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Characterization of alkaline phosphatase activity in seminal plasma and in fresh and frozen–thawed stallion spermatozoa

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ABSTRACT

Alkaline phosphatase (AP) has been studied in several situations to elucidate its role in reproductive biology of the male from different mammalian species; at present, its role in horse sperm physiology is not clear. The aim of the present work was to measure AP activity in seminal plasma and sperm extracts from freshly ejaculated as well as in frozen–thawed stallion spermatozoa and to verify whether relationship exists between AP activity and sperm quality parameters. Our data on 40 freshly ejaculated samples from 10 different stallions demonstrate that the main source of AP activity is seminal plasma, whereas sperm extracts contribution is very low. In addition, we found that AP activity at physiological pH (7.0) is significantly lower than that observed at pH 8.0, including the optimal AP pH (pH 10.0). Alkaline phosphatase did not exert any effect on sperm–oocyte interaction assessed by heterologous oocyte binding assay. Additionally, we observed a thermal stability of seminal plasma AP, concluding that it is similar to that of bone isoforms. Positive correlations were found between seminal plasma AP activity and sperm concentration, whereas a negative correlation was present between both spermatozoa extracts and seminal plasma AP activity and seminal plasma protein content. A significant decrease in sperm extract AP activity was found in frozen–thawed samples compared with freshly ejaculated ones ($n = 21$), concomitantly with the decrease in sperm quality parameters. The positive correlation between seminal plasma AP activity measured at pH 10 and viability of frozen–thawed spermatozoa suggests that seminal plasma AP activity could be used as an additional predictive parameter for stallion sperm freezability. In conclusion, we provide some insights into AP activity in both seminal plasma and sperm extracts and describe a decrease in AP after freezing and thawing.

1. Introduction

Alkaline phosphatase (AP) is an enzyme that catalyzes the detachment of phosphate groups from several substrates [1]; it is present in male genital tract fluids, and its activity has been revealed in semen from various mammalian species [2]. The presence of AP in male genital secretions suggests that it could have a role in mammalian reproduction and in particular in sperm metabolism [3], even if a definite, clear conclusion has not been reached so far.

In bull seminal plasma, the activity of the enzyme has been correlated to fertility and sperm concentration [4], whereas in stallions, it resulted in a promising tool to

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determine whether ejaculation failure is due to either azoospermia or a blockage along the genital tract [5] and it was linked to sperm quality [6].

Other recent studies on AP in stallion seminal plasma furnish data on the different levels of activity in separated semen fractions [7], as well as on its possible role as an indicator of sperm longevity in fractionated semen [8].

The aforementioned studies evaluated the activity in seminal plasma after removing spermatozoa, or in the whole ejaculate, thus indirectly giving information on AP activity of the sperm cells [5]. In a recent study on boar semen [9], we measured the activity of AP from spermatozoa as well as the functional change it undergoes after sperm capacitation; the overall conclusion was that it could play a role in the control of sperm function. There are no data on AP activity in sperm extracts after the freezing–thawing process, excepting for those on frozen ram spermatozoa by Salamon and Maxwell [10] who reported a loss in phosphatase activity after freezing and thawing, thus indicating that the enzyme activity could be used as an additional parameter for detecting cryoinjuries.

On the basis of the questions raised by the aforementioned studies, the present work was aimed at describing AP kinematic properties (thermal inactivation test) in stallion seminal plasma and determining the possible correlations between AP and sperm quality parameters and sperm function as well as its possible predictive role in freezability of stallion semen.

2. Materials and methods

The experiment was approved by the Ethic-Scientific Committee of Alma Mater Studiorum, University of Bologna.

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

2.1. Semen collection and preparation

Forty ejaculates from 10 fertile stallions (nine trotter breed and one Connemara), aging from 5 to 25 years, were sampled. The stallions were housed individually, and semen was sampled between February and April 2014 by an artificial vagina (Missouri model) equipped with a disposable liner with an in-line filter; ejaculates were collected on a phantom and in the presence of an estrous mare.

Semen concentration was evaluated using a hemocytometer chamber (Thoma).

Two aliquots of semen were kept undiluted: the first one was used to obtain seminal plasma and the second one to obtain spermatozoa for AP activity assay. A third aliquot of semen was diluted in the Kenney extender at a concentration of 40×10^6 spermatozoa/mL for semen quality analysis (sperm motility, viability, mitochondrial membrane integrity).

Twenty-one ejaculates, collected in the same period of time from four of the aforementioned stallions, were used for analysis of AP activity in frozen–thawed spermatozoa in comparison with fresh ones. Part of the ejaculates was immediately diluted in the Kenney extender for subsequent freezing procedure, while part was kept undiluted to obtain seminal plasma and spermatozoa for AP activity determination.

2.2. AP activity in seminal plasma, freshly ejaculated and frozen–thawed spermatozoa

Protein concentration in seminal plasma and sperm extracts was measured by Bradford's method [11] using BSA as the protein standard.

Seminal plasma was obtained by two subsequent centrifugations of the undiluted sperm aliquot at $12{,}000 \times g$ for 15 minutes at 4 °C. The resultant supernatant was observed at the microscope to check the absence of either spermatozoa or their fragments.

Sperm cells (2×10^9 spermatozoa/mL; both freshly ejaculated and frozen–thawed) were washed twice in PBS ($900 \times g$ for 3 minutes at room temperature). Subsequently, the sperm pellet was sonicated in PBS and subsequently centrifuged at $12{,}000 \times g$ for 15 minutes at 4 °C; finally, the pellet was discarded and the supernatant was analyzed for AP activity.

The enzyme activity was assayed as described by Bucci et al. [9]. Briefly, AP activity was measured by a spectrophotometric assay that monitors the absorbance change at 405 nm as para-nitrophenylphosphate (pNPP, colorless) is converted to para-nitrophenol (yellow). Alkaline phosphatase was assayed in 50-mM Tris-HCl buffer, at 25 °C at different pH (7.0, 8.0, and 10.0). Fifty microliters of seminal plasma or sperm extracts were used, and the absorbance at 405 nm was measured at 1 minute and 30 minutes before the addition of pNPP to evaluate the nonenzymatic variations of absorbance; no interferences were recorded in any case. In the case of seminal plasma analyzed at pH 10.0, it was diluted 1:10 in Tris buffer. The reaction was started by the addition of 10-mM pNPP, and each measurement was run in duplicate and averaged. Enzyme activity was expressed as specific activity, nmol/min/mg of protein.

To better characterize the seminal plasma isoform, AP thermoinhibition was carried out at 60 °C, as described by Iyer et al. [12] with some modifications. The samples were kept at 60 °C for 0, 15, 30, 1, 75, 90, 105, 120, 150, 180 minutes and then placed in ice, and the activity was measured within 30″ at pH 8.0. The same assay was also performed on the thermostable AP from the bovine intestinal mucosa as a control.

2.3. Evaluation of sperm viability, motility, and mitochondrial activity

Twenty-five microliters of semen was incubated with 2 μL of a 300-μM solution of propidium iodide (PI) and 2 μL of a 10-μM solution of SYBR green-14, both obtained from the live/dead sperm viability kit (Molecular Probes, Inc., Eugene, OR, USA) for 5 minutes at 37 °C in the dark. Ten microliters of the stained suspensions was placed on clean microscope slides and carefully overlaid with coverslips, and at least 200 spermatozoa per sample were observed under a Nikon Eclipse E600 epifluorescence microscope (Nikon Europe BV, Badhoevedorp, The Netherlands). Spermatozoa stained with SYBR-14 but not with PI were

considered as viable (SYBR-14$^-$/PI$^-$). Spermatozoa of both SYBR-14$^+$ and PI$^+$ and those of SYBR-14$^-$/PI$^+$ were considered with damaged membranes or dead.

For each sample, an aliquot (25 μL) of semen was incubated with 2 μL of a 300-μM PI stock solution, 2 μL of a 10-μM SYBR green-14 stock solution, both obtained from the live/dead sperm viability kit (Molecular Probes, Inc.), and 2 μL of a 150-μM JC-1 solution, for 20 minutes at 37 °C in the dark. Ten microliters of the sperm suspension was then placed on a slide, and at least 200 spermatozoa per sample were scored using the aforementioned microscope. Spermatozoa stained with SYBR green-14 and not stained with PI were considered as viable. Spermatozoa of SYBR$^+$ and PI$^+$ and those of SYBR$^-$/PI+ were considered as cells with nonintact membrane or dead. JC-1 monomers emit a green fluorescence in mitochondria with low membrane potential, whereas emission of a bright red-orange fluorescence in case of multimer formation (J-aggregates) in mitochondria indicates high membrane potential. When an orange fluorescence was present in the midpiece, live spermatozoa were considered to have functional active mitochondria (SYBR$^+$/PI$^-$/JC-1$^+$).

Motility was measured using a computer-assisted sperm analysis system (CASA, Hamilton Thorne, IVOS Ver. 12, standard equine setup). Thousand-1000 cells were evaluated on each sample diluted to 30 × 10^6 sperm/mL using a fixed-height Leja Chamber SC 20-01-04-B (Leja, The Netherlands). Sperm motility end points assessed were percent of total motile spermatozoa (TM), percent of progressive spermatozoa (PM), curvilinear velocity (VCL), and average path velocity (VAP). The setting parameters of the program were the followings: frames per second: 60, number of frames: 45, threshold path velocity: 30 μm/s, straightness threshold: 50.

2.4. Effect of AP on heterologous oocyte binding

Porcine oocytes maturation was performed as reported by Galeati et al. [13]. Briefly, ovaries were obtained from prepubertal gilts at a local abattoir and transported to the laboratory within 1 hour. Cumulus–oocyte complexes (COCs) were aspirated from 4- to 6-mm follicles using an 18-ga needle attached to a 10-mL disposable syringe. Under a stereomicroscope, intact COCs were selected and transferred into a Petri dish (35 mm; Nunclon, Denmark) prefilled with 2 mL of modified PBS supplemented with 0.4% BSA. After three washes in NCSU 37 supplemented with 5.0 mg/mL of insulin, 0.57-mM cysteine, 10 ng/mL of EGF, 50-mM β-mercaptoethanol, and 10% porcine follicular fluid (IVM medium), groups of 50 COCs were transferred to a Nunc four-well multidish containing 500 μL of the same medium per well and cultured at 39 °C in a humidified atmosphere of 5% CO2/7% O2 in air. For the first 22 hours of IVM, the medium was supplemented with 1.0-mM dbcAMP, 10 IU/mL of eCG (Folligon; Intervet, Boxmeer, The Netherlands), and 10 IU/mL of hCG (Corulon; Intervet). For the last 22 hours, the COCs were transferred to fresh maturation medium.

Heterologous oocyte binding assay was conducted as described by Balao da Silva [14]. Briefly, semen collected from three of the previously described stallions was washed twice in modified Tyrode's medium pH 7.4 for 2.5 minutes at 800 × *g*; the pellet was resuspended in the same medium at 1 × 10^6 spermatozoa/mL, and aliquots of 500 μL were capacitated in the presence or absence of AP (1.25 or 2.5 IU/mL) for 1 hour at 38.5 °C in 95% humidity and 5% CO_2 atmosphere. Subsequently, 50 matured denuded oocytes were added to each well. After 1 hour of coincubation at 38.5 °C in 95% humidity and 5% CO2 in air, the oocytes were washed three times in PBS containing 0.4% BSA with a wide-bore glass pipette, fixed in 4% paraformaldehyde for 15 minutes at room temperature in the dark, and stained with 8.9-μM Hoechst 33342. Cells were washed twice in PBS, individually placed in droplets of Vectashield (Vector Laboratories, Burlingame, CA, USA) on a slide, and covered with a coverslip. The number of spermatozoa attached to each oocyte was assessed by using the aforementioned microscope and was expressed as mean number of spermatozoa per oocyte.

2.5. Sperm cryopreservation

After dilution in the Kenney extender, semen was centrifuged in a glass conical tube at 600 × *g* for 20 minutes; the supernatant was partially removed, and the sperm pellet was resuspended in Heitland's extender supplemented with 3% egg yolk and 3% glycerol [15] to a final concentration of 150 × 10^6 sperm/mL. Resuspended semen was loaded in 0.5-mL straws, kept at +4 °C for 2 hours, and then frozen on liquid nitrogen vapors for 20 minutes 6 cm above the liquid nitrogen level.

2.6. Statistical analysis

Data were analyzed using R version 3.0.3. (Copyright 2014, The R Foundation for Statistical Computing), and significance was set at $P < 0.05$ unless otherwise specified.

Data were assayed for normal distribution using the Shapiro–Wilk test; in case of nonnormal distribution, data were handled by a log transformation to match normality. Subsequently, an ANOVA test was used to assess differences between the AP activity at pH 7.0, 8.0, and 10.0. The paired samples *t* test was used for comparison of the sperm quality parameters and AP activity in samples before and after thawing.

To test correlation between sperm quality parameters and AP activity, Spearman's rank test was used.

As for the oocyte binding assay, data were tested by a general mixed-effects model, setting the treatment as a fixed effect and the horse as a random effect. Linear regression was used to examine relationship between AP activity in seminal plasma and postthawing sperm viability [16].

The results are expressed as mean ± standard deviation.

3. Results

Sperm concentration, motility (TM, PM, VAP, VCL), viability, and mitochondrial membrane integrity as well as seminal plasma and sperm extracts protein concentration are reported in Table 1.

Table 1
Sperm quality parameters in freshly ejaculated spermatozoa.

Parameter	Mean ± SD
Concentration ($\times 10^6$/mL)	197 ± 125
TM (%)	69.3 ± 20.0
PM (%)	45.0 ± 12.7
VAP (μm/s)	102.0 ± 22.5
VCL (μm/s)	190.1 ± 41.8
Viability (%)	71.4 ± 13.8
Seminal plasma protein (mg/mL)	12.4 ± 10.4
Sperm extracts protein (mg/mL)	2.3 ± 1.0

The results are expressed as mean ± standard deviation (SD, n = 40).
Abbreviations: PM, progressive motility; TM, total motility; VAP, average path velocity; VCL, curvilinear velocity.

Sperm quality parameters from each stallion are presented in Supplementary Table 1.

Alkaline phosphatase activity in both seminal plasma and sperm extracts is significantly different depending on the three analyzed pH. A significant increase is evident from pH 7.0 to 8.0 ($P < 0.001$) as well as from pH 8.0 to 10.0 ($P < 0.001$), as reported in Table 2.

Different AP activities in both seminal plasma and sperm extract were found among stallions, and data from single subjects are shown in Supplementary Table 1.

The thermal inhibition test, carried out on seminal plasma AP activity, showed a decrease because of heat exposure in a time-dependent manner, whereas the activity of the intestinal AP isoform was not affected by incubation at 60 °C. In fact, seminal plasma AP lost 45% activity after 1 minute and 15 seconds at 60 °C, reaching the 80% after 1 minute and 45 seconds, and was completely inactivated after 3 minutes (Fig. 1).

Alkaline phosphatase activity in frozen–thawed sperm extracts significantly decreased ($P < 0.01$ for pH 7.0 and 8.0 and $P < 0.05$ for pH 10 measurements) in comparison with the relative freshly ejaculated sample. The results on AP activity are shown in Table 2.

Alkaline phosphatase activity in sperm extracts from freshly ejaculates and frozen–thawed spermatozoa from each stallion is presented in Supplementary Table 2.

Sperm quality parameters significantly changed after freezing (Table 3). Motility parameters (TM, PM, VAP, and VCL) significantly ($P < 0.01$) decreased after freezing, and the same trend was evident in viability and mitochondrial activity ($P < 0.01$); on the contrary, sperm protein content was not affected by the freezing process.

Table 2
Alkaline phosphatase (AP) activity in seminal plasma and spermatozoa extract at different pH.

Sample	Specific activity (nmol/min/μg protein)		
	pH 7.0	pH 8.0	pH 10.0
AP activity seminal plasma (n = 40)	11.1 ± 10.5[a]	68.2 ± 48.5[b]	503.2 ± 419.4[c]
AP activity sperm extracts (n = 40)	2.7 ± 2.3[a]	7.5 ± 6.9[b]	102.9 ± 78.7[c]
AP activity before freezing (n = 21)	2.6 ± 2.0[d]	6.8 ± 4.7[d]	58.3 ± 46.2[e]
AP activity after freezing /thawing (n = 21)	0.9 ± 0.6[d]	3.0 ± 1.6[d]	19.9 ± 11.6[e]

Data are expressed as mean ± standard deviation.
Lowercase superscript letters (a, b, c) indicate significant differences for $P < 0.001$.
[d] Indicates significant differences within a column for $P < 0.01$.
[e] Indicates significant differences within a column for $P < 0.05$.

The results on sperm quality parameters from each stallion are presented in Supplementary Table 2.

The oocyte binding assay did not show any difference between the control group and those treated with different AP concentrations (1.25 and 2.5 IU/mL).

The control group scored 13.85 ± 14.4 sperm bound per oocyte, whereas AP 1.25 scored 15.17 ± 13.04 and AP 2.5 scored 14.44 ± 14.26.

Spearman's correlations between AP activity in seminal plasma and sperm extracts and sperm quality parameters in freshly ejaculated spermatozoa are shown in Table 4.

Seminal plasma AP activities measured at pH 7.0, 8.0, and 10.0 were positively correlated. In addition, sperm extracts AP activity measured at pH 7.0, 8.0, and 10.0 showed positive correlation. Alkaline phosphatase activity in seminal plasma was positively correlated with sperm concentration and negatively with seminal plasma protein concentration.

Table 5 shows the correlation between AP activity in sperm extracts and sperm quality parameters in frozen–thawed stallion spermatozoa. Although in fresh sperm extracts, no correlation was found between motility and AP activity from sperm extracts, in frozen–thawed samples, positive correlations between AP activity in sperm extracts at pH 7.0 and 10.0 and total motility and progressive motility were recorded.

Correlations between freshly ejaculated stallion sperm AP activity (in seminal plasma and sperm extracts) and sperm quality parameters and frozen–thawed ones are presented in Supplementary Table 3.

Alkaline phosphatase activity in seminal plasma measured at pH 10.0 and 7.0, as well as total motility and progressive motility in freshly ejaculated spermatozoa, was correlated with sperm viability after thawing.

Multiple regression generated a statistical model with only one explanatory variable (AP activity in seminal plasma measured at pH 10.0) explaining 26.5% (adjusted R^2) of the variation in sperm viability after thawing.

4. Discussion

This study was aimed at determining AP activity in stallion seminal plasma and sperm extracts, and at defining AP relationship with some sperm quality parameters.

Our results are reported as specific activity, expressed as nmoles of substrate transformed in 1 minute for milligram of protein in the sample. This is, in our opinion, the best way to express the activity, as it permits to delineate the substrate consumption in relation to the effective protein content of the sample, that can considerably differ among both stallions (see Supplementary Table 1; intersubject variability) and samples from the same subject (intrasubject variability). Therefore, this measurement is standardized and is not affected by the different concentrations of protein (and enzyme) in the ejaculate.

To better define the properties of seminal plasma AP, we performed a thermal inhibition test, as the enzyme's isoforms show significantly different sensitiveness to heat.

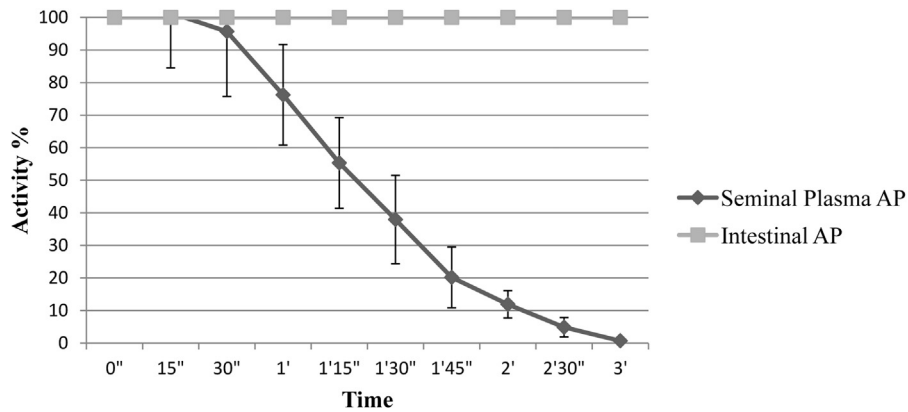

Fig. 1. Thermal inactivation of seminal plasma alkaline phosphatase (AP) activity. Alkaline phosphatase activity was inhibited by heat (60 °C) for different time periods. Error bars represent 1 SD. SD, standard deviation.

Alkaline phosphatase in horse seminal plasma is easily and rapidly inhibited by heat, as reported in Figure 1. Recently, we observed that AP from boar seminal plasma is quite more resistant, losing 100% activity only after 10 minutes at 60 °C [9]; on the basis of these data and those reported for boars and humans [12,17], we could infer that seminal plasma AP in stallions is very similar to bone isoforms.

This is the first report that approached the study of AP activity respecting the physiological environment of stallion semen. The enzyme activity at pH 7.0 is very low, in both seminal plasma and sperm extracts. This fact seems to be conflicting with other reports [5,6], stating that AP activity in stallion semen is very high. Anyway, our data on AP activity reveal that it parallels the increase of pH, thus confirming that, at least at pH 8.0 and 10.0, AP activity in stallion semen is very high. The intriguing question risen from these data regards the role of AP in seminal plasma and sperm extracts of physiological stallion semen pH (near 7.0). Turner and McDonnell [5] studied the activity of the enzyme in extracts from the testis, epididymis, and ampulla, as well as from ampullary fluid and epididymal fluid. The highest activity was found in fluids from both the epididymis and ampulla, thus letting to hypothesize that the enzyme is secreted in these compartments; in particular, epididymal fluid activity is 50 fold the mean activity registered in seminal plasma.

Table 3
Sperm quality parameters in freshly ejaculated and frozen–thawed spermatozoa.

Parameter	Freshly ejaculated sperm	Frozen/thawed sperm
TM (%)	73.8 ± 18.2	28.8 ± 15.5[a]
PM (%)	47.4 ± 11.2	22.5 ± 13.4[a]
VAP (μm/s)	107.1 ± 16.2	88.2 ± 14.6[a]
VCL (μm/s)	195.9 ± 30.0	157.9 ± 25.5[a]
JC1-positive live (%)	63.1 ± 13.9	31.8 ± 12.5[a]
Viability (%)	74.8 ± 12.6	41.1 ± 13.1[a]
Protein sperm extracts (mg/mL)	2.6 ± 1.1	2.2 ± 1.1

Data are expressed as mean ± standard deviation (n = 21).
Abbreviations: PM, progressive motility; TM, total motility; VAP, average path velocity; VCL, curvilinear velocity.
[a] Indicates significant difference within a row for $P < 0.001$.

In a previous work, we hypothesized that AP could play a role in maintaining porcine spermatozoa quiescent, preventing a premature capacitation [9]. In horse sperm, on the basis of ours and other's [5] data, AP likely plays a role on spermatozoa metabolism and activity during their transit from testicles to the ampullae, whereas sperm dilution in seminal plasma dramatically reduces the enzyme activity and therefore its possible involvement in sperm metabolism. It should also be stressed that spermatozoa, during natural mating, are diluted in seminal plasma for a brief period of time, after which they enter the female genital tract. In this region, the environment is completely different and spermatozoa gradually lose seminal plasma to begin the capacitation process that leads to sperm–oocyte fusion and fertilization [18]. This process, in the mare, takes from 0.5 to 4 hours.

To reinforce this hypothesis, the heterologous oocyte binding assay showed no difference between untreated spermatozoa under capacitating condition and spermatozoa treated with AP at two different doses. We may infer that the addition of AP to a capacitating medium with a neutral pH (7.4) is not effective in modifying heterologous oocyte binding, considered a good parameter to assess sperm capacitation in stallion [14].

Alkaline phosphatase activity of seminal plasma is significantly higher than that of sperm extracts (at all pH tested, 7.0, 8.0, and 10.0), with a minimum mean ratio respectively of 5:1 at pH 10.0 and a maximum of 9:1 at pH 8.0. This is not surprising: other authors [5] showed that the contribution of spermatozoa to the whole ejaculate's activity is very low if compared to that of seminal plasma. Those researchers, anyway, obtained an indirect indication of AP activity from spermatozoa, as they subtracted the activity of unprocessed semen samples to that of seminal plasma, whereas in our study, we pointed out the effective AP activity from sperm cell extracts.

The role of AP attached to sperm surface is more intriguing: in boar spermatozoa, we found a significant decrease in AP activity after *in vitro* capacitation [9]. It should be highlighted that AP activity at physiological pH in

Table 4
Spearman correlations between sperm quality parameters and alkaline phosphatase (AP) activity in seminal plasma and sperm extracts in freshly ejaculated samples.

	AP_P7	AP_P8	AP_P10	AP_S7	AP_S8	AP_S10	Conc	PlProt	SPProt	TM	PM	VAP	VCL	Live	Livepos
AP_P7	1														
AP_P8	0.95*	1													
AP_P10	0.90*	0.88*	1												
AP_S7	0.14	0.17	0.27	1											
AP_S8	0.49*	0.51*	0.47*	0.74*	1										
AP_S10	0.37	0.49**	0.37	0.60*	0.91*	1									
Conc	0.59*	0.48*	0.53*	−0.05	0.01	−0.02	1								
PlProt	−0.56*	−0.60*	−0.50*	−0.13	−0.53*	−0.65*	0.18	1							
SPProt	0.49**	−0.25	−0.33	−0.09	−0.14	−0.61*	0.05	0.33**	1						
TM	−0.29	0.08	0.19	0.07	0.04	−0.27	0.31	0.33	0.10	1					
PM	0.19	0.35	0.50**	0.14	0.19	−0.21	0.33	0.03	−0.02	0.83*	1				
VAP	−0.33	−0.33	−0.42**	−0.27	−0.50	−0.59*	0.13	0.52*	0.17	0.41**	0.30	1			
VCL	−0.46**	−0.36	−0.52*	−0.25	−0.53**	−0.54**	0.14	0.49*	0.19	0.16	−0.04	0.89	1		
Live	0.20	0.18	0.18	−0.24	0.03	−0.11	0.10	0.12	−0.04	0.63*	0.61*	0.29	0.02	1	
Livepos	−0.09	−0.08	−0.12	−0.25	−0.26	−0.46**	0.01	0.40**	0.04	0.48*	0.38**	0.39**	0.26	0.60*	1

Data represent Spearman's rho coefficient.
*$P < 0.01$; **$P < 0.05$.
Parameters are reported as follows: AP_P7: AP activity in seminal plasma at pH 7; AP_P8: AP activity in seminal plasma at pH 8; AP_P10: AP activity in seminal plasma at pH 10; AP_S7: AP activity in sperm extracts at pH 7; AP_S8: AP activity in sperm extracts at pH 8; AP_S10: AP activity in sperm extracts at pH 10; Conc: sperm concentration; PlProt: seminal plasma proteins; SPProt: sperm extracts protein; TM; PM; VAP; VCL; Live: % live spermatozoa; Livepos: % live spermatozoa with high mitochondrial membrane activity.
Abbreviations: PM, progressive motility; TM, total motility; VAP, average path velocity; VCL, curvilinear velocity.

boars (8.0) is similar to that observed in this study in stallions at the same pH that is significantly higher than that registered at pH 7.0. In that study, we demonstrated that AP exerts a down regulatory activity in tyrosine protein phosphorylation during capacitation [9]. This action is intriguing, as under capacitating conditions, boar semen protein phosphorylation increases significantly, and it lets us to hypothesize that AP should be hashed down to permit capacitation in pig [9]. We do not have any experimental evidence that AP plays a role in stallion sperm capacitation, as it is deducible from heterologous oocyte binding, and its role (if any) during sperm transit in the male genital tract does not seem to be important. Recently, other authors [19] showed that stallion spermatozoa acquire capacitation by interacting with tubal epithelial cells: in particular, those authors found that tyrosine phosphorylation of spermatozoa is positively correlated to the increase in intracellular pH, strictly associated with the microenvironment induced by epithelial cells. Nevertheless, further studies are necessary to determine the possible role of AP during the capacitation process.

There is a great range of AP activity in both seminal plasma and sperm extracts; we found significant differences among horses in AP seminal plasma activity at pH 7.0 and 8.0, whereas no difference was recorded either at pH 10.0 or in AP activity from sperm extracts (See Supplementary Table 1.) The same trend between AP activity in seminal plasma and sperm extracts has been observed in boars [9]. The individual variation is well known in horses and pigs, thus it is not surprising that mean values of our samples are quite dispersed.

The relationship between sperm quality parameters and AP activity was investigated by a correlation test. As already described by Pesch et al. [6], we found a positive correlation between seminal plasma AP activity and sperm concentration. A high sperm concentration results from a lower

Table 5
Spearman correlation between alkaline phosphatase (AP) activity in sperm extracts and sperm quality parameters in frozen–thawed spermatozoa.

	AP_S7	AP_S8	AP_S10	Conc	SPProt	TM	PM	VAP	VCL	Live	Livepos
AP_S7	1										
AP_S8	−0.0992	1									
AP_S10	0.5767**	0.3299	1								
Conc	−0.1808	−0.037	−0.0515	1							
SPProt	0.2149	−0,6305*	−0.0283	0.0455	1						
TM	0,5717**	−0.1129	0.5988**	0.2861	0.1482	1					
PM	0.5994*	−0.1334	0.6102*	0.2861	0.17	0.9928*	1				
VAP	0,1948	−0.1817	0.1214	0.1215	0.4448**	0.5629*	0.5672*	1			
VCL	0.0847	−0.0234	−0.0368	−0.0468	0.3012	0.3225	0.3108	0.8912*	1		
Live	0.0785	−0.0208	−0.0221	0.3494	−0.1535	0.6665*	0.658*	0.3852	0.2442	1	
Livepos	0.2841	−0.1326	0.2966	0.0091	−0.0338	0.7666*	0.7581*	0.5138**	0.3455	0.6753*	1

Data represent Spearman's rho coefficient.
*$P < 0.01$; **$P < 0.05$.
Parameters are reported as follows: AP_S7: AP activity in sperm extracts at pH 7; AP_S8: AP activity in sperm extracts at pH 8; AP_S10: AP activity in sperm extracts at pH 10; Conc: sperm concentration; SPProt: sperm extracts protein; TM; PM; VAP; VCL; Live: % live spermatozoa; Livepos: % live spermatozoa with high mitochondrial membrane activity.
Abbreviations: PM, progressive motility; TM, total motility; VAP, average path velocity; VCL, curvilinear velocity.

secretion of seminal plasma by the accessory glands and, therefore, it is concomitant with a lower dilution of epididymal and testicle secretions which are rich of AP [5]. On the other hand, in our study, sperm extracts AP activity does not correlate with sperm concentration. We registered a negative correlation between seminal plasma protein concentration and AP activity in both seminal plasma and sperm extracts, excepting for sperm extracts activity at pH 7.0. These findings are difficult to interpret, and further studies are necessary to understand if they could be related to AP origin (secretion from ampullae and epididymides). No correlations between AP activity in seminal plasma and/or sperm extracts and viability were found, whereas a negative correlation between AP activity in seminal plasma and sperm extracts, measured at pH 10.0, and the two sperm velocity parameters, VCL and VAP, was recorded. This finding might deserve further insights, as it seems to indicate that high AP activity is related with lower velocity of the spermatozoa, but evidence of a similar effect at physiological pH is still lacking, thus letting the question open.

The second part of our work aimed at determining the possible involvement of AP activity in cryopreservation of stallion sperm, with particular attention to sperm cryodamage. Our results on sperm quality parameters (motility, viability, and mitochondrial activity) are consistent with those reported by others [20,21], thus delineating a significant loss in sperm quality after freezing and thawing procedure.

We found that AP activity in sperm extracts significantly decreases after freezing and thawing; similar results were reported for ram spermatozoa by Salamon and Maxwell [10], who observed that the phosphatase activity of ram semen after freezing and thawing decreases significantly. However, those authors did not specify whether it was alkaline or acid one. Alkaline phosphatase activity is related to protein dephosphorylation, and it possibly plays an important role in regulating this process, at least in pigs [9]. Evidence exists that tyrosine phosphorylation is activated after freezing and thawing of stallion spermatozoa and that the response of stallion sperm to capacitation stimuli is different after freezing and thawing [22]. In addition, tyrosine phosphorylation is one of the aspects of the so-called cryocapacitation [20], and its increase after freezing and thawing may be due to AP inhibition. The intimate mechanism of AP inactivation is still unclear: can be the inactivation a consequence of the membrane damage that leads to a loss of enzyme from the sperm surface? Cryoelution of surface proteins?

As for the correlation between frozen–thawed sperm quality parameters and AP activity of relative sperm extracts, only measurements at pH 10.0 and 7.0 (but not at pH 8.0) showed some correlations. A strong positive correlation between AP activity (at pH 7.0 and 10.0) and TM and PM is evident; this correlation is not present in the freshly ejaculated spermatozoa. We may suppose that this change is due to a perturbation of both motility parameters and AP activity because of the freezing-thawing process.

We also correlated AP activity in freshly ejaculated cells with sperm quality parameters of the relative frozen sample (presented in Supplementary Table 3). This correlation could possibly be useful for predicting sperm freezability based on AP activity in seminal plasma. The multiple linear regression showed that a part of the variability in sperm viability after thawing could be related to AP activity measured at pH 10 and that this parameter could be used for predicting, at least in part, the freezability of stallion semen. The linear relationship, in fact, links the amount of AP activity (measured at pH 10) in seminal plasma of the fresh ejaculate with the viability of postthawing spermatozoa indicating a direct proportionality between the parameters.

In conclusion, our data demonstrate for the first time the presence of AP on stallion sperm surface and describe the enzyme activity in both seminal plasma and sperm extracts at different pH.

Measurements at pH 7.0 indicates that AP in the ejaculate is not very active, letting thus unknown its role in the ejaculate. In contrast, the activity on the sperm surface, when reaching the uterotubal junction, could be enhanced by the higher pH in this part of the female genital tract. In addition, we found some interesting correlations between AP and sperm quality parameters. Finally, we observed a decrease of AP activity after the freezing-thawing process, which parallels that of both viability and sperm motility.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.theriogenology.2015.09.007.

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Supplementary Table 1
Alkaline phosphatase (AP) activity in seminal plasma and spermatozoa extract and sperm quality parameters in freshly ejaculated stallion spermatozoa.

Horse	n	AP_P7	AP_P8	AP_P10	AP_S7	AP_S8	AP_S10	PlProt	SPProt	TM	PM	VAP	VCL	Live	Livepos
1	1	15.7 ± NA	49.7 ± NA	291.2 ± NA	NaN	5.2 ± NA	NaN	11.6 ± NA	3.0 ± NA	NaN	NaN	NaN	NaN	41.2 ± NA	41.2 ± NA
2	2	53.3 ± NA	218.6 ± NA	1181.4 ± NA	2.2 ± 2.8	7.1 ± 3.8	102.7 ± 69.2	4.7 ± NA	2.4 ± 0.7	9.0 ± NA	6.0 ± NA	70.0 ± NA	170.3 ± NA	60.8 ± 28.1	14.5 ± 14.0
3	8	6.2 ± 3.4	33.5 ± 15.23	204.8 ± 128.3	1.7 ± 0.8	5.0 ± 4.2	49.6 ± 43.5	19.4 ± 13.1	2.5 ± 1.3	77.0 ± 7.2	47.0 ± 4.6	121.4 ± 14.7	223.6 ± 23.3	67.0 ± 10.3	62.2 ± 11.3
4	2	16.9 ± 0.7	81.0 ± 4.16	334.2 ± 135.9	1.4 ± 1.6	10.9 ± 2.7	144.6 ± 53.0	5.5 ± 1.0	1.5 ± 0.5	71.0 ± NA	55.0 ± NA	98.9 ± NA	162.0 ± NA	82.8 ± 2.0	73.7 ± 14.8
5	9	9.7 ± 8.9	47.3 ± 32.84	481.5 ± 455.2	2.6 ± 1.3	5.6 ± 5.1	76.5 ± 53.6	19.5 ± 13.0	2.6 ± 1.1	75.5 ± 10.3	46.0 ± 8.2	103.3 ± 19.0	195.8 ± 42.2	79.2 ± 9.0	71.0 ± 7.8
6	6	23.0 ± 13.3	107.8 ± 53.28	746.0 ± 405.4	3.6 ± 3.3	10.3 ± 9.5	87.1 ± 88.8	8.5 ± 2.8	2.1 ± 1.2	88.3 ± 3.9	58.2 ± 1.2	112.9 ± 10.6	197.2 ± 23.6	80.7 ± 8.7	70.5 ± 8.6
7	2	17.5 ± 1.4	85.6 ± 19.59	578.0 ± 328.7	8.6 ± NA	19.7 ± 22.0	183.0 ± 198.3	6.2 ± 1.3	2.0 ± 1.1	66.0 ± NA	52.0 ± NA	70.0 ± NA	139.2 ± NA	80.5 ± 3.3	72.6 ± 3.7
8	2	29.3 ± 3.1	127.0 ± 6.92	1077.3 ± 604.3	2.3 ± 3.1	8.3 ± 6.3	95.9 ± 77.1	9.1 ± 0.2	2.3 ± 0.5	65.0 ± NA	43.0 ± NA	78.0 ± NA	134.4 ± NA	76.0 ± 12.5	71.0 ± 19.5
9	6	9.7 ± 7.2	56.9 ± 42.35	410.7 ± 467.8	1.6 ± 1.2	5.7 ± 2.6	170.8 ± NA	5.6 ± 1.2	2.0 ± 0.8	45.6 ± 13.2	31.4 ± 7.6	99.4 ± 8.8	194.2 ± 30.1	56.2 ± 10.1	46.9 ± 10.8
10	2	8.0 ± 1.0	62.3 ± 27.73	171.3 ± 72.9	3.3 ± NA	12.4 ± 3.5	180.3 ± 25.0	5.48 ± 0.4	2.6 ± 0.9	53.0 ± NA	39.0 ± NA	33.8 ± NA	68.3 ± NA	72.1 ± 0.1	47.3 ± 10.3

Parameters are reported as follows: n: number of ejaculates; AP_S7: AP activity in sperm extracts at pH 7; AP_S8: AP activity in sperm extracts at pH 8; AP_S10: AP activity in sperm extracts at pH 10; Conc: sperm concentration; PlProt: seminal plasma proteins; SPProt: sperm extracts protein; TM; PM; VAP; VCL; Live: % live spermatozoa; Livepos: % live spermatozoa with high mitochondrial membrane activity.
Abbreviations: NA, not available; NaN, missing data; PM, progressive motility; TM, total motility; VAP, average path velocity; VCL, curvilinear velocity.

Supplementary Table 2
Alkaline phosphatase (AP) activity and sperm quality parameters in freshly ejaculated and frozen–and thawed spermatozoa.

Horse	n	AP_S7	AP_S8	AP_S10	SPProt	TM	PM	VAP	VCL	Live	Livepos
3	4	2.65 ± 1.85	6.24 ± 5.96	33.98 ± 17.82	3.09 ± 0.97	79.25 ± 5.91	49.00 ± 1.41	120.55 ± 16.77	219.95 ± 25.30	75.38 ± 3.30	61.06 ± 12.58
3FT	4	1.01 ± 0.31	2.17 ± 0.85	15.52 ± 4.35	3.25 ± 1.22	30.25 ± 7.93	23.25 ± 5.56	103.10 ± 9.64	190.80 ± 10.58	39.73 ± 5.36	35.14 ± 3.11
5	6	2.32 ± 0.85	6.38 ± 3.00	52.10 ± 42.02	2.98 ± 1.15	79.00 ± 7.01	48.83 ± 7.47	98.78 ± 19.45	180.18 ± 34.21	83.28 ± 2.28	71.45 ± 6.11
5FT	6	0.81 ± 0.54	2.59 ± 0.70	17.46 ± 3.86	2.65 ± 0.70	32.17 ± 16.19	24.00 ± 13.43	95.42 ± 12.45	166.33 ± 23.12	44.43 ± 15.43	30.77 ± 13.66
6	6	3.22 ± 3.06	8.26 ± 6.98	62.58 ± 41.45	2.18 ± 1.20	88.33 ± 3.93	58.17 ± 1.17	112.92 ± 10.61	197.15 ± 23.62	80.68 ± 8.68	70.53 ± 8.63
6FT	6	1.14 ± 0.78	3.05 ± 1.64	26.43 ± 17.83	2.05 ± 1.09	39.33 ± 13.32	33.00 ± 11.78	85.03 ± 6.14	144.67 ± 12.53	48.79 ± 13.71	38.21 ± 13.45
9	5	1.62 ± 1.24	5.94 ± 2.87	170.83 ± 17.82	2.07 ± 0.91	45.60 ± 13.20	31.40 ± 7.57	99.40 ± 8.85	194.20 ± 30.06	57.00 ± 11.09	45.82 ± 11.69
9FT	5	0.44 ± 0.52	4.07 ± 2.46	12.22 ± 4.35	1.19 ± 0.64	11.00 ± 4.58	7.40 ± 4.51	71.32 ± 9.23	137.24 ± 17.55	29.15 ± 4.53	22.74 ± 11.56

Parameters are reported as follows: n: number of ejaculates; AP_S7: AP activity in sperm extracts at pH 7; AP_S8: AP activity in sperm extracts at pH 8; AP_S10: AP activity in sperm extracts at pH 10; Conc: sperm concentration; PlProt: seminal plasma proteins; SPProt: sperm extracts protein; TM; PM; VAP; VCL; Live: % live spermatozoa; Livepos: % live spermatozoa with high mitochondrial membrane activity.
Abbreviations: FT, frozen adn thawed; PM, progressive motility; TM, total motility; VAP, average path velocity; VCL, curvilinear velocity.

Supplementary Table 3
Spearman correlation between alkaline phosphatase (AP) activity and sperm quality parameters in freshly ejaculated and frozen and–thawed spermatozoa.

	AP_P7	AP_P8	AP_P10	AP_S7	AP_S7F	AP_S8	AP_S8F	AP_S10	AP_S10F	Conc	ConcF	PlProt	SPProt	SPProtF	TM	TMF	PM	PMF	VAP	VAPF	VCL	VCLF	Live	LiveF	Livepos	LiveposF
AP_P7	1																									
AP_P8	*0.99*	1																								
AP_P10	*0.94*	*0.94*	1																							
AP_S7	**0.49**	0.45	0.38	1																						
AP_S7F	−0.09	−0.10	−0.11	−0.19	1																					
AP_S8	*0.63*	*0.59*	**0.55**	*0.77*	−0.03	1																				
AP_S8F	0.40	**0.45**	0.34	0.27	−0.10	**0.44**	1																			
AP_S10	**0.62**	**0.60**	**0.6**	0.3	−0.30	**0.60**	**0.50**	1																		
AP_S10F	0.07	0.04	0.10	−0.11	**0.58**	0.20	0.33	0.16	1																	
Conc	*0.63*	*0.59*	**0.56**	0.39	0.02	0.33	0.12	0.43	0.12	1																
ConcF	*0.60*	*0.55*	**0.55**	**0.56**	−0.18	**0.51**	−0.04	0.09	−0.05	*0.64*	1															
PlProt	*−0.60*	*−0.70*	*−0.63*	−0.07	0.15	−0.34	−0.37	−0.44	0.07	0.001	−0.02	1														
SPProt	**−0.47**	**−0.50**	**−0.56**	−0.01	0.11	−0.21	−0.15	*−0.64*	−0.05	−0.12	0.12	*0.58*	1													
SPProtF	*−0.59*	*−0.63*	*−0.59*	−0.15	0.21	−0.40	*−0.63*	*−0.65*	−0.03	−0.07	0.05	*0.80*	**0.51**	1												
TM	0.33	0.19	0.28	**0.47**	0.27	0.25	−0.31	−0.07	0.43	**0.44**	*0.60*	0.25	−0.06	0.36	1											
TMF	0.32	0.21	0.33	0.18	**0.57**	0.12	−0.11	0.004	**0.60**	**0.47**	0.29	0.28	−0.17	0.15	*0.70*	1										
PM	**0.53**	0.36	**0.55**	0.30	0.03	0.12	−0.02	0.22	0.30	**0.54**	**0.48**	0.13	−0.19	0.13	*0.80*	*0.69*	1									
PMF	0.31	0.22	0.34	0.18	*0.60*	0.12	−0.13	−0.01	*0.61*	**0.49**	0.29	0.26	−0.21	0.17	*0.71*	*0.99*	*0.68*	1								
VAP	−0.22	−0.27	−0.34	−0.04	0.40	−0.27	−0.21	−0.31	0.10	0.15	−0.15	0.38	0.11	0.38	0.36	**0.49**	0.36	**0.46**	1							
VAPF	−0.14	−0.23	−0.09	0.29	0.19	0.14	−0.18	−0.33	0.12	0.12	0.12	**0.60**	0.10	**0.44**	0.40	*0.56*	0.31	*0.57*	0.33	1						
VCL	−0.35	−0.30	**−0.46**	−0.01	0.41	−0.19	−0.07	−0.35	0.11	0.079	−0.23	0.29	0.23	0.26	0.10	0.28	−0.01	0.25	*0.87*	0.16	1					
VCLF	−0.24	−0.31	−0.16	0.22	0.08	0.16	−0.02	−0.25	−0.04	−0.01	−0.05	0.53	0.12	0.30	0.12	0.32	0.05	0.31	0.36	*0.89*	0.32	1				
Live	0.16	0.03	0.16	−0.06	0.45	0.002	−0.24	0.37	0.46	0.21	0.21	0.35	−0.10	0.28	**0.53**	*0.68*	**0.52**	*0.67*	0.22	0.32	−0.04	0.08	1			
LiveF	**0.51**	0.37	**0.55**	0.44	0.08	0.28	−0.02	0.05	−0.02	0.26	0.35	−0.01	−0.35	−0.2	*0.58*	*0.67*	*0.63*	*0.66*	0.23	0.39	0.01	0.24	**0.49**	1		
Livepos	−0.22	−0.30	−0.26	−0.29	0.41	−0.27	−0.17	−0.44	**0.53**	0.01	0.12	**0.55**	0.37	**0.50**	**0.45**	**0.47**	**0.40**	**0.46**	0.33	0.34	0.13	0.17	*0.61*	0.19	1	
LiveposF	0.15	0.08	0.16	0.14	0.28	−0.01	−0.13	−0.24	0.30	0.18	0.01	0.09	−0.26	−0.0	**0.45**	*0.77*	**0.44**	*0.76*	**0.48**	**0.51**	0.32	0.35	0.32	*0.68*	0.34	1

Data represent Spearman's rho coefficient; italicized values indicate $P < 0.01$; bold values indicate $P < 0.05$.
Parameters are reported as follows: AP_P7: AP activity in seminal plasma at pH 7; AP_P8: AP activity in seminal plasma at pH 8; AP_P10: AP activity in seminal plasma at pH 10; AP_S7: AP activity in sperm extracts at pH 7; AP_S8: AP activity in sperm extracts at pH 8; AP_S10: AP activity in sperm extracts at pH 10; SPProt: sperm extracts protein; TM; PM; VAP; VCL; Live: % live spermatozoa; Livepos: % live spermatozoa with high mitochondrial membrane activity. "F" letter following the parameters name refers to frozen–thawed spermatozoa.
Abbreviations: PM, progressive motility; TM, total motility; VAP, average path velocity; VCL, curvilinear velocity.