Temperature-dependent regulation of sperm motility of Ijima's copper pheasants (*Syrmaticus soemmerringii ijimae*), one of 'near threatened' species

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ABSTRACT

In order to conserve the copper pheasants, one of the Japanese 'near threatened' species, the knowledge on the sperm characteristics is the inevitable issue. Therefore, temperature-dependent regulation of copper pheasant sperm motility was investigated in comparison with that of domestic fowl spermatozoa. Motility of intact spermatozoa from both species was markedly affected by temperature. During incubation at 30°C, copper pheasant spermatozoa showed around 60-70% motility, but became almost immotile when the temperature was raised to 40°C. Then, when the temperature of the sperm suspension was subsequently cooled to 30°C, the spermatozoa regained their motility. The motility of domestic fowl spermatozoa showed a similar pattern. Temperature also affected the motility of both demembranated copper pheasant and domestic fowl spermatozoa in the same way. The motility of intact copper pheasant and domestic fowl spermatozoa at 30°C was unaffected following the addition of 2 mM $CaCl_2$, 100 nM calyculin A, an inhibitor of protein phosphatase-type 1 (PP1), or 4 mM diB-cAMP, respectively, compared with those with no effectors. However, the presence of 10 μM ML-7, a selective inhibitor of myosin light chain kinase (MLCK), inhibited motility of spermatozoa from both species. At 40°C, the presence of $CaCl_2$ or calyculin A restored the motility of spermatozoa from both species, but the addition of diB-cAMP or ML-7 could not prevent the immobilization of spermatozoa. At 30°C in the presence of ATP, the motility of demembranated copper pheasant spermatozoa was over 60% but was inhibited following the addition of 10 μM ML-7; a similar pattern was found with demembranated domestic fowl sperm motility. The motility of demembranated spermatozoa from both species was inhibited following the addition of 2 mM EGTA to the reactivation

medium at 30°C, but restored by the subsequent addition of 4 mM $CaCl_2$. These results suggest that copper pheasant sperm motility might be regulated by similar mechanisms to that of domestic fowl spermatozoa: i.e., the balance of Ca^{2+}/MLCK or an MLCK-like protein-dependent phosphorylation and PP1-dependent dephosphorylation. The similarity in physiological regulation of spermatozoa from both species shows that extensive technology developed for artificial breeding of the domestic fowl might be applicable to captive breeding of copper pheasants.

Keywords: avian spermatozoa, immobilization, temperature, flagellar movement, phosphorylation, calcium

1. Introduction

The copper pheasants (*Syrmaticus soemmerringii*) are native to Japan and inhabit coniferous forest and adjoining mixed forest with dense undergrowth and grassy hillsides in mountainous regions at elevations below around 1,300 m. Their diet consists mainly of acorns and mast of such forest trees, which are gathered from the forest floor. They also eat many kinds of insects, earthworms and crabs (Johnsgard, 1999). Due to ongoing habitat loss, limited range and overhunting in some areas, the copper pheasant is evaluated as 'Near Threatened' on the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species (IUCN Red List, 2008).

The Ijima (*Syrmaticus soemmerringii ijimae*) is distributed in southern Kyushu and is one of five subspecies. The male is easily recognized by the large white patch on the lower back and rump. He has a beautiful long tail (around 70-80cm), about twice the length of his body. As its name suggests,

the colour of the male is a coppery reddish brown which with the tiniest ray of light shines more like gold. The female is a very pretty bird, although she lacks the iridescence and long tail of her male counterpart. She is heavily flecked with many shades of brown and black with a white stripe under her eye and, on some birds, red can be seen round the eye too. As with most hens, her colouring gives her excellent camouflage (Downie and Hunter, 2009).

Copper pheasant and domestic fowl (*Gallus gallus domesticus*) are the same order (*Galliformes*) and family (*Phasianidae*), but different genus and species. Unlike domestic fowl, the breeding season of copper pheasant is short with rapid and robust reproductive organs response at the onset of the season (from middle of March to the end of May in Miyazaki, Japan). Copper pheasants respond to the same semen collection and insemination techniques that are used for domestic fowl: i.e., the abdominal massage method is the most common method for semen collection. The quantity of semen ranged from 0.01 to 0.02 ml per ejaculation: rather small quantity compared with domestic fowl (0.5-0.8 ml), but more concentrated (approximately 10 x 10^9 cells/ml) than domestic fowl semen (approximately 3.5 x 10^9 cells /ml) (Maru et al., 1966; Smyth, 1968; Sugimoto et al., 2001).

Abundant evidence has shown that flagellar movement of spermatozoa is based on the active sliding of microtubules as a result of ATP hydrolysis by dynein ATPase of the outer doublet microtubules (for review, see Satir, 1979; Warner and Mitchell, 1980; Gibbons, 1982; Mohri, 1993). Although this basic axonemal mechanism is fairly well understood, many factors and mechanisms of its regulation have still to be clarified. For example, unlike mammalian spermatozoa, domestic fowl spermatozoa display the characteristic phenomenon of reversible temperature-dependent immobilization: in simple salt solutions, they become immotile at the avian body temperature of 40-41^{o}C, but motility is restored by decreasing the

temperature or by the addition of Ca^{2+} at 40°C (Munro, 1938; Ashizawa and Nishiyama, 1978; Wishart and Ashizawa, 1987; Ashizawa et al., 1989a; Thomson and Wishart, 1989, 1991; Ashizawa et al., 1994a). Ca^{2+} is the major stimulatory factor in body fluids such as seminal plasma, or the fluid of the female ovarian pocket taken at the time of ovulation, in which spermatozoa can maintain their motility even at 40°C (Ashizawa and Wishart, 1987, 1992). The axoneme and/or accessory cytoskeletal components themselves appear to be directly involved in the temperature-dependent regulatory system, since the motility of demembranated spermatozoa is, as with intact spermatozoa, negligible at 40°C and restored at 30°C (Ashizawa et al., 1989a, b, 2000). However, the intracellular molecular mechanisms involved in immobilization and restoration of motility are still unknown.

From invertebrate to vertebrate species, intracellular cascade systems for the regulation of sperm motility are triggered by several factors, mainly second messengers such as Ca^{2+} and cyclic nucleotides, which seem to converge upon the phosphorylation and dephosphorylation of proteins via several kinds of protein kinases and phosphatases which regulate axonemal movement (for review, see Tash and Means, 1983; Brokaw, 1987; Lindemann and Kanous, 1989; Morisawa, 1994).

Even although copper pheasant and domestic fowl are the same order and family, no information is available concerning the characteristics of copper pheasant sperm motility, especially whether they display the same characteristic phenomenon of reversible temperature-dependent immobilization at body temperature as those of domestic fowl spermatozoa. If so, what kinds of factors are involved in the regulation of sperm motility? In the following experiment, therefore, attempts were made to investigate the effects of temperature on the motility of Ijima's copper pheasant spermatozoa compared with that of domestic fowl spermatozoa, using some stimulators

and inhibitors of signal transduction pathways.

2. Materials and methods

2.1. Animals and preparation of spermatozoa

Ijima's copper pheasant males were obtained from eggs collected from the same nest of grassy hillside in Miyazaki Prefecture, Japan, and then incubated and hatched in incubator and raised for more than one year with permission from Miyazaki Prefectural government. Commercial White Leghorn roosters (Dekalb strain) were purchased from Amuse Poultry Breeding Farm, Miyazaki, Japan. All birds were housed in individual cages and fed *ad libitum* on a commercial breeder diet. Roosters were exposed to a photoperiod of 14 h light : 10 h darkness, but copper pheasant males were exposed to natural conditions of light, so semen could be collected during only breeding season (from March to May).

Semen was collected by the method of Bogdonoff and Shaffner (1954). Samples of semen pooled from four to six males were diluted approximately tenfold in 150 mM NaCl with 20 mM TES (*N*-Tris-[hydroxymethyl]-methyl-2-aminoethanesulfonic acid) at pH 7.4 and centrifuged at 700 **g** for 13 min at room temperature (20-25^{o}C). The washed spermatozoa were reconstituted in the same buffer to give a final concentration of approximately 1 x 10^{9} cells/ml.

2.2. Chemicals

Adenosine 5'-triphosphate (ATP), dibutyryl adenosine 3':5'-monophosphate (diB-cAMP; a cell-permeable cAMP analog), dithiothreitol, 1-(5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-7, a cell-permeable selective inhibitor of myosin light

chain kinase), potassium glutamate, TES and Triton X-100 were purchased from Sigma Chemical Co. (St Louis, MO, USA). Calyculin A, a cell-permeable inhibitor of protein phosphatase-type 1 and -type 2A, was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals were of reagent grade from Nacalai Tesque, Inc. (Kyoto, Japan).

2.3. Analysis of motility of spermatozoa

The intact copper pheasant and domestic fowl sperm preparations were incubated aerobically in a water bath at 30°C for 6 min, and then transferred to another water bath and incubated at 40°C for 9 min. After that, they were re-incubated at 30°C for 9 min. Finally, they were moved to 40°C again and incubated for 9 min. Overall, the samples were incubated for 33 min, including two periods at each of 30°C and 40°C. During incubation, sperm motility were measured every 3 min at each temperature. The effects of the addition of $CaCl_2$, calyculin A, an inhibitor of protein phosphatase-type 1 and -type 2A, ML-7, a selective inhibitor of myosin light chain kinase (MLCK) and diB-cAMP were also examined at 30°C and 40°C to be compared to controls with no effectors. The inhibition constant (K_i) values of calyculin A for protein phosphatase-type 1 and -type 2A are around 2 nM and 0.5-1 nM, respectively (Ishihara et al., 1989) and the K_i value of ML-7 for MLCK is around 0.3 μM (Saitoh et al., 1987). Ordinarily, 10-100-fold higher concentrations are required for whole cells. Diluent for the measurement of intact sperm motility was TES/NaCl buffer described above.

The demembranated copper pheasant and domestic fowl sperm samples were also incubated at 30°C then 40°C, twice over and sperm motility was measured every 3 min at each temperature, as for intact spermatozoa. Demembranation and reactivation of spermatozoa were performed according to the method described previously (Ashizawa et al., 1989b). The extraction

197 medium used consisted of 0.1% (v/v) Triton X-100, 200 mM sucrose, 25 mM
198 potassium glutamate, 1 mM $MgSO_4$, 1 mM dithiothreitol and 20 mM
199 Tris-HCl buffer (pH 7.9). The reactivation medium consisted of 0.5 mM
200 ATP, 200 mM sucrose, 25 mM potassium glutamate, 1.5 mM $MgSO_4$, 1 mM
201 dithiothreitol, 20 mM Tris-HCl buffer (pH 7.9). To examine the effects of
202 Ca^{2+} and MLCK, the time courses of motility following the addition of EGTA,
203 $CaCl_2$ and ML-7 were also examined at 30°C.
204 The suspension of spermatozoa was placed into a microscope slide
205 chamber (Sekisui Chemical Co., Ltd., UR-157 type, Tokyo, Japan) on a
206 thermostatically-controlled warm plate, and the motility of spermatozoa was
207 recorded by videomicroscopy (magnification on the 12-inch black and white
208 monitor was approximately x 600) at 30°C or 40°C (Katz and Overstreet,
209 1981). Measurements were made on a total of 200-300 spermatozoa,
210 distributed uniformly among three or more fields, to determine the percentage
211 of vigorously motile spermatozoa. The percentage of vigorously motile
212 spermatozoa was defined as ‘motility’ in this paper.
213
214 *2.4. Statistical analysis*
215 All statistical analyses were carried out by the Statistical Analysis
216 System R software package (R Development Core Team, 2008). For the
217 evaluation of differences, the percentages of motility were analyzed by the
218 generalized linear model (GLM, binomial error distribution) procedure
219 followed by the Tukey test for non-parametric multiple comparisons (Ryan,
220 1960).
221
222 **3. Results**
223
224 *3.1. Effects of temperature on the motility of copper pheasant and domestic*

fowl spermatozoa

Motility of both intact copper pheasant and domestic fowl spermatozoa was markedly affected by the temperature (Fig. 1). During the first incubation at 30°C, copper pheasant spermatozoa showed around 60-70% motility, and even after a second incubation at 30°C, a similar proportion of motile spermatozoa was found. In contrast, these spermatozoa lost their motility quickly at 40°C. Temperature also affected the motility of demembranated copper pheasant spermatozoa as well as those of domestic fowl (Fig. 2). Motility was maintained vigorously at 30°C but remained almost negligible at 40°C, then when the temperature of the sperm suspension was subsequently cooled to 30°C, the spermatozoa regained their motility.

3.2. Effects of various effectors on the motility of copper pheasant and domestic fowl spermatozoa

Figure 3 shows sperm motility at 10 min after the addition of various effectors. The motility of intact copper pheasant and domestic fowl spermatozoa at 30°C was unaffected 10 min after the addition of 2 mM $CaCl_2$, 100 nM calyculin A or 4 mM diB-cAMP, respectively, compared with controls with no effectors. However, the presence of 10 μM ML-7 inhibited copper pheasant and domestic fowl sperm motility significantly ($P<0.05$) (Fig. 3A). At 40°C, the motility of intact copper pheasant and domestic fowl spermatozoa was negligible. However, the presence of $CaCl_2$ or calyculin A permitted restoration of motility, but the addition of diB-cAMP or ML-7 could not prevent the immobilization of spermatozoa from either species (Fig. 3B).

The time-courses of demembranated copper pheasant and domestic fowl motility at 30°C, following various additions are shown in Figs. 4 and 5. In the presence of ATP, the motility of demembranated copper pheasant

spermatozoa without ML-7 was over 60%. However, inhibition of motility of demembranated spermatozoa was observed following the addition of 10 μM ML-7. Similar effects were obtained for domestic fowl sperm motility (Fig. 4).

In the reactivation medium, $CaCl_2$ was not added at the beginning of incubation, but a 'trace/contaminated' calcium ion was present at around 100 nM, since EGTA was not added to the medium. Therefore, copper pheasant spermatozoa and domestic fowl spermatozoa were able to move in this condition. However, neither copper pheasant spermatozoa nor domestic fowl spermatozoa were motile following the addition of 2 mM EGTA to the reactivation medium. However, spermatozoa regained their motility after the subsequent addition of 4 mM $CaCl_2$ (Fig. 5).

4. Discussion

In general, it is expected that the rate of cellular functions such as motility should become faster as the temperature is raised. Whilst this is true for domestic fowl spermatozoa suspended in a simple salt solution as the temperature is raised from 20°C to 35°C, beyond this temperature sperm motility decreases, so that at the avian body temperature of 40-41°C, the spermatozoa are completely immotile. This characteristic phenomenon was first reported by Munro (1938) who also noted that when the temperature of the sperm suspension was subsequently cooled to 30°C, the spermatozoa regained their motility, so that the phenomenon is known as reversible, temperature-dependent motility immobilization (Munro, 1938; Ashizawa and Nishiyama, 1978; Ashizawa et al., 1989a).

The present study clearly showed that copper pheasant spermatozoa, the same order and family, but different genus and species from domestic

fowl, display the same characteristic phenomenon as those of domestic fowl spermatozoa (Fig. 1). The pattern of temperature-dependent inhibition was also found in drake spermatozoa and, partially, in turkey spermatozoa. However, no such temperature-dependent inhibition was found in spermatozoa from Japanese quail and Houbara bustard (Wishart and Wilson, 1999). These observations suggest that such immobilization might not simply occur at the same classification in taxonomic ranks, since quail and domestic fowl/copper pheasant are the same order (*Galliformes*) and family (*Phasianidae*), and turkey is the same order (*Galliformes*) but different family (*Meleagrididae*) compared with domestic fowl/copper pheasant. On the other hand, drake (order: *Anseriformes*, family: *Anatidae*) and bustard (order: *Gruiformes*, family *Otididae*) are completely different from domestic fowl/copper pheasant. Difference in sperm motility profiles were also observed during sperm maturation in the testis of the domestic fowl, pigeon and drake on the one hand, and in the passerine song sparrow on the other. Domestic fowl spermatozoa, when removed from the testis were able to display some motility (Ashizawa and Sano, 1990). Testicular spermatozoa from pigeon and drake were also shown to have the capacity for motility, although not spermatozoa from the passerine song sparrow (Bedford, 1979). The proportion of intact testicular spermatozoa from the domestic fowl was shown to be around 75% of that of ejaculated spermatozoa and their velocity around 50%. However, for demembranated spermatozoa, the proportion of motile and velocity of testicular spermatozoa were each around 10% of ejaculated spermatozoa, suggesting that the immaturity of testicular spermatozoa resides mainly in their axonemal response to added ATP (Ashizawa and Sano, 1990).

If copper pheasant spermatozoa could remain motile at 40^{o}C after removing the plasma membrane, it was suspected that the plasma membrane

and/or cytosol might be involved in the immobilization of copper pheasant spermatozoa at 40°C. However, the motility of demembranated spermatozoa was, as with intact spermatozoa, almost negligible at 40°C (Fig. 2). These results suggest that the mechanisms involved in the immobilization of copper pheasant spermatozoa at 40°C do not involve the plasma membrane and/or cytosol, but the axoneme and/or accessory cytoskeletal components themselves as found for those of domestic fowl spermatozoa.

In the present study, when 2 mM $CaCl_2$ was added, the motility of intact copper pheasant and domestic fowl spermatozoa at 30°C was unaffected, respectively, compared with the control (no addition of effectors) (Fig. 3A). However, at 40°C, the presence of $CaCl_2$ permitted restoration of motility (Fig. 3B). Therefore, Ca^{2+} seems to play a key role in the stimulation of motility of copper pheasant and domestic fowl spermatozoa at the avian body temperature. Furthermore, the motility of intact domestic fowl spermatozoa was inhibited at both 30°C and 40°C by loading spermatozoa with the intracellular calcium chelator BAPTA/AM, but the inhibition could be released instantly by the addition of excess calcium to the medium (Ashizawa et al., 1994a). The locus of action of calcium is likely to be the axoneme and/or accessory cytoskeletal components, since the present study showed that in demembranated copper pheasant and domestic fowl spermatozoa at 30°C, the addition of 2 mM EGTA to sequester 'contaminant' calcium, arrests the motility, and then spermatozoa regained their motility after the subsequent addition of 4 mM $CaCl_2$ (Fig. 5).

Substantial evidence implicates a major role for protein phosphorylation and dephosphorylation systems in the regulation of mammalian sperm movement, and the predominant system involves protein kinase A (PKA), which is dependent on the second messenger, cyclic AMP (cAMP), and has been identified in several species (for review, see Tash and

Means, 1983; Brokaw, 1987; Lindemann and Kanous, 1989; Tash, 1989; Majumder et al., 1990; Morisawa, 1994). However, the addition of diB-cAMP could not prevent the immobilization of both copper pheasant and domestic fowl spermatozoa at 40°C (Fig. 3B). Furthermore, the motility of demembranated domestic fowl spermatozoa at 40°C could not be restored by the addition of cAMP (Ashizawa et al., 1989b, 1992). The addition of a PKA substrate peptide did not inhibit the motility of demembranated domestic fowl spermatozoa at 30°C (Ashizawa et al., 1995a, b). Thus, it seems unlikely that the phosphorylation of protein(s) by PKA is involved in the maintenance of copper pheasant and domestic fowl sperm motility.

As described above, calcium seems to have significant effects on copper pheasant and domestic fowl sperm motility. Instead of PKA, axonemal and/or accessory cytoskeletal myosin light chain kinase (MLCK), or an MLCK-like protein, has been proposed to be a candidate of calcium-dependent protein kinase in domestic fowl spermatozoa, since the motility of both intact and demembranated domestic fowl spermatozoa at 30°C decreased markedly following the addition of ML-9, a specific inhibitor of MLCK (Ashizawa et al., 1995b). Moreover, it may be possible that the phosphorylation of a 30-kDa protein which may be a substrate for MLCK or an MLCK-like protein might be necessary for the activation of domestic fowl sperm motility at 30°C, since the addition of an MLCK substrate peptide decreased the phosphorylation levels of this protein and inhibited the motility of demembranated spermatozoa (Ashizawa et al., 1995a). It is interesting to note that in the present work, the presence of 10 μM ML-7, another specific inhibitor of MLCK, inhibited copper pheasant and domestic fowl sperm motility at 30°C (Fig. 3A), and at 40°C, the addition of ML-7 could not prevent the immobilization of both spermatozoa (Fig. 3B). Furthermore, inhibition of motility of demembranated copper pheasant and domestic fowl

spermatozoa was observed following the addition of 10 μM ML-7 (Fig. 4). Thus, phosphorylation by MLCK or an MLCK-like protein might be necessary for the activation of not only domestic fowl spermatozoa, but also copper pheasant sperm motility.

If phosphorylation is required for the activation of flagellar motility, then dephosphorylation of proteins by specific regulatory phosphatases should reduce sperm motility. Such regulatory serine/threonine protein phosphatases are classified into four main types: PP1; PP2A; PP2B and PP2C (Cohen, 1989). With regard to dog spermatozoa, it has been proposed that inhibition of sperm motility by high concentrations of Ca^{2+} may be due to the activation of PP2B (calcineurin), a calcium-dependent protein phosphatase (Tash et al., 1988). In contrast, PP1 might be dominant in the temperature-dependent inhibition of flagellar movement of domestic fowl spermatozoa at 40°C, since the motility of demembranated domestic fowl spermatozoa at 40°C was stimulated by the addition of calyculin A or okadaic acid, specific inhibitors of PP1 and PP2A, and inhibitors 1 and 2, small heat-stable proteins which inhibit PP1 activity only (Ashizawa et al., 1994b). In addition, the motility of demembranated domestic fowl spermatozoa at 30°C decreased markedly following the addition of recombinant PP1 supplemented with Mn^{2+}. This inhibition was not restored by the addition of cAMP (Ashizawa et al., 1997). The present study showed that the temperature-dependent immotility of copper pheasant spermatozoa at 40°C was restored after the addition of calyculin A. These results suggest that PP1-mediated dephosphorylation of some proteins of copper pheasant spermatozoa may also be involved in the inhibition of motility.

In conclusion, copper pheasant sperm motility might be regulated by similar mechanisms to domestic fowl spermatozoa: i.e., the balance of Ca^{2+}/MLCK or an MLCK-like protein-dependent phosphorylation and

PP1-dependent dephosphorylation. The similarity in physiological regulation of spermatozoa from both species suggests that extensive technology developed for artificial breeding of the domestic fowl might be applicable to captive breeding of Ijima's copper pheasants, one of the Japanese 'near threatened' species.

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Legends for Figures

Fig. 1. The time course of motility of intact copper pheasant (○) and domestic fowl (●) spermatozoa during sequential incubation in TES/NaCl buffer (pH 7.4) at 30°C (0 to 6 and 18 to 24 minutes) and 40°C (9 to 15 and 27 to 33 minutes). Each point represents the mean (±S.E.M.) of five samples of spermatozoa. $*^{\#}P<0.05$ compared with value at 0 minute incubation of each spermatozoa.

Fig. 2. The time course of motility of demembranated copper pheasant (□) and domestic fowl (■) spermatozoa during sequential incubation in the reactivation medium at 30°C (0 to 6 and 18 to 24 minutes) and 40°C (9 to 15 and 27 to 33 minutes). Each point represents the mean (±S.E.M.) of five samples of spermatozoa. $*^{\#}P<0.05$ compared with value at 0 minute incubation of each spermatozoa.

Fig. 3. Motility of intact copper pheasant and domestic fowl spermatozoa at 10 minutes after the addition of various effectors (2 mM $CaCl_2$, 100 nM calyculin A, 4 mM diB-cAMP and 10 μM ML-7) in TES/NaCl buffer (pH 7.4) at 30°C (A) and 40°C (B). Each value represents the mean (±S.E.M.) of five samples of spermatozoa. Values with different superscripts differ significantly ($P<0.05$) from each other.

Fig. 4. The time course of motility of demembranated copper pheasant (■, □) and domestic fowl (●, ○) spermatozoa during incubation in the reactivation medium at 30°C following addition of 10 μM ML-7 at arrow (○, □) or without ML-7 (control) (●, ■). Each point represents the mean (±S.E.M.) of five samples of spermatozoa. $*^{\#}P<0.05$ compared with value when no inhibitor

602 was added (control) at each period.

603

604 **Fig. 5.** The time course of motility of demembranated copper pheasant (○)
605 and domestic fowl (●) spermatozoa during incubation in the reactivation
606 medium at 30°C following addition of 2 mM EGTA and 4 mM $CaCl_2$ at
607 arrows. Each point represents the mean (±S.E.M.) of five samples of
608 spermatozoa. $*^{\#}P<0.05$ compared with value at 0 minute incubation of each
609 spermatozoa.

610

611

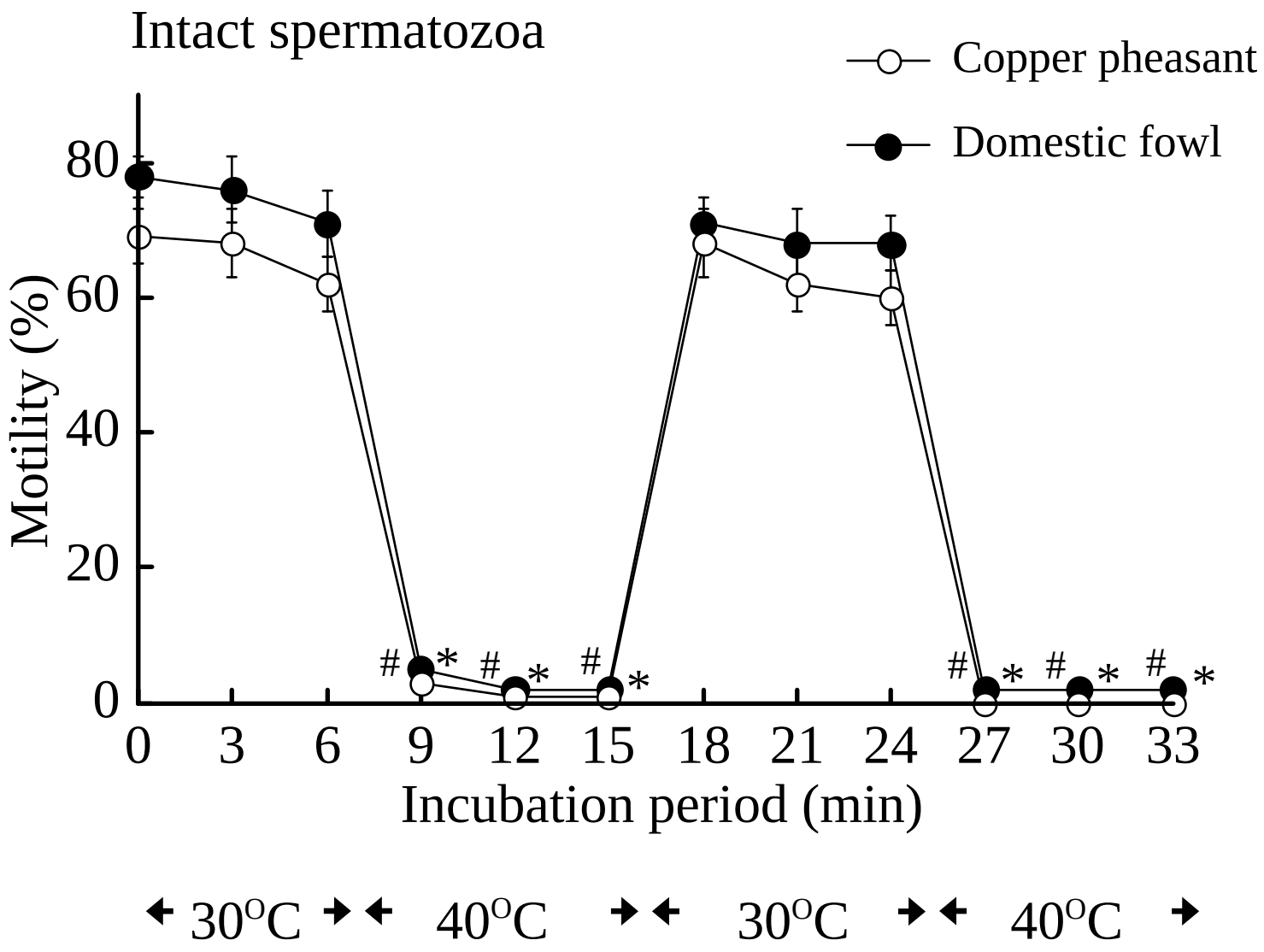
Intact spermatozoa
Copper pheasant
Domestic fowl
Motility (%)
80
60
40
20
0
0
3
6
9
12
15
18
21
24
27
30
33
Incubation period (min)
30°C
40°C
30°C
40°C

Fig. 1.

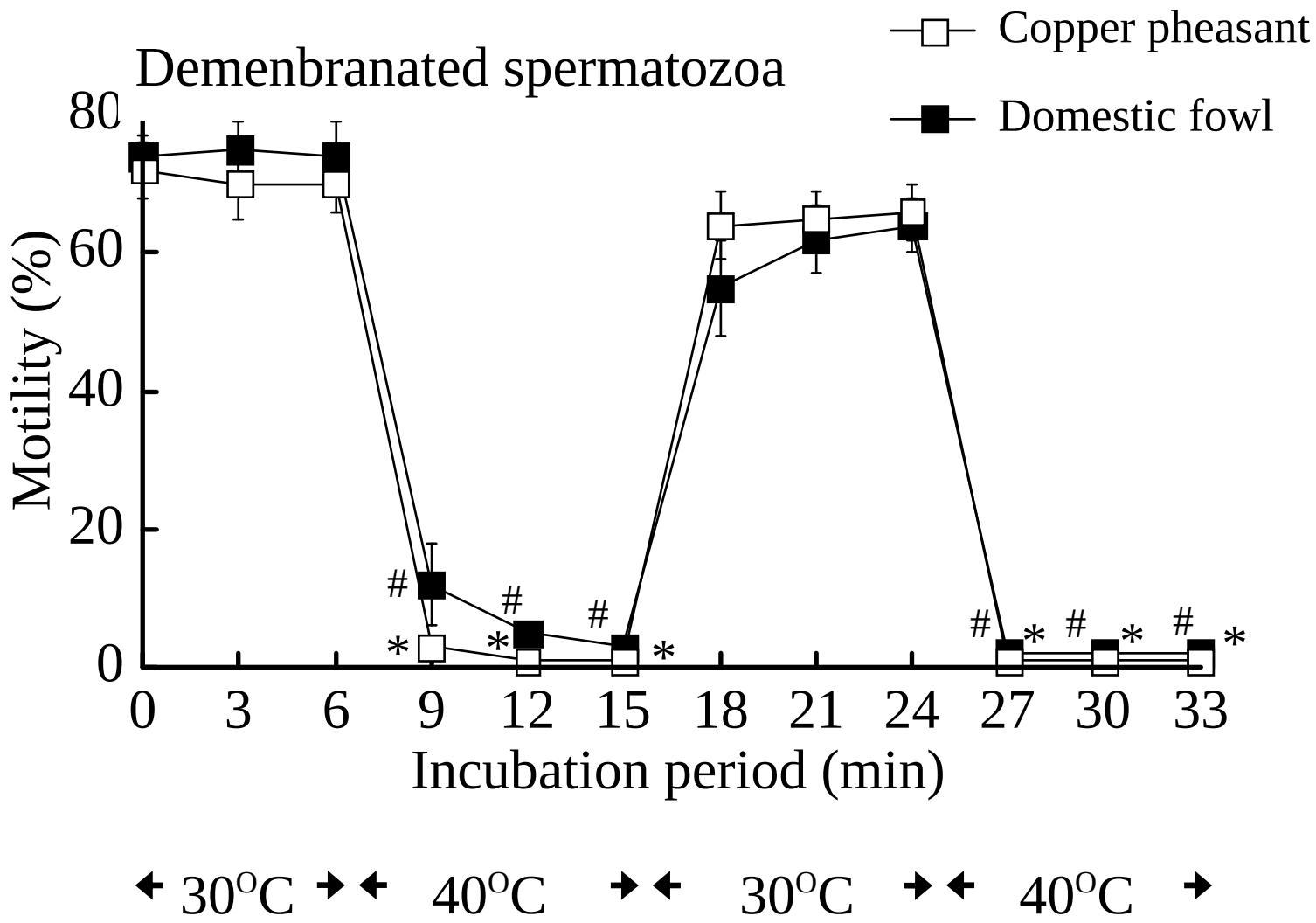
Demenbranated spermatozoa
Copper pheasant
Domestic fowl
Motility (%)
80
60
40
20
0
0
3
6
9
12
15
18
21
24
27
30
33
Incubation period (min)
30°C
40°C
30°C
40°C

Fig. 2.

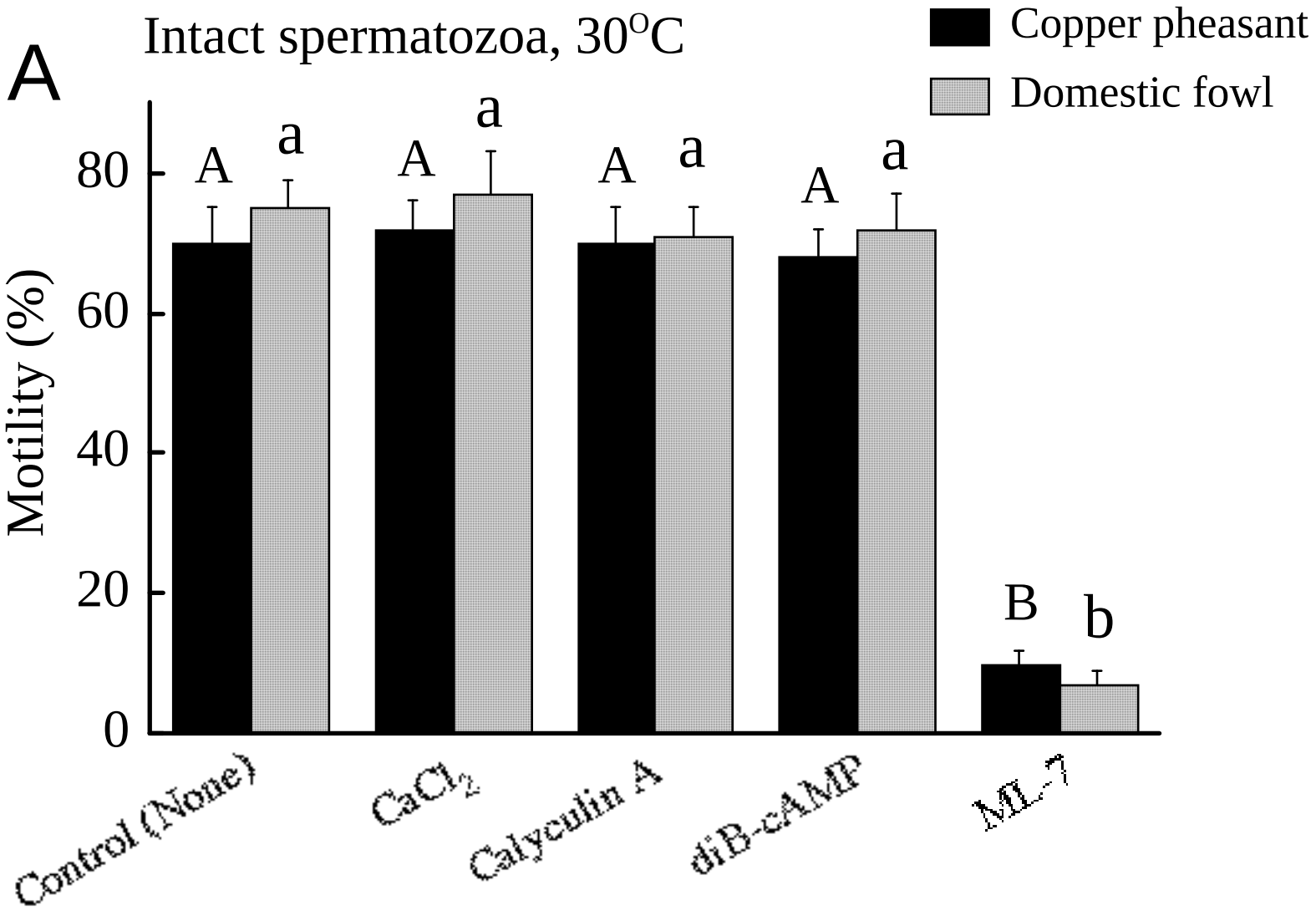
A
Intact spermatozoa, 30°C
Copper pheasant
Domestic fowl
Motility (%)
0
20
40
60
80
A
a
A
a
A
a
A
a
B
b
Control (None)
CaCl2
Calyculin A
diB-cAMP
ML-7

Fig. 3A.

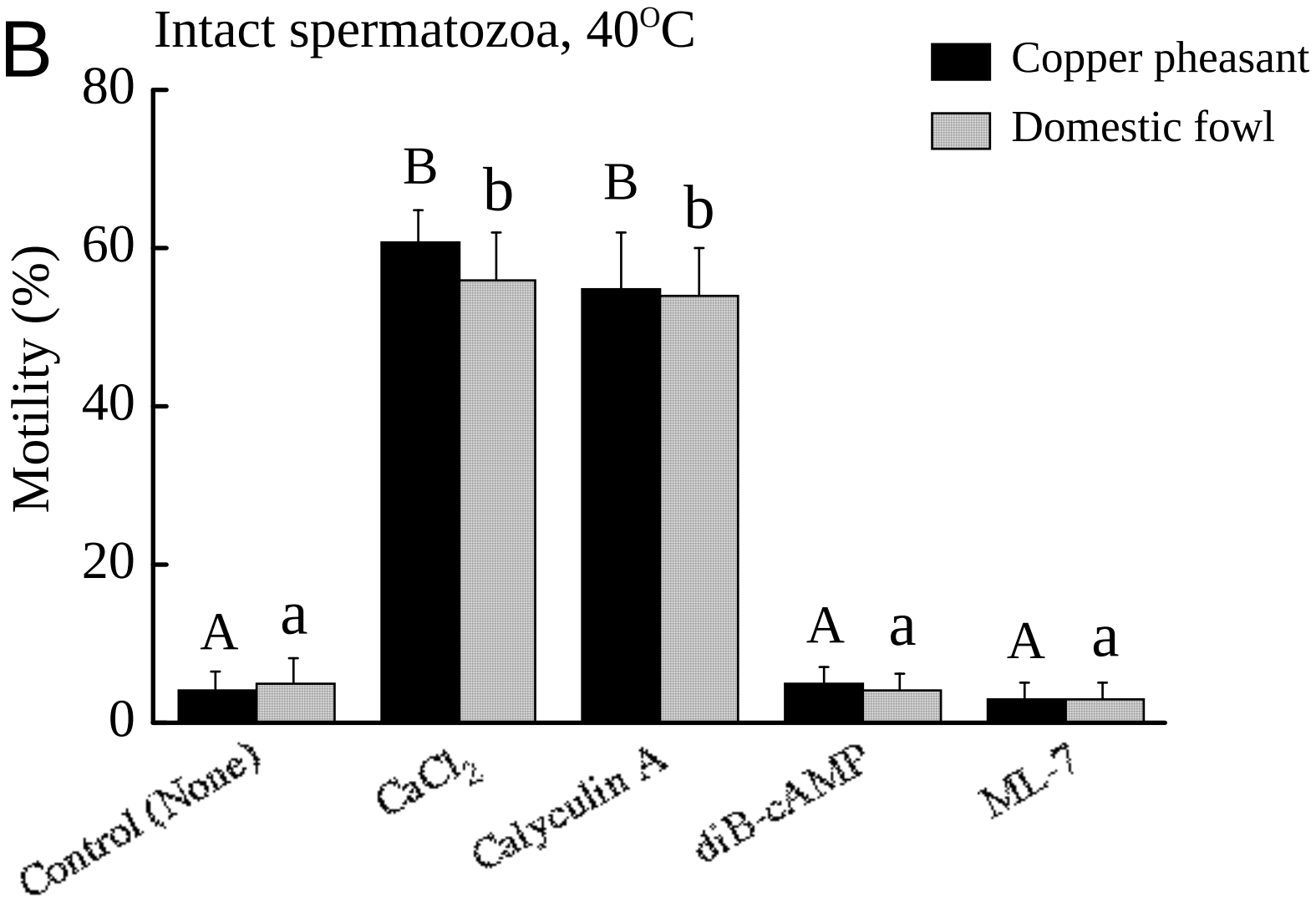
B
Intact spermatozoa, 40°C
Copper pheasant
Domestic fowl
Motility (%)
80
60
40
20
0
A
a
B
b
B
b
A
a
A
a
Control (None)
CaCl2
Calyculin A
diB-cAMP
ML-7

Fig. 3B.

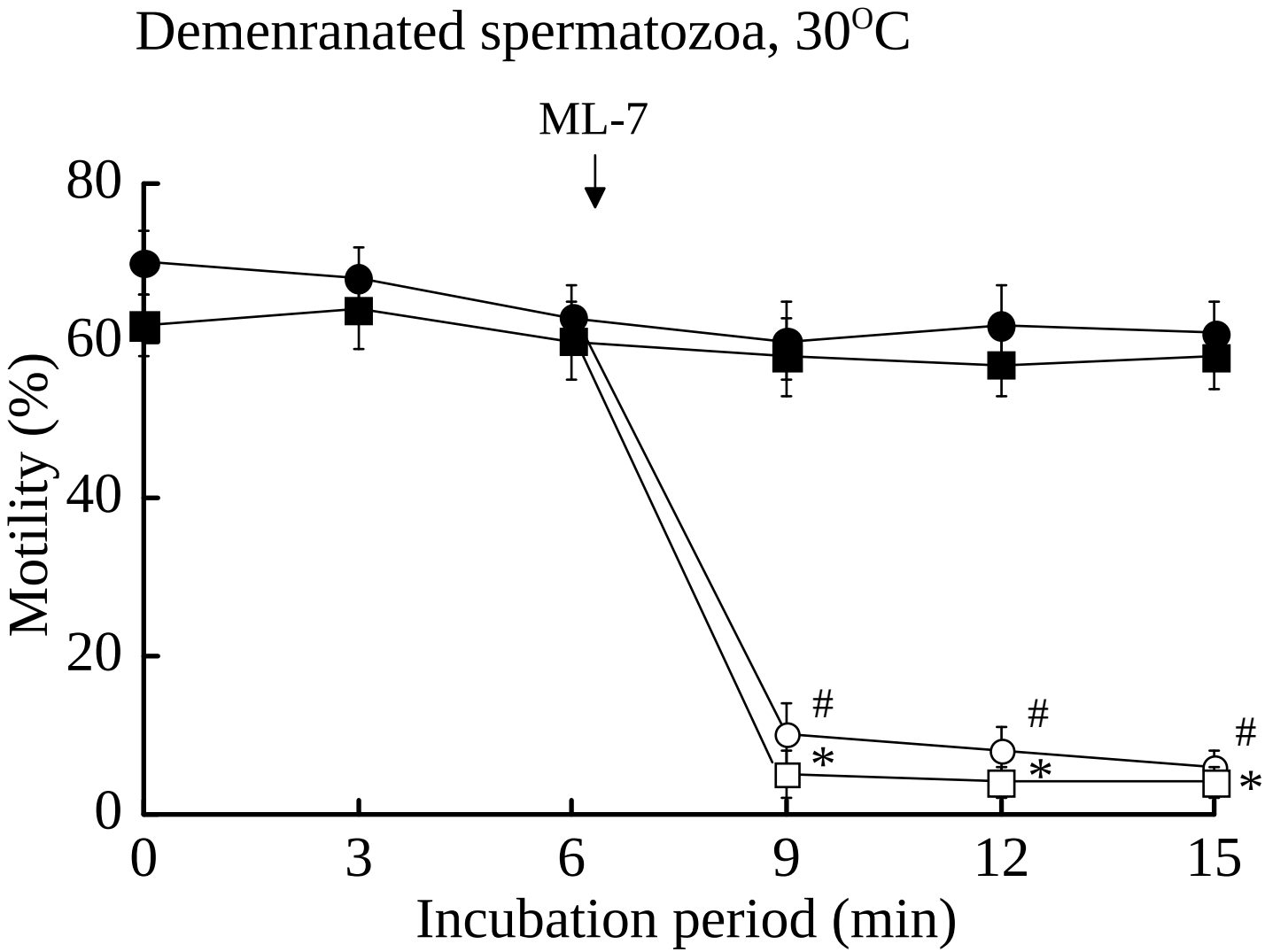
Demenranated spermatozoa, 30°C
ML-7
Motility (%)
80
60
40
20
0
0
3
6
9
12
15
Incubation period (min)
#
#
#
*
*
*

Fig. 4.

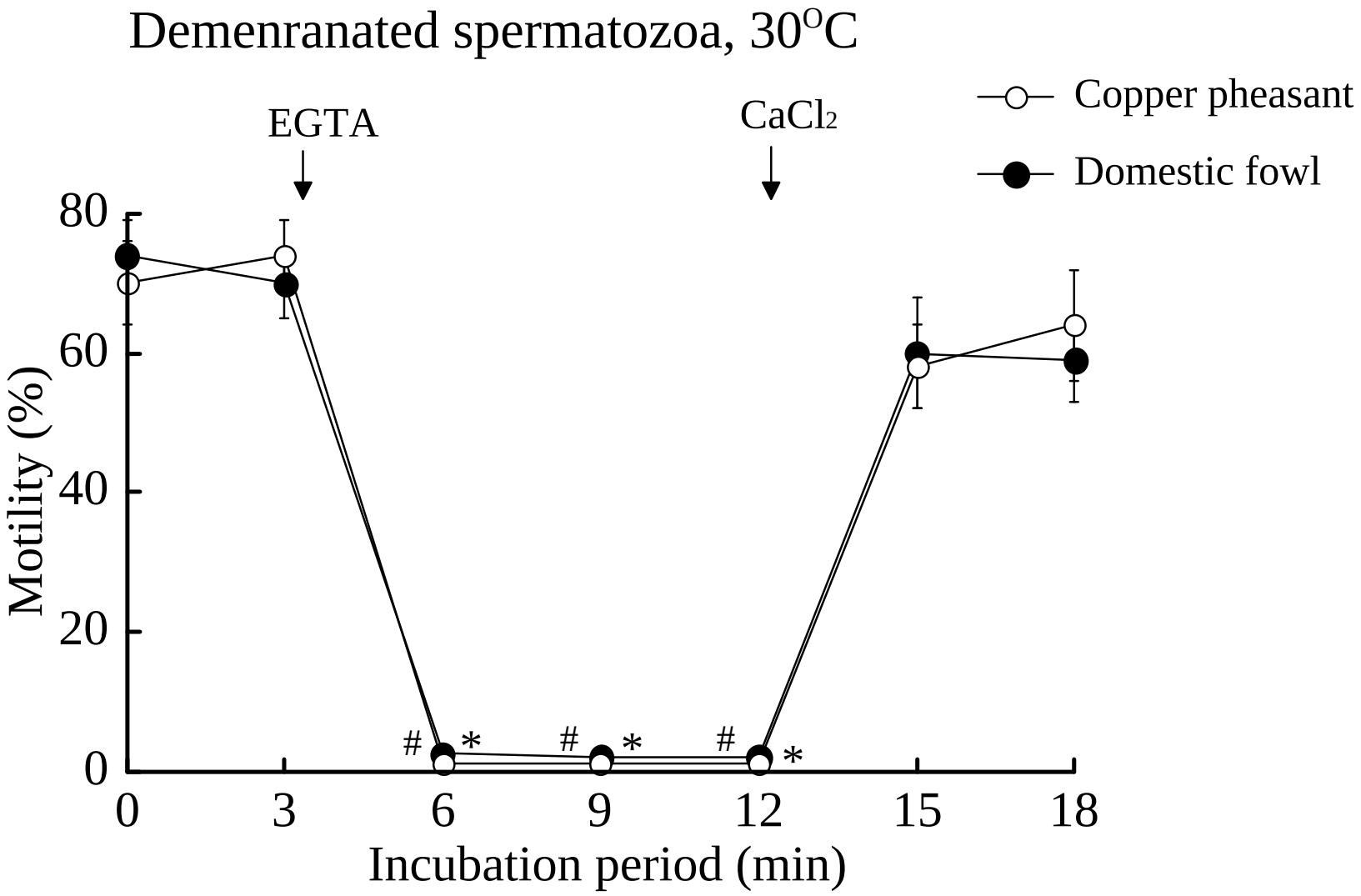
Demenranated spermatozoa, 30°C
Copper pheasant
Domestic fowl
EGTA
CaCl2
Motility (%)
80
60
40
20
0
0
3
6
9
12
15
18
Incubation period (min)
#
*
#
*
#
*

Fig. 5.