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 determine safe concentrations or even eliminate from sperm diluents. Increasing understanding of these characteristics are likely to be applicable in the preparation of sperm diluents and activating solutions, and to improve the management of gametes for artificial propagation. In the loach, many kinds of solutions have been used to dilute sperm including modified Ringer solution (Suzuki *et al*., 1985), Kurokura's solution (Fujimoto *et al*., 2008; Yasui *et al*., 2008 and 2009) and NaCl 0.9% (unpublished data). For cryopreservation, we previously used potassium solution (Yasui *et al*., 2008 and 2009) and a sodium-based solution (Yasui *et al*., 2010), both containing methanol as the cryoprotectant. Most of these solutions were empirically produced or based on previous studies of other fish species. However, we do not know which component is necessary, or if it presents potentially detrimental effects. Nevertheless, it is important to produce such solutions that are appropriate for the loach in order to optimize sperm maintenance and fertilization success.

Consequently, the aim of the present study was to evaluate the toxicity of some components commonly used in sperm diluents that may affect both sperm and subsequent fertilization success in the loach. This study will allow us to prepare non-toxic extenders, cryo-solutions, and to manage sperm dilution ratios in order to maximize fertilization rates in the aquaculture setting.

2. Materials and Methods

2.1 Gamete sampling and evaluation of sperm motility

Wild parental fishe were obtained from Iwamizawa city (Hokkaido Island, Japan) during the spawning season (June - August). Gamete maturation was achieved as described in our previous work (Fujimoto *et al.*, 2004) with a single dose of human chorionic gonadotropin (Aska Pharmaceuticals, Tokyo - 100 I.U. per male and 500 I.U. per female) injected intraperitonially. After 10 to 12 h at 27°C, fish were anesthetized in 2-phenoxyethanol solution (0.1%) and oocytes were collected in 2 mL tubes by stripping (approximately 1.5 mL of oocytes). Sperm was collected using glass hematocrit tubes and transferred immediately to a 2 mL tube containing 800 µL of immobilizing solution (Table 1) and gently mixed by vortexing.

In order to evaluate sperm motility, 1 µL of sperm was pipetted onto a glass slide previously coated with 0.1% BSA solution to prevent sperm attachment to the surface. Activation was performed by a 20-fold dilution in distilled water, and sperm motility was observed under the microscope at 400x magnification. Sperm motility was captured by a digital camera (Olympus C-3040) connected to a microscope, and projected onto an LCD screen (LCD-13A1, Sanyo, Japan). Video sequences of sperm motility were obtained using a VHS recorder (VC-HF920, Sharp, Japan). From video sequences, we measured the percentage of total motility, progressive motility and the duration of motility, following the criteria used in our previous work (Yasui *et al.*, 2008 and 2009; Fujimoto *et al.*, 2008). Total motility denoted any type of sperm movement (locally motile, circling or forward motility), while progressive motility regards only to those cells exhibiting forward movement. The duration of sperm motility was measured from activation until ~5% of progressive motility.

2.2 The effects of ions and cryoprotectants upon motility parameters

In this experiment we evaluated the motility characteristics of sperm suspensions at different concentrations of ions (sodium, calcium, magnesium and potassium) and cryoprotectants (methanol; MeOH, dimethyl acetamide; DMA and dimethyl sulfoxide; DMSO). Sperm from five males were pooled in 800 µL of immobilizing solution and the volume was equally divided into eight tubes (200 µL) followed by centrifugation at 2200 X g for 120 sec. The supernatant was removed and the sperm pellet was re-suspended in 60 µL of various concentrations of NaCl, $MgCl_2$, KCl and $CaCl_2$. This procedure lasted less than 10 minutes from mixing and sperm evaluation and resulted in concentrations of magnesium, potassium and calcium at 0, 25, 50, 100 and 200 mM (Table 1). For each solution, the sodium content was manipulated to provide the same theoretical osmolarity of 600 mOsm L^{-1} (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 significantly reduced hatching rates. Potassium at 1.25 mM yielded hatching rates of 33.1%, and at 2.5 mM, the same parameter decreased to just 12.5%. At 5 mM, fertilization rate was only 4.9% and at the highest concentration (10 mM), just 0.5% of eggs hatched. Calcium and magnesium also caused a severe reduction in hatching at the highest concentrations (5.2% and 26.2 %, respectively).

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

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

4. Discussion

In the present study, we demonstrate that sperm motility parameters were affected by the addition of calcium, magnesium and DMSO. Calcium has previously been reported to exhibit negative effects upon the sperm of some fish species (Christen *et al*., 1987; Cosson *et al*., 1991). Divalent cations such as calcium and magnesium have the ability to induce membrane fusion or adhesion (Takeda and Kasamo, 2002, Ahkong *et al*., 1975; Ohki *et al*., 1985). Consequently, the reason for sperm aggregation observed in media that contained high concentrations of calcium and magnesium in the present study could be attributed, at least in part, to membrane fusion/adhesion effects related to these ions. In fish, sperm fusion or adhesion was induced by calcium (Araki *et al*., 1995), high pH treatments (Ueda *et al*., 1988), and polyethylene glycol (Ueda *et al*., 1986, Kirankumar and Pandian, 2004). Our present data also indicate that we can add magnesium to this list. The presence of sperm aggregates, combined with poor sperm motility, reduced the accuracy of sperm motility evaluation. Evaluation of sperm motility requires the observation of an adequate number of cells in the viewing field, and sperm cells must be sufficiently dispersed to be analyzed individually (Billard and Cosson, 1992). So, some of our sperm motility data may be considered inaccurate, especially in calcium and magnesium treatments at 5 and 10 mM in which many sperm aggregates were observed and then reduced the accuracy during evaluation of motility parameters.

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

We observed that motility parameters were not affected by potassium treatments but fertilization rates were reduced. Similar results have been reported in other teleost species. In the common carp (*Cyprinus carpio*), for example, reduced fertilization rates were observed from 5 mM of potassium (Saad and Billard 1987). In our present study, potassium concentrations of just 1.25 mM led to reduced fertilization rates. Previously, Lahnsteiner *et al.* (2003) also observed reduced fertilization rates in *Chalcalburnus chalcalburnus*, when a KCl-based solution was used as the fertilization medium. The hatching rate of this species was 98.7% in a NaCl-based solution, but the rate decreased to just 1.6% in a KCl - based solution. The authors hypothesized that potassium solutions could exert negative effects upon eggs or sperm. Potassium has a strict relationship with the transportation of ions through cell membranes. Consequently, high potassium concentrations or an unbalance on sodium/potassium relationship in extracellular media may affect the transportation of other ions necessary for fertilization and/or egg development.

Historically, potassium ions have proved to be a highly controversial issue for fish gametes. In the case of salmonid sperm, potassium is important in order to keep cells immotile and to maintain their viability (Gatti *et al.*, 1990; Benau and Terner, 1980) as in the case of cyprinids (Morisawa *et al.*, 1983). Additionally, Linhart *et al.* (2008) used potassium solutions in order to improve sperm motility and showed that even activated sperm were able to re-initiate motility following incubation in a high potassium solution. In herring (*Clupea pallasi*), sperm remained immotile at low concentration of potassium, whilst the addition of 9 mM of potassium led to some spermatozoa immediately entering the micropyle (Yanagimachi *et al*., 1992). On the other hand, it is also important to consider that potassium exerts deleterious effects upon fertilization, as discussed above.

We conclude that high potassium concentrations should be avoided in the preparation of extenders or activating media for fish species, with the exception of species where sperm maintenance is specifically driven by potassium, as is the case of salmonids or sturgeon species (see review by Alavi and Cosson, 2006).

We also evaluated the toxicity of the predominantly successful cryoprotectants for fish sperm, using the optimal range for most species (5-20% - see review by Billard and Zhang, 2001 and our studies on loach described by Yasui *et al*., 2008, 2009 and 2010). At the highest concentration (20%), DMA (2.16 M) presented decreased fertilization rates although methanol (4.96 M) did not affect sperm motility and fertilization rates. Such comparison suggests that the decreased fertilization rates were caused by the cryoprotectant but not specifically related to molar concentration. Specific ions and cryoprotectants, and their relative concentrations, may cause severe physiological effects including the influx of cryoprotectants into the cell (Mazur *et al*., 1984), altered membrane characteristics causing the induction of membrane fusion (Araki *et al*., 1995, Ueda *et al*., 1986, 1988), dehydration, ionic signalling and egg activation (Coward *et al*., 2002), the removal of sperm attractants in the region of the micropyle (Yanagimachi *et al*., 1992), and other important mechanisms relating to fish gametes and successful fertilization. However, we still do not know which mechanism underlies the observed effects in loach gametes.

Based in our findings, ions and cryoprotectants may exert negative effects upon the fertilization of loach eggs via two mechanisms. Firstly, ions and cryoprotectants may damage sperm cells and cause reduced motility, thereby reducing the chances of successful penetration into the egg micropyle. Secondly, these factors may reduce fertilization rates, but without reducing sperm motility. The mechanism by which potassium may exert such inhibitory actions remains unknown and requires future studies. Based on the discussion above and considering that molar concentration is the main factor inhibiting the loach sperm motility, non-toxic components (such as NaCl) are preferable for preparation of sperm diluents and optimize the fertilization success. However, further studies are necessary to evaluate the effects of longer sperm storage periods. In addition, we recommend that methanol should be used for the preparation of cryo-solutions for loach since it is an effective cryoprotectant for this species (Yasui et al., 2008, 2009) and in addition as observed here, methanol do not affect sperm characteristics and subsequent fertilization rates.

In conclusion, ions and cryoprotectants may affect both, sperm motility and fertilization ability. We also showed with potassium treatments that sperm motility is not always an effective predictor of fertilization success. Our findings suggest that a balanced 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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6. References

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Table 1. Sperm diluents composed with KCl, NaCl, $CaCl_2$ and $MgCl_2$. All diluents had the same theoretical osmolarity at 600 mOsm L^{-1}. Immobilizing solution (IS, Kurokura et al., 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)

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

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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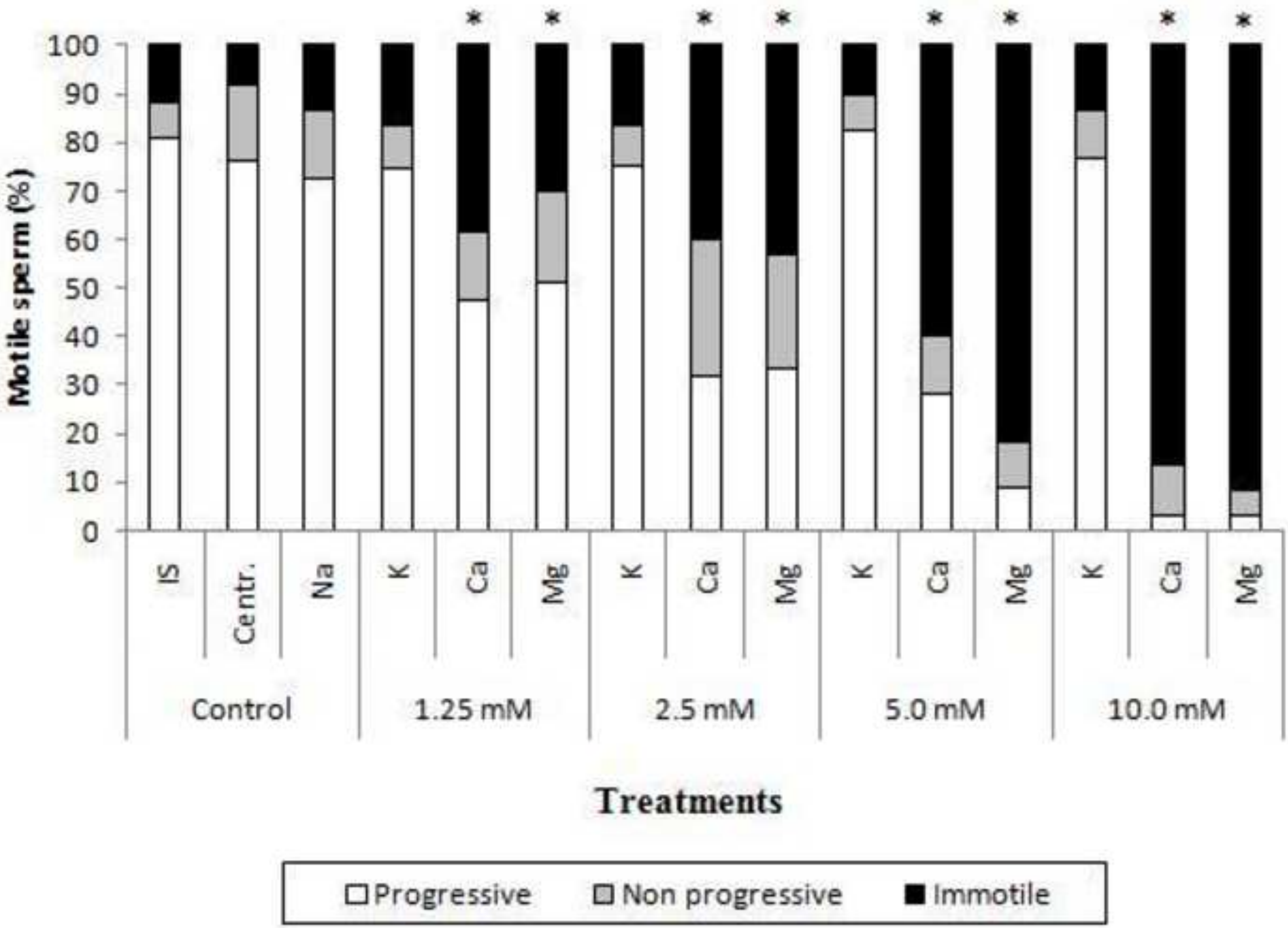

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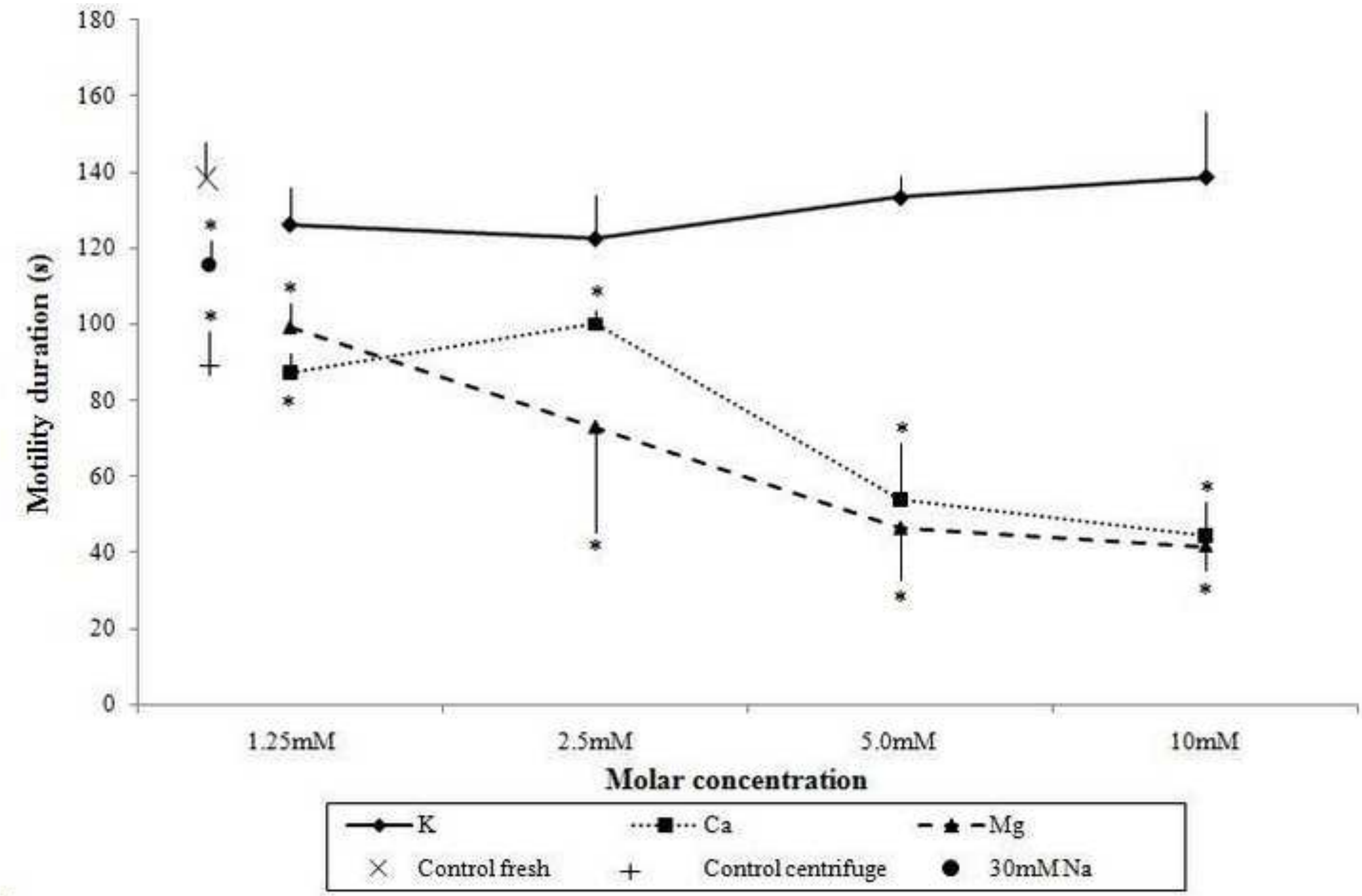

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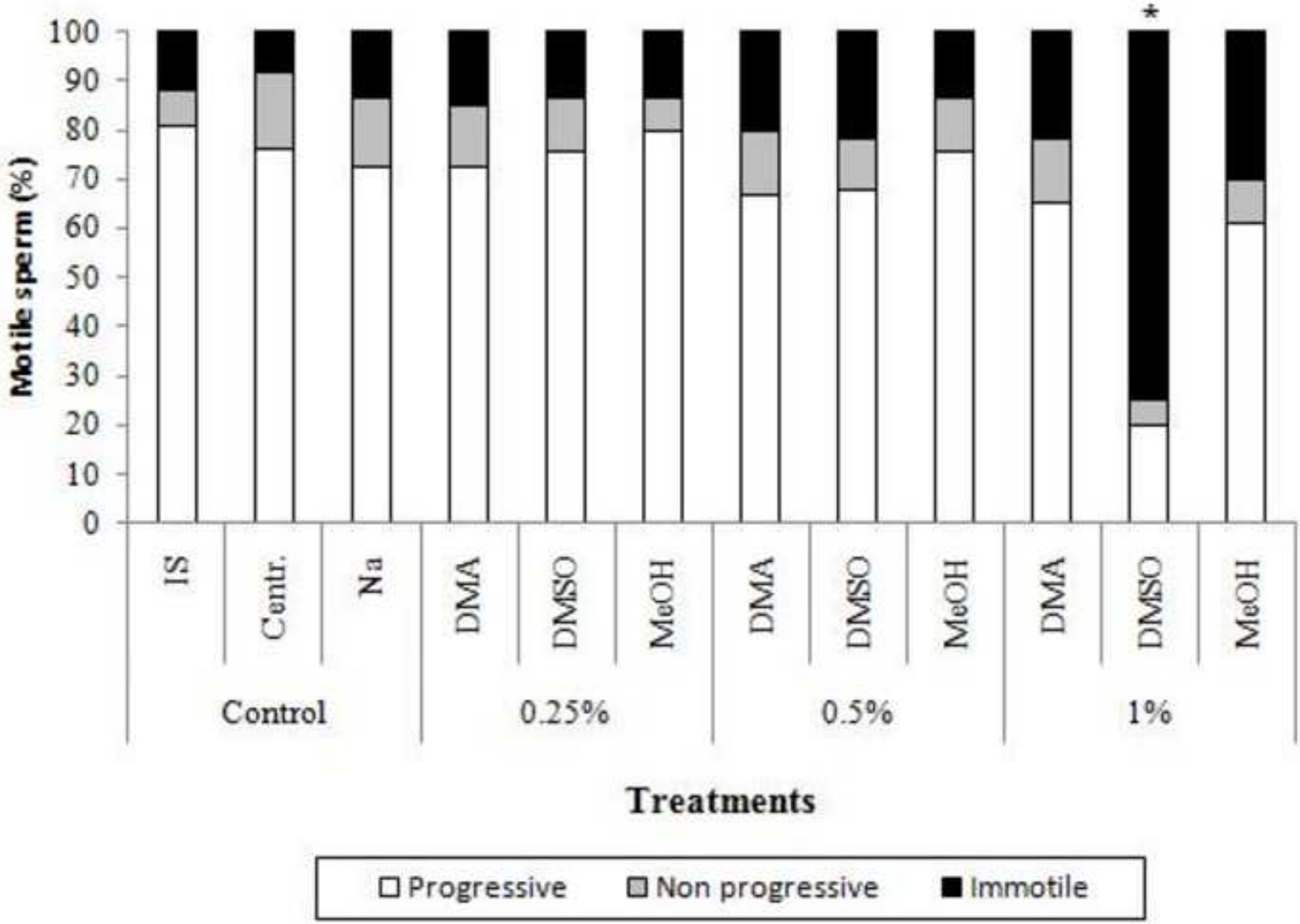

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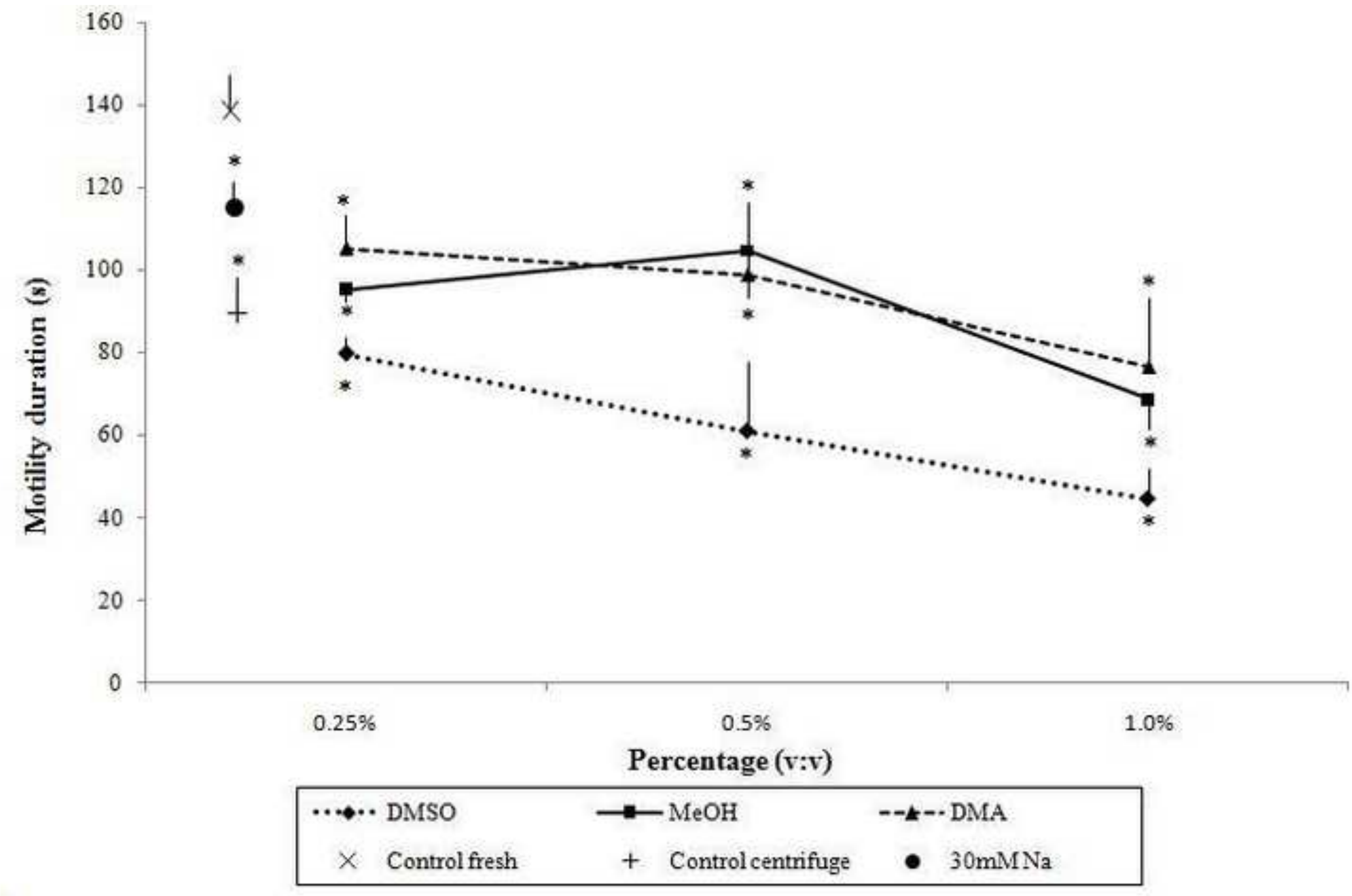

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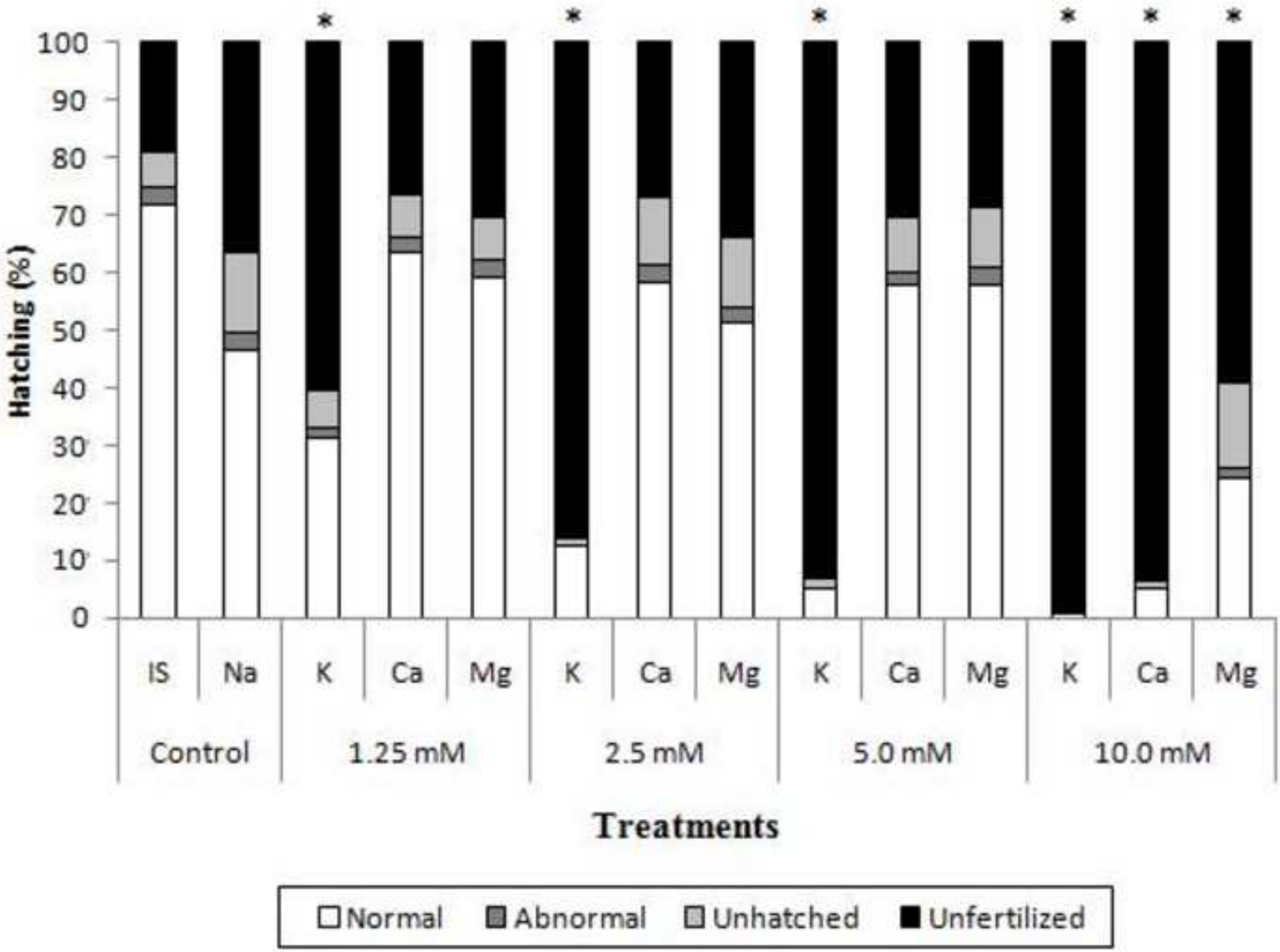

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