magnesium at concentrations of 1.25, 2.5, 5.0 and 10 mM) as sperm diluents upon sperm motility and fertilization success in the loach *Misgurnus anguillicaudatus* sperm. Our results demonstrated that DMSO (at 1%) decreased sperm motility whilst calcium and magnesium ions (from 2.5 mM) induced sperm aggregation and reduced sperm motility. Reduced fertilization rates were observed with potassium (from 1.25 mM), calcium (at 10 mM), magnesium (at 10 mM), DMA (at 1%), and DMSO (at 1%). We conclude that specific ions and cryoprotectants, and their relative concentrations caused effect upon loach gametes. These data are important to consider for the preparation of sperm diluents and activating solutions in order to manage gamete quality for artificial propagation.

Keywords: cryoprotectant, fertilization, fish, gametes, ion, osmolarity.

1. Introduction

The application of *in vitro* fertilization (IVF) for species of fish is important for the reproductive manipulation of species that do not spawn spontaneously under cultured conditions. Hatchery procedures such as artificial incubation and larval rearing, in combination with IVF, may greatly increase hatching rate and the survival of resultant juveniles (Woynarovich and Horváth, 1980; Ciereszko *et al.*, 2000). The potential benefits are highly relevant since IVF would optimize the large-scale production of seeds for aquaculture. Furthermore, IVF permits the application of biotechnology related to chromosome set manipulation (Arai, 2001) and the utilization of cryopreserved sperm (Billard and Zhang, 2001).

The general hatchery procedure for IVF in fish involves mixing sperm with the oocyte mass, followed by activation using water (the so-called 'dry method'). Following activation, spermatozoa become motile and enter the oocyte via the micropyle in order to achieve fertilization (Linhart *et al.*,1995; Hart, 1990). Following this, the water volume is increased and the fertilized eggs are incubated in well-aerated water in order to promote hatching (Woynarovich and Horváth, 1980, Legendre *et al.*, 1996). The external media used to activate sperm, may affect important sperm characteristics (Morisawa *et al.*, 1983; Litvak and Trippel, 1998; Wojtczak *et al.*, 2007; Yoshida and Nomura, 1973), fertilization success (Ketola *et al.*,

1988; Saad and Billard, 1987) and subsequent embryo development (Gonzal *et al.*, 1987; Molokwu and Okpokwasili, 2002; Silva *et al.*, 2003). During fish IVF, the ionic components of the fertilization media is influenced by the water source, ovarian fluid and the sperm.

Fish sperm is commonly diluted in physiological media (known as 'extenders'), as this optimizes sperm usage (Scott and Baynes, 1980; Rodina *et al.*, 2004; Linhart *et al.*, 1987). The composition of extenders must be carefully determined in order to optimize sperm viability and fertilization success. Another method frequently employed in fish IVF is to use cryopreserved sperm. In such cases, diluents often contain cryoprotectants such as methanol and DMSO that may also exert detrimental effects upon gametes (Kopeika *et al.*, 2003). Sperm cryopreservation is particularly interesting for genebanking in the loach as individuals such as natural clones, polyploids and hybrids are important for academic research and aquaculture (Arai *et al.*, 2001).

In our previous studies, we succeeded in cryopreserving loach sperm with high rates of sperm motility and subsequent fertilization (Yasui *et al.*, 2008 and 2009). However, we observed that fertilization rates were severely reduced if cryopreserved sperm were not sufficiently diluted (Unpublished data). This fact suggests that some components from sperm diluents may be toxic at a given concentration and must be diluted sufficiently to improve fertilization. However, although we confirmed the importance of sperm dilution in order to optimize fertilization, it is still necessary to identify which components are toxic and

76 determine safe concentrations or even eliminate from sperm diluents. Increasing
77 understanding of these characteristics are likely to be applicable in the preparation of sperm
78 diluents and activating solutions, and to improve the management of gametes for artificial
79 propagation. In the loach, many kinds of solutions have been used to dilute sperm including
80 modified Ringer solution (Suzuki *et al.*, 1985), Kurokura's solution (Fujimoto *et al.*, 2008;
81 Yasui *et al.*, 2008 and 2009) and NaCl 0.9% (unpublished data). For cryopreservation, we
82 previously used potassium solution (Yasui *et al.*, 2008 and 2009) and a sodium-based solution
83 (Yasui *et al.*, 2010), both containing methanol as the cryoprotectant. Most of these solutions
84 were empirically produced or based on previous studies of other fish species. However, we do
85 not know which component is necessary, or if it presents potentially detrimental effects.
86 Nevertheless, it is important to produce such solutions that are appropriate for the loach in
87 order to optimize sperm maintenance and fertilization success.
88 Consequently, the aim of the present study was to evaluate the toxicity of some
89 components commonly used in sperm diluents that may affect both sperm and subsequent
90 fertilization success in the loach. This study will allow us to prepare non-toxic extenders,
91 cryo-solutions, and to manage sperm dilution ratios in order to maximize fertilization rates in
92 the aquaculture setting.
93
94 **2. Materials and Methods**

95

96 *2.1 Gamete sampling and evaluation of sperm motility*

97

98 Wild parental fishe were obtained from Iwamizawa city (Hokkaido Island, Japan)
99 during the spawning season (June - August). Gamete maturation was achieved as described
100 in our previous work (Fujimoto *et al.*, 2004) with a single dose of human chorionic
101 gonadotropin (Aska Pharmaceuticals, Tokyo - 100 I.U. per male and 500 I.U. per female)
102 injected intraperitonially. After 10 to 12 h at 27°C, fish were anesthetized in
103 2-phenoxyethanol solution (0.1%) and oocytes were collected in 2 mL tubes by stripping
104 (approximately 1.5 mL of oocytes). Sperm was collected using glass hematocrit tubes and
105 transferred immediately to a 2 mL tube containing 800 µL of immobilizing solution (Table 1)
106 and gently mixed by vortexing.
107 In order to evaluate sperm motility, 1 µL of sperm was pipetted onto a glass slide
108 previously coated with 0.1% BSA solution to prevent sperm attachment to the surface.
109 Activation was performed by a 20-fold dilution in distilled water, and sperm motility was
110 observed under the microscope at 400x magnification. Sperm motility was captured by a
111 digital camera (Olympus C-3040) connected to a microscope, and projected onto an LCD
112 screen (LCD-13A1, Sanyo, Japan). Video sequences of sperm motility were obtained using a
113 VHS recorder (VC-HF920, Sharp, Japan). From video sequences, we measured the

114 percentage of total motility, progressive motility and the duration of motility, following the
115 criteria used in our previous work (Yasui *et al.*, 2008 and 2009; Fujimoto *et al.*, 2008). Total
116 motility denoted any type of sperm movement (locally motile, circling or forward motility),
117 while progressive motility regards only to those cells exhibiting forward movement. The
118 duration of sperm motility was measured from activation until ~5% of progressive motility.
119
120 *2.2 The effects of ions and cryoprotectants upon motility parameters*
121
122 In this experiment we evaluated the motility characteristics of sperm suspensions at
123 different concentrations of ions (sodium, calcium, magnesium and potassium) and
124 cryoprotectants (methanol; MeOH, dimethyl acetamide; DMA and dimethyl sulfoxide;
125 DMSO). Sperm from five males were pooled in 800 µL of immobilizing solution and the
126 volume was equally divided into eight tubes (200 µL) followed by centrifugation at 2200 X g
127 for 120 sec. The supernatant was removed and the sperm pellet was re-suspended in 60 µL of
128 various concentrations of NaCl, $MgCl_2$, KCl and $CaCl_2$. This procedure lasted less than 10
129 minutes from mixing and sperm evaluation and resulted in concentrations of magnesium,
130 potassium and calcium at 0, 25, 50, 100 and 200 mM (Table 1). For each solution, the sodium
131 content was manipulated to provide the same theoretical osmolarity of 600 mOsm L^{-1}
132 (calculated by the ionic content of each salt). The loach sperm is generally maintained at

physiological concentrations (~300mOsm) but we increased the solute concentration to evaluate a larger range of toxicity for all ions. However, the molar concentration of 600 mOsm is not toxic for loach eggs or sperm. To evaluate the effects of cryoprotectants, a similar procedure was performed, but the pellet was re-suspended in immobilizing solution containing MeOH, DMA and DMSO at 5, 10 and 20% (v:v) (Table 2). Diluted sperm were then observed under the microscope following activation using water (20-fold dilution) and sperm parameters assessed as previously described. Following sperm activation, potassium, calcium and magnesium concentrations were 1.25; 2.5; 5.0 and 10.0 mM and the concentration of cryoprotectants were 0.25, 0.5 and 1%. As control groups, we used three types of sperm. The first was simply diluted in immobilizing solution, the other was centrifuged using the same procedure described above and then re-suspended using IS, and the third one was centrifuged and then re-suspended in NaCl solution.

2.3 Fertilization and hatching success using diluted sperm

In this experiment, loach eggs were inseminated using the same sperm suspensions containing ions and cryoprotectants evaluated above. Experiments were performed in triplicate, each using different females and different sperm pools (5 males for each pool).

Batches of approximately 120 oocytes (40 µL) were pipetted onto plastic Petri dishes (90 mm diameter) covered by a plastic film. Each egg batch was inseminated with 50 µL of diluted sperm, which was sufficient to cover all eggs. Gamete activation was performed by the addition of 950 µL of distilled water (DW), resulting in a 20-fold sperm dilution. The gamete suspension was then gently mixed by hand. After 5 min, the inseminated eggs were transferred to Petri dishes without plastic film and the water volume increased to about 50 mL. The incubation was performed in the Petri dishes at 20°C until hatching.

Fertilized eggs (at the blastula stage) were counted approximately 4 h post fertilization (hpf) and hatched fry were counted after 60 hpf (Fujimoto *et al.*, 2006). Dead eggs, denoted by a white appearance, were collected at 10 to 12 h intervals. Fertilization, hatching rates and the percentage of normal and abnormal embryos were calculated based upon the initial number of eggs.

2.4 Statistics

All data were performed in triplicate and results are shown as mean ± SD. All data were tested using the Lilliefors' test for normality. Comparisons were performed using analysis of variance, followed by Tukey's multiple range test ($P<0.05$). Motility parameters

of fresh and treated sperm were compared using a paired t-test ($P<0.05$). Statistical analysis was performed with SAEG software version 9.1 (SAEG, 1997).

3. Results

Sperm parameters

Immotile sperm, and sperm exhibiting progressive motility and non-progressive motility are shown in Fig. 1. The percentage of progressive cells was 80.9% for intact fresh sperm, 76.3% for centrifuged fresh sperm and 72.3% for sperm treated with 30 mM NaCl. None of these values were significantly different ($P>0.05$). Potassium maintained sperm quality at increasing concentrations. Average progressive motility was 74, 75%, 82% and 76% for the respective concentrations of potassium at 1.25, 2.5, 5 and 10 mM. Calcium and magnesium treatments significantly reduced sperm motility at all concentrations. Some fused or adhered cells were observed from 1.25 mM, and from 2.5 mM we could observe reduced sperm motility and some sperm aggregates. At the highest concentration of these ions, the percentage of progressive sperm was very low and many sperm aggregates were detected, which reduced the accuracy of motility estimation.

Motility duration was 140 s for fresh sperm and 115 s for sperm treated with 30 mM NaCl (Fig. 2). Centrifugation reduced the duration of motility to 89 s. Potassium concentrations did not affect the duration of motility, ranging from 122.6 s to 138.7 s. However, calcium and magnesium at a concentration of 1.25 mM reduced motility duration to 87.3 s and 99 s, respectively. At 10 mM, duration was 44 s and 41 s for calcium and magnesium, respectively.

The addition of cryoprotectants did not reduce motility parameters at any concentration, except for DMSO at 1%, in which progressive motility was 19% (Fig. 3). The other treatments maintained progressive motility between 61 (1% MeOH) and 79.5% (0.25% MeOH).

DMSO also significantly reduced the duration of progressive motility to 0.5% (Fig. 4). The duration of progressive motility was reduced at all concentrations of cryoprotectants and the duration ranged from 104.7 (MeOH at 0.5%) to 44.67 s (DMSO at 1%).

Fertilization rates

The effect of ions and cryoprotectants upon fertilization and hatching rates are shown in Fig. 5. When eggs were inseminated using sperm diluted in immobilizing solution or NaCl, the hatching rates were 75.1% and 49.5%, respectively. All potassium concentrations

206 significantly reduced hatching rates. Potassium at 1.25 mM yielded hatching rates of 33.1%,
207 and at 2.5 mM, the same parameter decreased to just 12.5%. At 5 mM, fertilization rate was
208 only 4.9% and at the highest concentration (10 mM), just 0.5% of eggs hatched. Calcium and
209 magnesium also caused a severe reduction in hatching at the highest concentrations (5.2% and
210 26.2 %, respectively).

211 Cryoprotectants reduced hatching rates only at the highest concentrations of DMA
212 (28.3%) and DMSO (46.8%) (Fig. 6). Control groups, in which the sperm was diluted in
213 immobilizing solution yielded fertilization rates of 78.2%.

214 In all treatments, the percentage of abnormal embryos remained unaffected by either
215 ions or cryoprotectants.

216

217 **4. Discussion**

218

219 In the present study, we demonstrate that sperm motility parameters were affected by
220 the addition of calcium, magnesium and DMSO. Calcium has previously been reported to
221 exhibit negative effects upon the sperm of some fish species (Christen *et al.*, 1987; Cosson *et*
222 *al.*, 1991). Divalent cations such as calcium and magnesium have the ability to induce
223 membrane fusion or adhesion (Takeda and Kasamo, 2002, Ahkong *et al.*, 1975; Ohki *et al.*,
224 1985). Consequently, the reason for sperm aggregation observed in media that contained high

225 concentrations of calcium and magnesium in the present study could be attributed, at least in
226 part, to membrane fusion/adhesion effects related to these ions. In fish, sperm fusion or
227 adhesion was induced by calcium (Araki *et al.*, 1995), high pH treatments (Ueda *et al.*, 1988),
228 and polyethylene glycol (Ueda *et al.*, 1986, Kirankumar and Pandian, 2004). Our present data
229 also indicate that we can add magnesium to this list. The presence of sperm aggregates,
230 combined with poor sperm motility, reduced the accuracy of sperm motility evaluation.
231 Evaluation of sperm motility requires the observation of an adequate number of cells in the
232 viewing field, and sperm cells must be sufficiently dispersed to be analyzed individually
233 (Billard and Cosson, 1992). So, some of our sperm motility data may be considered
234 inaccurate, especially in calcium and magnesium treatments at 5 and 10 mM in which many
235 sperm aggregates were observed and then reduced the accuracy during evaluation of motility
236 parameters.

237 Fertilization rates were reduced by the addition of DMSO, magnesium, calcium and
238 potassium. Lower fertilization success with DMSO, calcium and magnesium treatments may
239 be explained by reduced sperm motility, a parameter that is related with fertilization success
240 (see reviews by Rurangwa *et al.*, 2004; Cosson *et al.*, 2004).

241 We observed that motility parameters were not affected by potassium treatments but
242 fertilization rates were reduced. Similar results have been reported in other teleost species. In
243 the common carp (*Cyprinus carpio*), for example, reduced fertilization rates were observed

244 from 5 mM of potassium (Saad and Billard 1987). In our present study, potassium
245 concentrations of just 1.25 mM led to reduced fertilization rates. Previously, Lahnsteiner *et al.*
246 (2003) also observed reduced fertilization rates in *Chalcalburnus chalcalburnus*, when a
247 KCl-based solution was used as the fertilization medium. The hatching rate of this species
248 was 98.7% in a NaCl-based solution, but the rate decreased to just 1.6% in a KCl - based
249 solution. The authors hypothesized that potassium solutions could exert negative effects upon
250 eggs or sperm. Potassium has a strict relationship with the transportation of ions through cell
251 membranes. Consequently, high potassium concentrations or an unbalance on
252 sodium/potassium relationship in extracellular media may affect the transportation of other
253 ions necessary for fertilization and/or egg development.
254 Historically, potassium ions have proved to be a highly controversial issue for fish
255 gametes. In the case of salmonid sperm, potassium is important in order to keep cells
256 immotile and to maintain their viability (Gatti *et al.*, 1990; Benau and Terner, 1980) as in the
257 case of cyprinids (Morisawa *et al.*, 1983). Additionally, Linhart *et al.* (2008) used potassium
258 solutions in order to improve sperm motility and showed that even activated sperm were able
259 to re-initiate motility following incubation in a high potassium solution. In herring (*Clupea*
260 *pallasi*), sperm remained immotile at low concentration of potassium, whilst the addition of 9
261 mM of potassium led to some spermatozoa immediately entering the micropyle (Yanagimachi

262 *et al.*, 1992). On the other hand, it is also important to consider that potassium exerts
263 deleterious effects upon fertilization, as discussed above.
264 We conclude that high potassium concentrations should be avoided in the preparation
265 of extenders or activating media for fish species, with the exception of species where sperm
266 maintenance is specifically driven by potassium, as is the case of salmonids or sturgeon
267 species (see review by Alavi and Cosson, 2006).
268 We also evaluated the toxicity of the predominantly successful cryoprotectants for fish
269 sperm, using the optimal range for most species (5-20% - see review by Billard and Zhang,
270 2001 and our studies on loach described by Yasui *et al.*, 2008, 2009 and 2010). At the highest
271 concentration (20%), DMA (2.16 M) presented decreased fertilization rates although
272 methanol (4.96 M) did not affect sperm motility and fertilization rates. Such comparison
273 suggests that the decreased fertilization rates were caused by the cryoprotectant but not
274 specifically related to molar concentration. Specific ions and cryoprotectants, and their
275 relative concentrations, may cause severe physiological effects including the influx of
276 cryoprotectants into the cell (Mazur *et al.*, 1984), altered membrane characteristics causing
277 the induction of membrane fusion (Araki *et al.*, 1995, Ueda *et al.*, 1986, 1988), dehydration,
278 ionic signalling and egg activation (Coward *et al.*, 2002), the removal of sperm attractants in
279 the region of the micropyle (Yanagimachi *et al.*, 1992), and other important mechanisms

280 relating to fish gametes and successful fertilization. However, we still do not know which
281 mechanism underlies the observed effects in loach gametes.
282 Based in our findings, ions and cryoprotectants may exert negative effects upon the
283 fertilization of loach eggs via two mechanisms. Firstly, ions and cryoprotectants may damage
284 sperm cells and cause reduced motility, thereby reducing the chances of successful
285 penetration into the egg micropyle. Secondly, these factors may reduce fertilization rates, but
286 without reducing sperm motility. The mechanism by which potassium may exert such
287 inhibitory actions remains unknown and requires future studies. Based on the discussion
288 above and considering that molar concentration is the main factor inhibiting the loach sperm
289 motility, non-toxic components (such as NaCl) are preferable for preparation of sperm
290 diluents and optimize the fertilization success. However, further studies are necessary to
291 evaluate the effects of longer sperm storage periods. In addition, we recommend that
292 methanol should be used for the preparation of cryo-solutions for loach since it is an effective
293 cryoprotectant for this species (Yasui et al., 2008, 2009) and in addition as observed here,
294 methanol do not affect sperm characteristics and subsequent fertilization rates.
295 In conclusion, ions and cryoprotectants may affect both, sperm motility and
296 fertilization ability. We also showed with potassium treatments that sperm motility is not
297 always an effective predictor of fertilization success. Our findings suggest that a balanced
298 combination of specific contents should be evaluated to prepare sperm diluents in order to

optimize both sperm motility and fertilization success, and such combination is probably species-specific.

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Yasui, G.S., Fujimoto, T., Arai, K. 2010. Restoration of the loach, *Misgurnus anguillicaudatus*, from cryopreserved diploid sperm and induced androgenesis. Aquaculture 308, S140-S144.

Yoshida, T., Nomura, M., 1972. A substance enhancing sperm motility in the ovarian fluid of brown trout, Bull. Jpn. Soc. Sci. Fish. 30, 1073.

437 Table 1. Sperm diluents composed with KCl, NaCl, $CaCl_2$ and $MgCl_2$. All diluents had the

438 same theoretical osmolarity at 600 mOsm L^{-1}. Immobilizing solution (IS, Kurokura et al.,

439 1984) served as a control group.

Treatment	**Sperm diluents**	**Concentration after activation**
Control	Immobilizing solution – IS: 128.4 mM NaCl, 2.7 mM KCl, 1.4 mM $CaCl_2$, 2.4 mM $NaHCO_3$ (pH: 8.20)	6.42 mM NaCl, 0.135 mM KCl, 0.07 mM $CaCl_2$, 0.12 mM $NaHCO_3$ (pH: 7.12)
NaCl	300 mM NaCl (pH: 6.73)	15 mM NaCl (pH: 5.69)
1.25 mM KCl	25 mM KCl, 275 mM NaCl (pH: 6.69)	1.25 mM KCl, 13.75 mM NaCl (pH: 5.67)
2.5 mM KCl	50 mM KCl, 250 mM NaCl (pH: 6.65)	2.5 mM KCl, 12.5 mM NaCl (pH: 5.65)
5 mM KCl	100 mM KCl, 200 mM NaCl (pH: 6.72)	5 mM KCl, 10 mM NaCl (pH: 5.72)
10 mM KCl	200 mM KCl, 100 mM NaCl (pH: 6.61)	10 mM KCl, 5 mM NaCl (pH: 5.77)
1.25 mM $CaCl_2$	262.5 mM NaCl, 25 mM $CaCl_2$ (pH: 6.51)	13.125 mM NaCl, 1.25 mM $CaCl_2$ (pH: 5.64)
2.5 mM $CaCl_2$	225 mM NaCl, 50 mM $CaCl_2$ (pH: 6.47)	11.25 mM NaCl, 2.5 mM $CaCl_2$ (pH: 5.68)
5 mM $CaCl_2$	150 mM NaCl, 100 mM $CaCl_2$ (pH: 6.20)	7.5 mM NaCl, 5 mM $CaCl_2$ (pH: 5.57)
10 mM $CaCl_2$	200 mM $CaCl_2$ (pH: 5.75)	10 mM $CaCl_2$ (pH: 5.6)
1.25 mM $MgCl_2$	262.5 mM NaCl, 25 mM $MgCl_2$ (pH: 6.59)	13.125 mM NaCl, 1.25 mM $MgCl_2$ (pH: 5.71)
2.5 mM $MgCl_2$	225 mM NaCl, 50 mM $MgCl_2$ (pH: 6.49)	11.25 mM NaCl, 2.5 mM $MgCl_2$ (pH: 5.66)
5 mM $MgCl_2$	150 mM NaCl, 100 mM $MgCl_2$ (pH: 6.2)	7.5 mM NaCl, 5 mM $MgCl_2$ (pH: 5.67)

10 mM $MgCl_2$	200 mM $MgCl_2$ (pH: 5.28)	10 mM $MgCl_2$ (pH: 5.51)

440

441 Table 2. Sperm diluents composed with immobilizing solution (IS) and cryoprotectants. The
442 equivalent molar concentrations of the cryoprotectants, and pH, are given in parentheses.
443 Sperm diluted in IS (without cryoprotectants) was used as control group.

444

Treatment	**Sperm diluents**	**Concentration after activation**
Control	Immobilizing solution – IS: 128.4 mM NaCl, 2.7 mM KCl, 1.4 mM $CaCl_2$, 2.4 mM $NaHCO_3$ (pH: 8.20)	6.42 mM NaCl, 0.135 mM KCl, 0.07 mM $CaCl_2$, 0.12 mM $NaHCO_3$ (pH: 7.12)
0.25% DMA	5% DMA in IS (0.54 M, pH: 7.67)	0.25% (0.03 M, pH: 7.05)
0.25% DMSO	5% DMSO in IS (0.7 M, pH: 8.20)	0.25% (0.04 M, pH: 7.12)
0.25% MeOH	5% MeOH in IS (1.24 M, pH: 8.10)	0.25% (0.06 M, pH: 7.13)
0.5% DMA	10% DMA in IS (1.08 M, pH: 7.58)	0.5% (0.05 M, pH: 6.99)
0.5% DMSO	10% DMSO in IS (1.41 M, pH: 8.26)	0.5% (0.07 M, pH: 7.05)
0.5% MeOH	10% MeOH in IS (2.47 M, pH: 8.26)	0.5% (0.12 M, pH: 7.04)
1% DMA	20% DMA in IS (2.16, pH: 7.49)	1% (0.11 M, pH: 6.98)
1% DMSO	20% DMSO in IS (2.82 M, pH: 8.32)	1% (0.14 M, pH: 7.07)
1% MeOH	20% MeOH in IS (4.94 M, pH: 8.37)	1% (0.25 M, pH: 7.09)

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Figures legends:

Fig. 1. Motility parameters of loach sperm at increasing concentrations of potassium, calcium and magnesium. Sperm were centrifuged and re-suspended in sodium-based solutions (600 mOsm L^{-1}) containing each ion and activated using a 20-fold dilution with distilled water. As control groups, we used an intact group, a group re-suspended in IS and another that was re-suspended in NaCl. Molar concentration refers to the final concentration of each ion after sperm activation. Asterisks above columns denote significant differences in progressive motility when compared with sperm diluted in immobilizing solution using Tukey's multiple range test ($P<0.05$).

Fig. 2. Duration of progressive motility in loach sperm at increasing concentrations of potassium, calcium and magnesium. Sperm were diluted in sodium-based solutions (600 mOsm L^{-1}) containing each component and activated using a 20-fold dilution with distilled water. As control groups, we used an intact group, a group re-suspended in IS and another that was re-suspended in NaCl. Molar concentration refers to the final concentration of each ion after sperm activation. Asterisks denote significant differences in progressive motility when compared with sperm diluted in immobilizing solution by the Tukey's multiple range test ($P<0.05$).

Fig. 3. Motility parameters of loach sperm at increasing concentrations of cryoprotectants. Sperm were diluted with immobilizing solution containing cryoprotectants and activated using a 20-fold dilution with distilled water. Percentages refer to the final amount of each cryoprotectant after sperm activation. As control groups, we used an intact group, a group re-suspended in IS and another that was re-suspended in NaCl. Asterisks above columns

denote significant differences in progressive motility when compared with sperm diluted in immobilizing solution, as determined by Tukey's multiple range test ($P<0.05$).

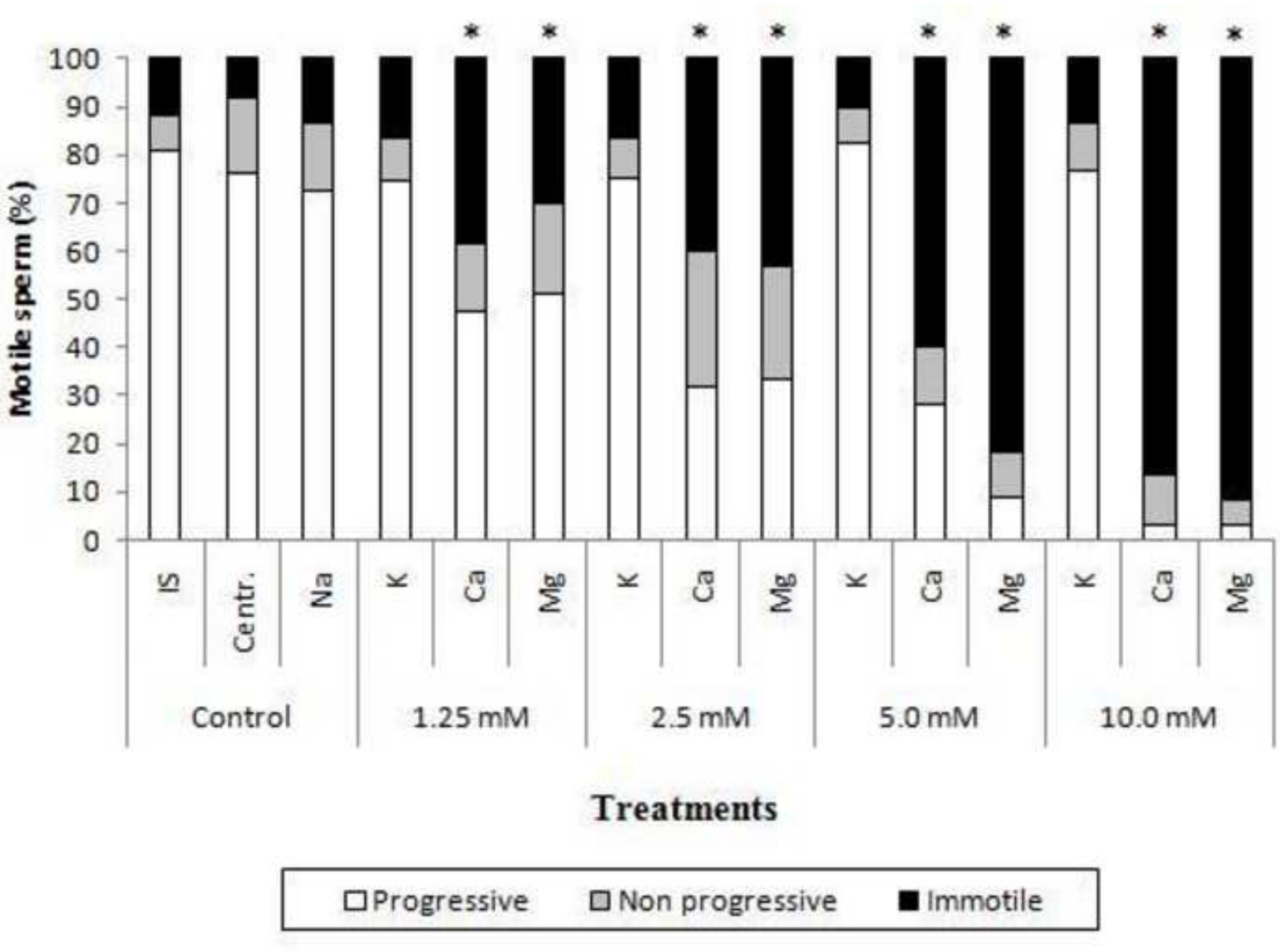
Fig. 4. Duration of loach sperm progressive motility with increasing concentrations of cryoprotectant. Sperm were diluted in immobilizing solution containing cryoprotectants and activated using a 20-fold dilution with distilled water. Percentages refer to the final amount of each cryprotectant after sperm activation. As control groups, we used an intact group, a group re-suspended in IS and another that was re-suspended in NaCl. Asterisks denote significant differences in progressive motility when compared sperm diluted in immobilizing solution by Tukey's multiple range test ($P<0.05$).

Fig. 5. Fertilization and hatching rates of loach eggs at increasing concentrations of potassium, calcium and magnesium ions. Sperm were diluted in sodium-based solutions (600 mOsm L^{-1}) containing each ion, which were poured on egg masses and activated using a 20-fold dilution with distilled water. As control groups, we used a group re-suspended in IS and another that was re-suspended in NaCl. Asterisks above columns denote significant differences in progressive motility when compared with sperm diluted in immobilizing solution, by Tukey's multiple range test ($P<0.05$).

Fig. 6. Fertilization and hatching rates of loach eggs with increasing concentrations of DMSO, MeOH and DMA. Sperm were diluted in immobilizing solution containing each cryoprotectant, which was poured onto egg masses and activated using a 20-fold dilution with distilled water. Asterisks above columns denote significant differences in hatching rates when compared with sperm diluted in immobilizing solution (control), by Tukey's multiple range test ($P<0.05$).

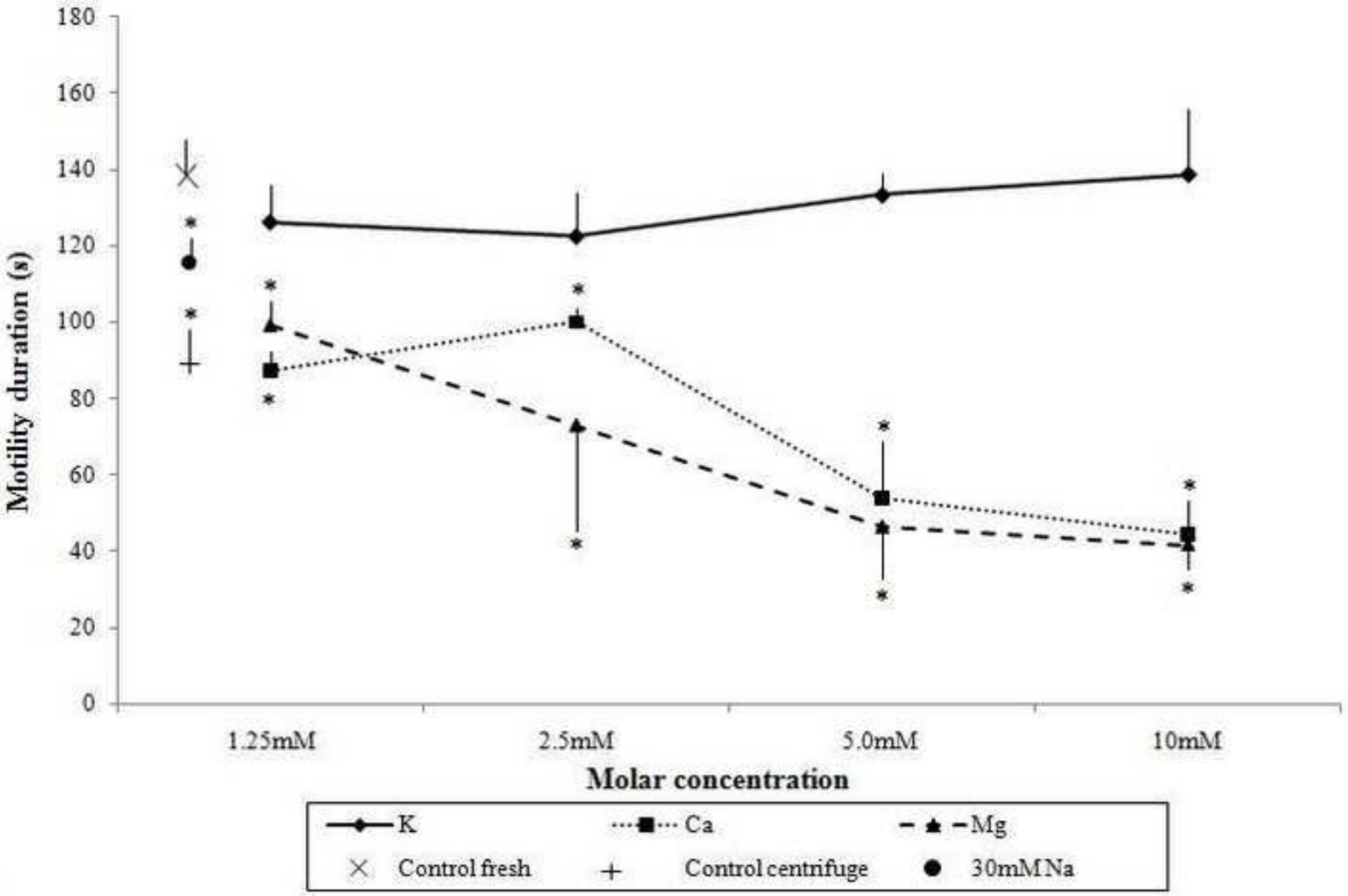
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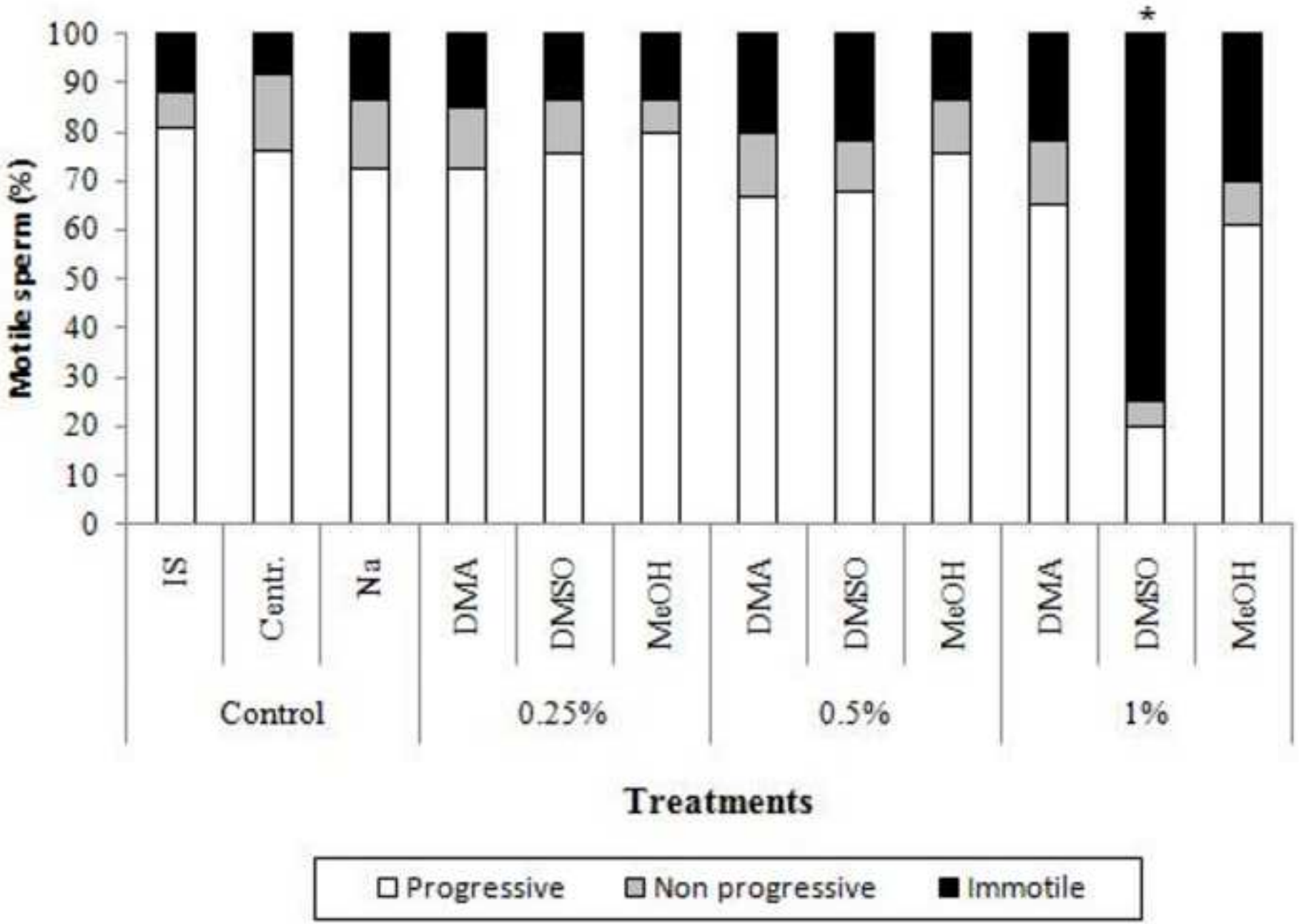
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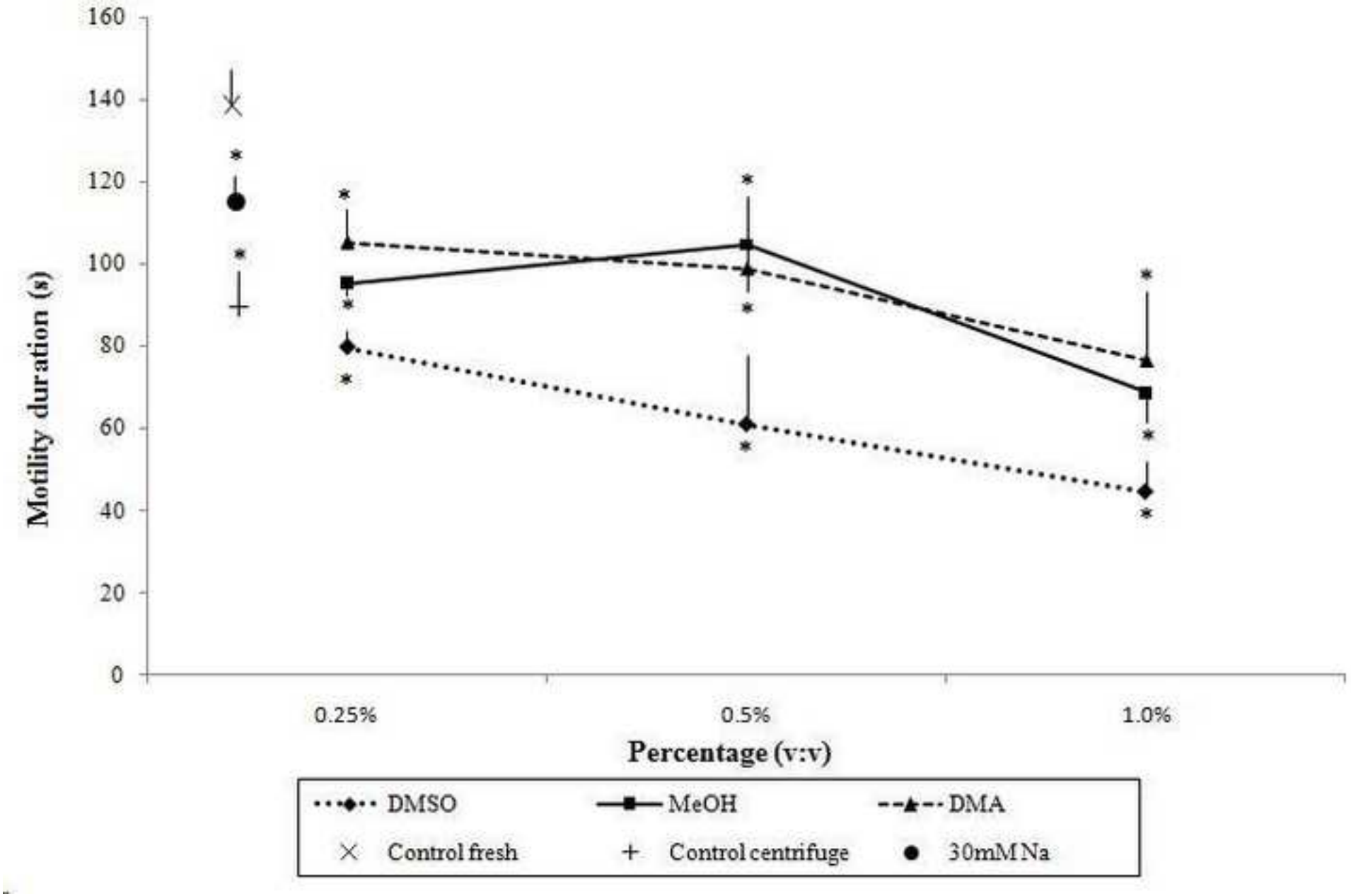
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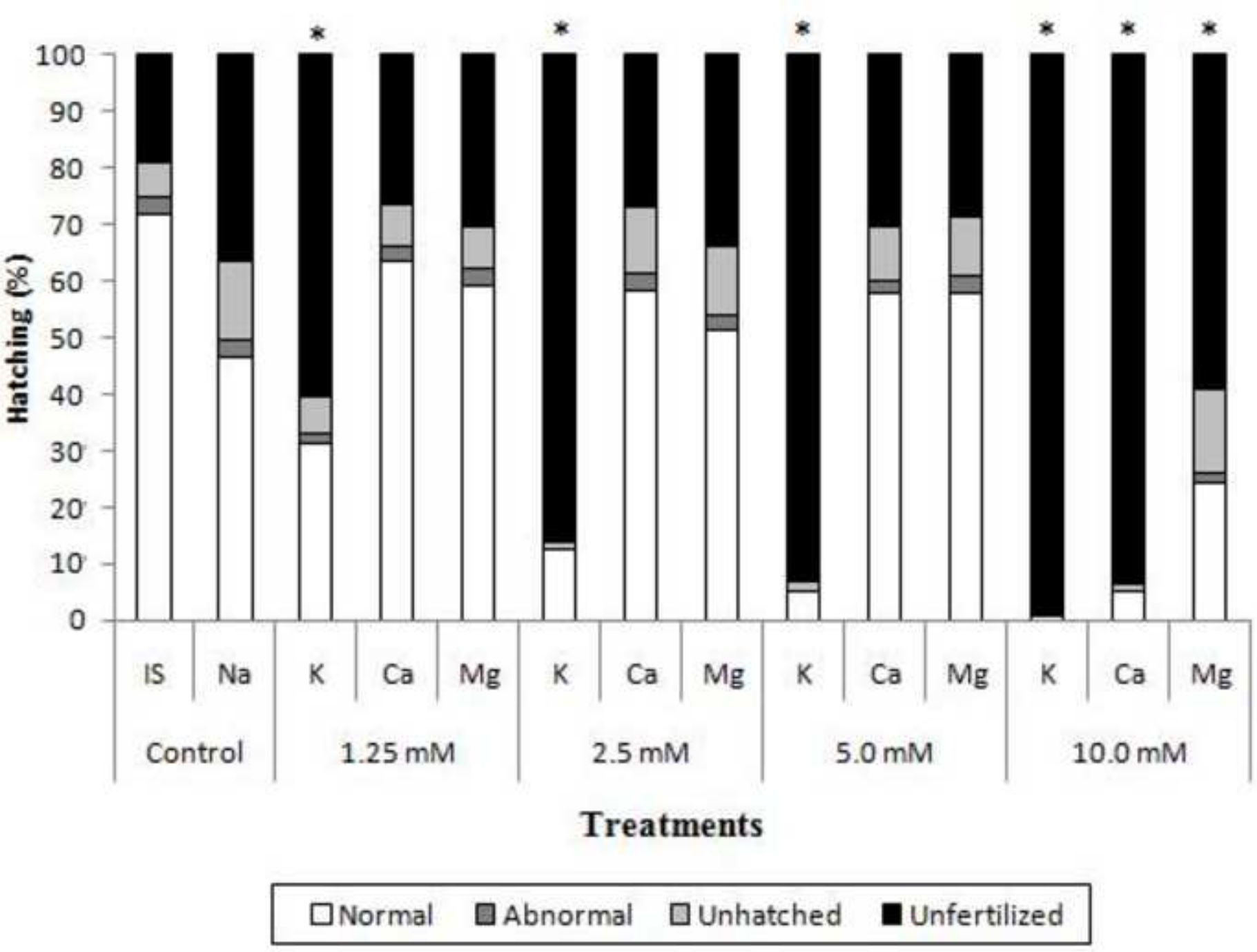
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