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5

6 **Alkaline phosphatase added to capacitating medium enhances horse sperm-zona pellucida binding**

7

8 Abstract

9 Alkaline phosphatase (AP) is present in equine seminal plasma and spermatozoa, but its functional role is not fully
10 understood yet. Being that sperm-oocyte interaction in equine species has been demonstrated to be enhanced at a slightly
11 basic pH, this work aimed at verifying whether exogenous alkaline phosphatase exerts any role on stallion spermatozoa
12 and sperm-oocyte interaction at different pHs (7.4; 8.0; 9.0).

13 Stallion spermatozoa were capacitated in Tyrode's medium at pH 7.4, 8.0 and 9.0 for 4 h at 38°C, 5% CO₂ with 2.5 IU
14 AP (AP group) or without AP (CAP group); viability with mitochondrial activity, motility and acrosome integrity were
15 measured. In addition, a homologous binding assay was carried out: stallion spermatozoa were capacitated 1 h at 38°C,
16 5% CO₂ with 2.5 IU AP (AP group) or without AP (CAP group). Oocytes were then added to sperm suspensions and co-
17 incubated for 1 h.

18 Our results indicate that AP at pH 9.0 significantly increases the percentage of living cells with active mitochondria
19 whereas it significantly reduces the percentage of acrosome-damaged cells at pH 8.0. No significant differences were
20 registered in motility parameters. The homologous binding assay showed a strong effect of AP, that increased the number
21 of sperm bound to the oocyte's zona pellucida at all pHs tested.

22 In conclusion, AP can induce some modifications on sperm membranes thus enhancing their capacity to bind to the zona
23 pellucida of equine oocytes.

24

25 KEYWORDS

26

27 Alkaline phosphatase; pH; Capacitation; Equine spermatozoa; Zona-binding

28

29 HIGHLIGHTS

30

31 Alkaline phosphatase (AP) added to capacitating medium at different pH AP exerts positive effects on semen parameters
32 after capacitation at elevated pH

33 AP enhances homologous oocyte binding in equine species

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35

36 1. INTRODUCTION

37

38 The presence and the activity of alkaline phosphatase (AP) in horse semen have been widely demonstrated; in particular,
39 some Authors [1;2] documented the importance of this enzyme for clinical purposes (determining ejaculation failure) as
40 well as a factor linked to sperm quality.

41 Other researchers better defined the distribution of AP activity in the different fractions of ejaculated semen [3],
42 highlighting a higher activity in the sperm rich fraction, compared with sperm poor or pre-spermatogenic fractions. These
43 results, together with those by Turner and McDonnell [1], indicate that AP originates from epididymal and ampullary
44 fluids. Kareskoski et al. [4] confirmed the high activity of AP in the sperm rich fraction and showed a positive correlation
45 between AP activity and sperm concentration. In a recent study [5] we delineated the activity of AP also in pig
46 spermatozoal extracts, showing that it is significantly lower if compared with the seminal plasma one. A similar result
47 was obtained by Turner and McDonnell [1] who indirectly measured AP activity in spermatozoa. In addition, we
48 demonstrated that AP activity is highly influenced by medium pH and that it could represent a parameter for predicting
49 sperm quality after freezing [6]. Anyway, the role of the cell surface attached enzyme is still unclear. Different studies
50 [5,7] showed that, in pig, sperm surface phosphatase could play a crucial role in sperm function, in particular in sperm
51 capacitation, sperm-oocyte interaction and fertilization [5]. Other evidences of a possible involvement of AP in sperm
52 function are reported by Glogowski et al. [8] who showed that AP could be inhibited by theophyllines which are enhancers
53 of the capacitation process in pig.

54 Leemans and co-workers [9] demonstrated that the optimal condition for stallion sperm capacitation may be reached by
55 increasing the environmental pH; oviductal epithelial cells may be responsible for this modification by secreting
56 intracellular alkaline vesicles. In addition, Loux et al. [10] demonstrated that hyperactivation of horse sperm could be
57 induced by increasing the environmental pH, even though this does not seem to be the main mechanism involved in
58 stallion sperm capacitation.

59 Basing on the information from these studies, the aim of the present work was to determine the effect of exogenous
60 alkaline phosphatase on:

- 61 • stallion sperm parameters (such as viability and mitochondrial activity, motility and acrosome integrity) under
62 capacitating condition in different pH media;
- 63 • sperm-zona pellucida binding in media with different pH.

64

65

66 2. MATERIALS AND METHODS

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68 All the reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise specified.

69

70 2.1. *Experimental design*

71

72 Thyrode's [11] modified medium at three different pHs (7.4, 8.0 and 9.0, pH adjusted by NaOH) was used as capacitating
73 medium as reported by Bucci et al [12].

74 Three experimental groups were set up: freshly ejaculated spermatozoa washed and diluted in Tyrode's medium (F),
75 capacitated spermatozoa (CAP), and capacitated spermatozoa in presence of 2.5 IU/mL of AP (from bovine intestinal
76 mucosa) (AP 2.5 group).

77 The subsequent sets of parameters were assayed for each experimental group:

- 78 • Sperm viability and mitochondrial activity;
- 79 • Acrosome integrity;
- 80 • Sperm motility

81 In addition, a zona-sperm binding assay was set up incubating washed spermatozoa for 1 hour under capacitating
82 condition with (AP group) or without (CAP group) 2.5 IU/mL alkaline phosphatase.

83

84 2.2. *Semen collection and preparation*

85

86 Ejaculates from four fertile stallions, aging 5 -25 years, were used. Stallions were housed individually at the National
87 Institute of Artificial Insemination, University of Bologna . The ejaculates were collected with a Missouri artificial vagina
88 equipped with a disposable liner and an inline filter to avoid eventual gelatinous fraction to be collected (Nasco, Fort
89 Atkinson, WI, USA) on a phantom and in presence of an estrous mare.

90 Sperm concentration was evaluated using a hemocytometer chamber (Thoma).

91 Semen was diluted in Kenney [13] extender pH 6.8 at a final concentration of 30×10^6 spermatozoa /mL and sent to the
92 laboratory within 1h.

93 Diluted spermatozoa were washed twice ($900 \times g$ for 2 min) and resuspended in capacitating medium at three different
94 pHs (7.4; 8.0 and 9.0); an aliquot was immediately analyzed for viability with mitochondrial activity, motility and
95 acrosome integrity (F group). Another aliquot was incubated for 4 h at 38.5°C , 5% CO_2 in absence (CAP group) or in
96 presence (AP group) of 2.5 IU AP. After incubation, viability and mitochondrial activity, motility and acrosome intactness
97 were assayed; each assay was performed 8 times (twice for each stallion) for all different media (pH 7.4, 8.0 and 9.0)

98

99 *2.3. Mitochondrial activity and viability and acrosome integrity assay*

100

101 For each sample, an aliquot (25 μL) of semen was incubated with 2 μL of a 300 μM propidium iodide (PI) stock solution,
102 2 μL of a 10 μM SYBR green-14 stock solution, both obtained from the live/dead sperm viability kit (Molecular Probes,
103 Inc, Eugene, OR, USA) and 2 μL of a 150 μM JC-1 solution, for 20 min at 37°C in the dark. Ten μL of the sperm
104 suspension were then placed on a slide and at least 200 spermatozoa per sample were scored using a Nikon Eclipse E 600
105 epifluorescence microscope.

106 Spermatozoa stained with SYBR green-14 and not stained with PI were considered as viable. Spermatozoa SYBR positive
107 and PI positive and those SYBR negative / PI positive were considered as dead or with non-intact membrane. JC-1
108 monomers emit a green fluorescence in mitochondria with low membrane potential and a bright red-orange fluorescence
109 in case of polymer formation (J-aggregates) when membrane potential is high. When an orange fluorescence was present
110 in the mid piece, live spermatozoa were considered to have functional active mitochondria (SYBR+/PI-/JC-1+).

111 Acrosome integrity was assessed by a FITC-conjugated lectin from *Pisum Sativum* (FITC-PSA) which labeled acrosomal
112 matrix glycoproteins. Spermatozoa were washed twice in PBS, resuspended in ethanol 95% and fixed/permeabilized at
113 4°C for 30 min. Samples were dried in heated slides and incubated with FITC-PSA solution (5 μg PSA-FITC/1ml H_2O)
114 for 15 min in darkness. After staining, samples were washed in PBS and mounted with Vectashield mounting medium
115 with propidium iodide (Vector Laboratories, Burlingame, CA, USA). The slides were then observed with a Nikon Eclipse
116 E 600 epifluorescence microscope (Nikon Europe BV, Badhoevedop, The Netherlands). The presence of a green
117 acrosomal fluorescence was considered indicative of an intact acrosome, while a partial or total absence of fluorescence
118 was considered to indicate acrosome disruption and/or acrosome reaction.

119

120 *2.4. Motility*

121

122 Motility was measured by a computer-assisted sperm analysis system, using the open source Image J CASA plugin as
123 described by Wilson-Leedy and Ingermann [15]. Sperm cells (30×10^6 sperm/mL) were evaluated using a fixed height
124 Leja Chamber SC 20-01-04-B (Leja, The Netherlands). Sperm motility endpoints assessed were: percent of total motile
125 spermatozoa (TM), percent of progressive spermatozoa (PM), curvilinear velocity (VCL) and mean velocity (VAP),
126 straight-line velocity (VSL), straightness (STR), linearity (LIN), beat cross frequency (BCF), lateral head displacement
127 (ALH) and wobble (WOB). The setting parameters of the program were the followings: frames per second 60, number of
128 frames 45, threshold path velocity 30 microns/sec, straightness threshold 75. These settings were chosen on the basis of
129 the Standard Operating Procedure of Italian Experimental Institute “Lazzaro Spallanzani” (Law 403/2000) for stallion
130 sperm analysis.

131

132 *2.5. Equine oocytes maturation and homologous oocyte binding assay*

133

134 *2.5.1. Collection and culture of cumulus oocyte complexes*

135

136 Horse ovaries were collected at a local abattoir and transported to the laboratory at 25°C in a thermos case (Cell Incubator,
137 IMV Technologies Italy). Upon arrival 2-3 h later, the ovaries were dissected free from connective tissue, rinsed with
138 25°C tap water and transferred to 0.9% (w/v) saline supplemented with 0.1% (v/v) penicillin/streptomycin. The cumulus-
139 oocyte complexes (COCs) were recovered by aspirating 5–30 mm follicles using a 19-gauge butterfly infusion set
140 connected to a vacuum pump (about 100 mmHg; KNF S.r.l, Italy). The fluid containing the COCs was collected into 250
141 ml glass flasks (Duran Group, Germany) and filtered through a 65 µm mesh nylon filter (EmSafe, Minitube, Germany).
142 COCs with at least 3–5 layers of cumulus investment were classified as compact (having a tight, complete compact
143 cumulus with a distinct, smooth hillock), expanded (having a granular or expanded cumulus), or denuded (having a partial
144 cumulus or only corona radiata) [16]; all types of COCs were used for this study. For IVM, groups of 25–30 COCs were
145 cultured for 26 h in 500 µl maturation medium in four-well plates (Scientific Plastic Labware, EuroClone, Italy) at 38.5°C
146 in a humidified atmosphere of 5% CO₂ in air. Maturation medium consists in the Dulbecco Modified Eagle Medium
147 Nutrient Mixture F-12 (DMEM-F12, Gibco, Life Technologies, Italy) supplemented with 10% (v/v) heat-inactivated
148 foetal calf serum (FCS; Gibco), ITS (insulin, transferrin, sodium selenite) supplement, 50 ng/ml epidermal growth factor,
149 100 ng/ml insulin-like growth factor 1, 10IU/mL equine chorionic gonadotropin (Folligon, Intervet, Italy), and 10 IU/mL
150 human chorionic gonadotropin (Corulon, Intervet).

151 At the end of the maturation period, the oocytes were denuded by gentle repeated pipetting in maturation medium.

152

153 2.5.2. *Homologous sperm-zona pellucida binding assay*

154 Homologous binding assay was carried out as described in [6,14] with some modifications. Briefly, spermatozoa were
155 washed twice (900 x g for 2 min) and incubated in Tyrode's medium at three different pHs (7.4, 8.0 and 9.0) for 1 h at
156 38.5°C, 5% CO₂) in absence (CAP group) or in presence (AP group) of 2.5 IU AP as already reported [6; 14].
157 Subsequently, spermatozoa were washed in fresh Tyrode's medium without AP and resuspended in the same medium to
158 obtain a concentration of 1.0 x 10⁶ spermatozoa/mL (total) and placed in 500µL wells. Matured denuded oocytes were
159 added to each well at a sperm suspension volume/oocyte ratio of 50 µL/oocyte. After 1 h of co-incubation, the oocytes
160 were washed three times in PBS 0.4% BSA with a wide bore glass pipette to remove the excess and unbound. The oocytes
161 were then fixed in 4% paraformaldehyde for 10 min at room temperature, washed in PBS and stained in the dark with
162 8.9 µM Hoechst 33342 for 10 min. Cells were washed twice in PBS, and individually placed in droplets of Vectashield
163 (Vector Laboratories) on a slide, and covered with a coverslip. The number of spermatozoa attached to each oocyte was
164 assessed by using a Nikon Eclipse E 600 epifluorescence microscope.

165 Homologous oocyte binding was repeated eight times (twice for each stallion). In total, 750 oocytes were used and divided
166 as follows: 113 for CAP pH 7.4; 115 for AP pH 7.4; 118 for CAP pH 8.0; 106 for AP pH 8.0; 142 for CAP pH 9.0; 156
167 for AP pH 9.0.

168

169 2.6. Statistical analysis

170

171 Data were analyzed using R version 3.0.3. (Copyright © 2014, The R Foundation for Statistical Computing) [17] and
172 significance was set at p<0.05 unless otherwise specified.

173 Results are expressed as mean ± standard deviation. Data were assayed for normal distribution using Shapiro-Wilk test;
174 the Levene test for homogeneity of variance was carried out. Subsequently an ANOVA test was used to assess differences
175 between treatments and pHs and their interaction. The Tukey Honest Significant Difference test was applied when due.

176 As for the quantification of the effect of AP on oocyte binding assay, a general linear model with Poisson distribution
177 was set up.

178

179 3. RESULTS

180

181 3.1. *Mitochondrial activity and acrosome integrity*

182 The percentage of viable cells with active mitochondria is summarized in Figure 1. Capacitation, both with and without
183 2.5 IU of AP, significantly reduces viable cells with active mitochondria as compared to F groups (p<0.05).

184 At pH 9.0, a significant increase in viable cells with active mitochondria induced by the addition of AP to the capacitating
185 medium was observed as compared to CAP group ($p < 0.05$).

186 The different pHs did not induce any difference between CAP groups.

187 The percentages of viable cells with active mitochondria in F groups were $67.8 \pm 7.3\%$, $67.2 \pm 7.4\%$ and $60.5 \pm 10\%$ at
188 pH 7.4, 8.0 and 9.0 respectively; in CAP groups they were $45.9 \pm 9.2\%$; $39.8 \pm 8.3\%$ and $38.1 \pm 8.1\%$ while in the AP
189 2.5 groups the percentages of spermatozoa with active mitochondria were $51.6 \pm 9.2\%$; $47.5 \pm 8.4\%$ and $48.8 \pm 7.1\%$ for
190 the three different pHs respectively.

191

192 3.2 Acrosome integrity

193

194 Capacitation in presence or absence of AP increased acrosome reacted cells in comparison with F group in each of the
195 media at different pH (Fig. 2; $p < 0.05$).

196 A significant reduction of acrosome reacted cells was evident at pH 8.0 in AP groups compared to CAP groups.

197 The percentages of acrosome reacted cells in F groups were $12.5 \pm 8.6\%$, $17.9 \pm 9.4\%$; $15.4 \pm 8.5\%$ at pH 7.4, 8.0 and
198 9.0 respectively. In the CAP groups the percentages of acrosome reacted cells were $32.9 \pm 6.5\%$; $32.5 \pm 9.1\%$; $37.1 \pm$
199 8.4% at pH 7.4, 8.0 and 9.0 respectively.

200 Finally, the percentage of acrosome reacted cells in AP group were: $24.0 \pm 6.3\%$; $29.0 \pm 10.3\%$; $32.0 \pm 8.9\%$ at the three
201 different pHs .

202

203 3.3. Sperm motility

204

205 Sperm motility parameters were not different between CAP and AP groups. Some parameters (total and progressive
206 motility, straightness beat cross frequency and linear velocity, VSL) showed a significant difference between F group and
207 capacitated one (in presence or absence of AP). The motility results are reported in Table 1.

208

209 3.4. Homologous oocytes binding

210

211 AP significantly increased ($p < 0.05$) the mean number of attached spermatozoa at all the pHs tested; no significant
212 difference was found between the different pHs (Fig. 3).

213

214 4. DISCUSSION

215

216 The role of alkaline phosphatase in sperm function is still unclear, particularly in stallion; this paper was aimed at
217 determining the effect of exogenous AP on stallion sperm function under capacitating conditions.

218 We studied the effects of exogenous AP on viability, mitochondrial activity, motility and acrosome integrity after
219 incubating spermatozoa for 4 h under capacitating condition at different pHs. Moreover, the effect of exogenous alkaline
220 phosphatase on sperm-zona pellucida binding in media with different pH was evaluated. Our results on mitochondrial
221 activity in living cells clearly show that the incubation in capacitating medium results in a decrease in the number of live
222 cells with active mitochondria. This is not surprising, as the capacitating condition leads to a metabolic activation of the
223 cell that could induce an energy resources depletion, membrane disruption and cell death [18]. Interestingly, no significant
224 differences were recorded between sperm cells incubated at pH 7.4, 8.0 and 9.0; environmental pH does not therefore
225 seem to interfere with capacitation-induced changes. However, AP reduces the percentage of dead spermatozoa after
226 capacitation at pH 9.0. This trend is also evident considering acrosome integrity: the number of acrosome intact cells is
227 higher in AP than in CAP group even if the difference is significant only at pH 8.0. Similar results have been observed in
228 boar sperm [5]; in that species, however, AP added during capacitation does not affect cell viability, while it significantly
229 reduces acrosome reacted cells. Furthermore, AP added during capacitation tends to reduce the number of capacitated
230 cells as well as tyrosine phosphorylation [5].

231 As above reported, an effect of the capacitation process is evident on some motility parameters (TMOT, PMOT, VSL,
232 BCF, STR), irrespective to pH or treatment with AP. Other parameters as VAP, VCL, ALH, LIN and WOB do not change
233 even after capacitation.

234 Alkaline phosphatase can play a role in pH-dependent sperm activation under capacitating condition; in that medium
235 alkalization highly enhances its activity. [6]. We demonstrated the enzyme is present on pig sperm surface; however,
236 its activity is lower than that observed in seminal plasma [5]. Molecules present in seminal plasma could play a very
237 different role from that exerted onto sperm surface: seminal plasma AP, in fact, can concur to prevent capacitation [5].
238 Anyway, it is reasonable to hypothesize that the enzyme could play a regulative role in the sperm-oocyte interaction:
239 follicular fluid of pre-ovulatory follicles has been recently demonstrated to contain alkaline vesicles that stimulate
240 hyperactivation and capacitation of stallion sperm [9,19]. This extracellular microenvironment with a slight alkaline pH
241 (7.9, as reported by Leemans et al., [19]) is more favorable for AP activity.

242 It should also be highlighted that capacitation is a very complex process [20] that induces several changes of the sperm
243 cell, involving membrane, motility as well as metabolism [21,22]. Some interesting studies [9, 23, 24] showed that equine
244 spermatozoa undergo some capacitation-related changes in response to the alkalization of the micro-environment, as it
245 seems to occur in the oviduct at the time of ovulation. This modification in environmental pH leads to an increase of the

246 intracellular pH (that, in species such as mouse and pig, could be achieved by adding bicarbonate to the capacitating
247 medium [25]) with a subsequent activation of the spermatozoa. Finally, as reported by many Authors [22,26],
248 mitochondria in stallion sperm seem to play a central role in modulating metabolism, and their preservation could be
249 crucial for an optimal sperm function.

250 We did not observe significant changes in sperm-related parameters due to capacitating conditions and different pH, but
251 the most interesting results are those related to sperm-ZP binding, which was clearly stimulated by AP, irrespective of
252 pH. In a previous work on pig spermatozoa [5] we observed a strong inhibitory effect of AP on fertilization rate, with a
253 consequent increase in normospermic zygotes; we hypothesized that AP could play a role in maintaining pig sperm
254 quiescent and that it should be “hashed up” to permit sperm-oocyte interaction and fertilization.

255 Conversely, AP exerted a positive effect on stallion sperm-ZP interaction in that it highly stimulated sperm binding with
256 zona pellucida after one hour of incubation under capacitating conditions, thus suggesting that AP could act at plasma
257 membrane level.

258 This aspect deserves an insight. In a recent work on stallion sperm [6] we did not observe any effect of AP on sperm
259 oocyte-ZP heterologous binding. It is therefore evident that the mechanism and/or the molecules involved in the
260 enhancement of the binding capacity are species-specific. As reported by Mugnier et al., [27], porcine zonae are probably
261 more selective and limit stallion spermatozoa binding; in addition, porcine zona protein composition has been
262 demonstrated to be different from the equine one in terms of localization and isoforms [27]. Therefore, it is reasonable
263 that the effect of AP has been reduced by these characteristics of porcine zona pellucida. Taken together, the overall
264 results indicate that AP could improve some sperm parameters (viable sperm with active mitochondria at pH 9.0;
265 acrosome integrity at pH 8.0) after 4 hours of incubation in capacitating condition and enhances cells ability to bind to
266 the zona pellucida. It should be stressed that this effect is exerted only with horse oocytes, and that the enzyme acts
267 specifically on some components of the outer membrane during sperm-oocyte interaction.

268

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271

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371 Table 1. Motility measures comparing treatments and pHs. Abbreviations: TMOT – total sperm motility; PMOT – Progressive sperm motility; VAP – average path velocity; VSL–
 372 straight line velocity; VCL – curvilinear velocity; ALH – amplitude of lateral head displacement; BCF– beat cross frequency; STR – straightness of track; LIN – linearity of track;
 373 WOB wobble. Data are reported as mean ± SD. Different superscripts represent significant difference for $p < 0.05$ between treatments within the same pH.

| | pH 7.4 | | | pH 8.0 | | | pH 9.0 | | |
|------|--------------------------|-------------------------|--------------------------|--------------------------|--------------------------|-------------------------|--------------------------|--------------------------|-------------------------|
| | F | CAP | AP | F | CAP | AP | F | CAP | AP |
| TMOT | 74.70±10.92 ^a | 9.26±3.35 ^b | 14.99±8.38 ^b | 73.05±11.86 ^a | 12.42±5.83 ^b | 16.09±9.36 ^b | 74.16±11.90 ^a | 19.05±16.46 ^b | 22.89±7.37 ^b |
| PMOT | 42.23±7.25 ^a | 3.38±2.94 ^b | 5.64±2.18 ^b | 36.07±3.93 ^a | 5.06±3.08 ^b | 3.89±4.86 ^b | 44.91±15.90 ^a | 7.32±3.62 ^b | 9.54±1.30 ^b |
| VAP | 84.10±15.24 | 77.51±33.04 | 83.15±4.78 | 99.08±20.01 | 74.15±25.72 | 82.46±21.38 | 102.49±31.36 | 95.68±31.14 | 92.58±19.06 |
| VSL | 63.78±9.18 ^a | 41.90±3.73 ^b | 48.16±13.17 ^b | 71.69±12.63 ^a | 40.18±15.34 ^b | 43.75±9.04 ^b | 75.14±13.91 ^a | 53.56±12.90 ^b | 53.76±4.40 ^b |
| VCL | 175.07±19.79 | 157.58±72.28 | 176.35±8.15 | 181.51±26.52 | 131.53±21.18 | 149.36±33.08 | 197.56±48.63 | 183.71±34.23 | 188.62±20.24 |
| ALH | 6.73±0.85 | 6.23±2.95 | 6.80±0.35 | 7.32±1011 | 5.33±1.20 | 6.08±1.35 | 7.79±1.97 | 7.29±2.06 | 6.95±1.38 |
| BCF | 28.78±1.61 ^a | 24.52±1.25 ^b | 25.10±1.29 ^b | 28.38±1.69 ^a | 24.94±1.95 ^b | 24.00±1.67 ^b | 30.40±0.30 ^v | 25.96±5.73 ^b | 22.89±0.97 ^b |
| STR | 74.33±2.52 ^a | 63.00±2.02 ^b | 62.0±2.01 ^b | 73.25±3.95 ^a | 63±3.56 ^b | 60.25±6.66 ^b | 75.00±7.00 ^a | 67.67±11.24 ^b | 64.33±8.50 ^b |
| LIN | 36.67±2.51 | 33.33±13.87 | 32±8.54 | 40±4.69 | 37.25±6.70 | 34.75±5.91 | 39±2 | 37.33±14.43 | 36.33±3.06 |
| WOB | 48.00±5.29 | 49.67±3.79 | 48.33±4.04 | 54.25±5.43 | 57.25±13.05 | 56.50±9.11 | 51.67±3.06 | 52.00±17.78 | 49.33±5.77 |

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Figure 1. Boxplot representing percentage of live stallion spermatozoa with active mitochondria depending on treatment (F, CAP, AP2.5) and pH: pH 7.4 (black boxes), pH 8.0 (dark grey boxes), and pH 9.0 (light grey boxes). Different superscripts represent significant difference for $p < 0.05$ between treatments within the same pH.

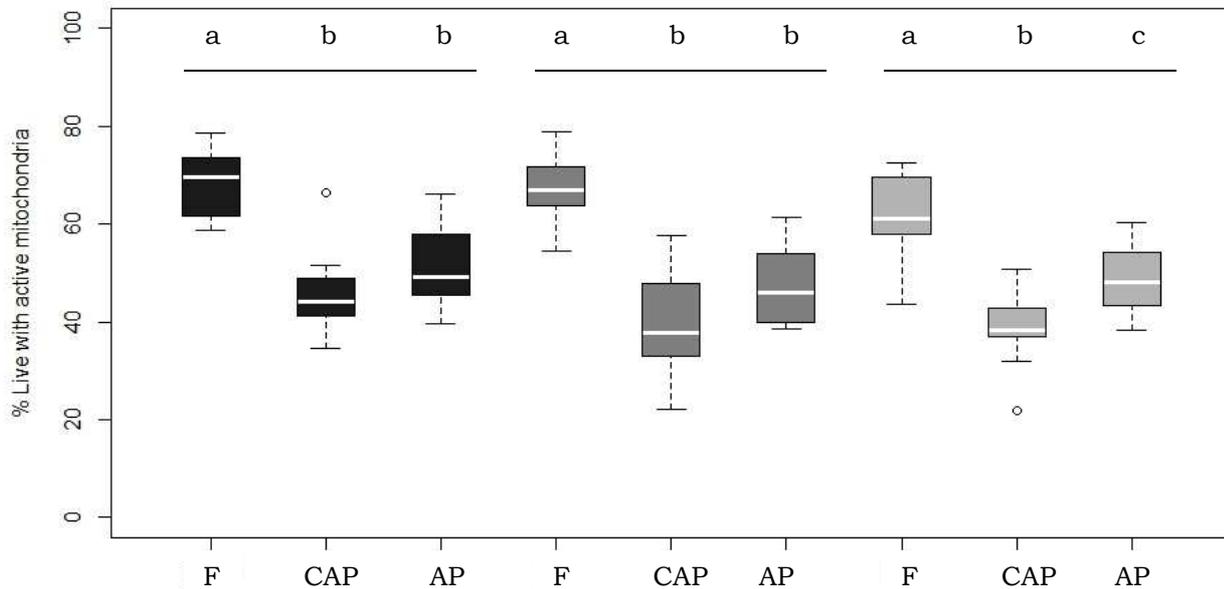


Figure 2. Boxplot representing percentage of acrosome reacted cells in the three groups (F, CAP, AP2.5) at different pH: pH 7.4 (black boxes), pH 8.0 (dark grey boxes), and pH 9.0 (light grey boxes). Different superscripts represent significant difference for $p < 0.05$ between treatments within the same pH.

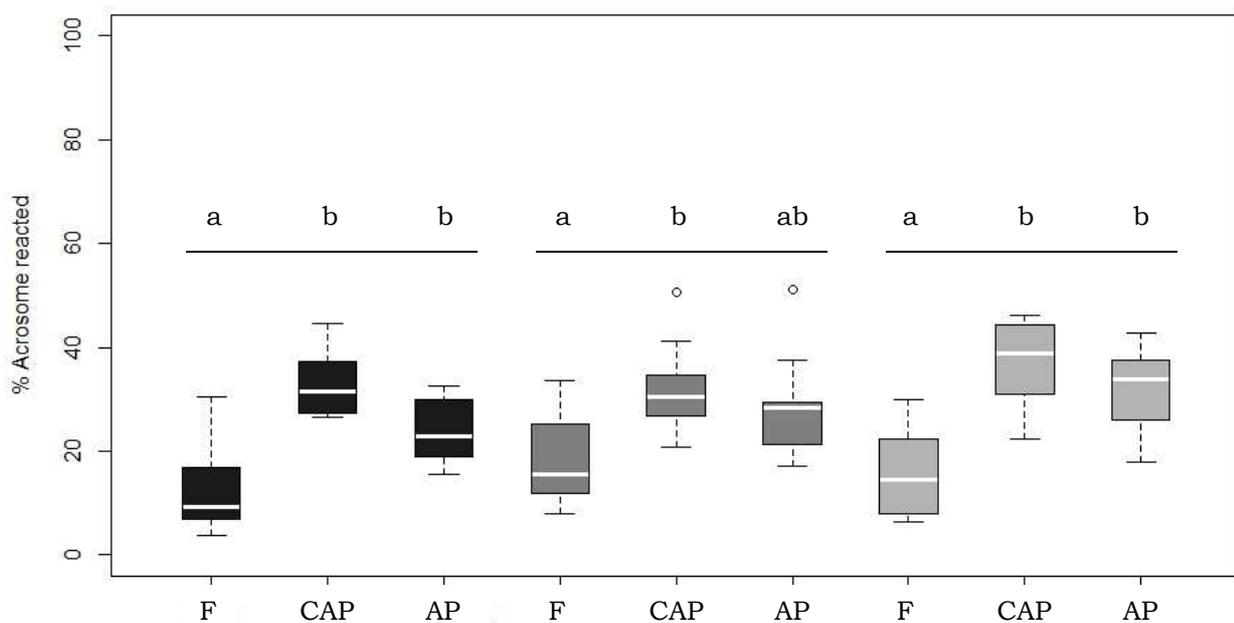


Figure 3. Boxplot representing the number of spermatozoa bound to the oocytes in the two groups (CAP, AP) at different pH: pH 7.4 (black boxes), pH 8.0 (dark grey boxes), and pH 9.0 (light grey boxes). Different letters represent significant difference for $p < 0.05$ between treatments within the same pH.

