Journal of Reproductive Immunology 131 (2019) 13–20

Contents lists available at ScienceDirect

Journal of Reproductive Immunology

journal homepage: www.elsevier.com/locate/jri

Antibacterial defense and sperm quality in boar ejaculates

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ARTICLE INFO

Keywords:
Bacterial killing activity
Boar semen quality
Boar semen preservation
Lysozyme
Reproductive male immunology

ABSTRACT

The aim of the present study was to characterize the lysozyme concentration and the bacterial killing activity (BKA) of boar seminal plasma against *E. coli* and *S. aureus* in 119 fertile Pietrain boars (aged: 18.1 ± 10.5 months). Lysozyme concentration was 2.4 ± 1.2 µg/ml in seminal plasma. More than 80% of the samples (97 of 119) showed BKA against *E. coli* or *S. aureus* or both bacterial strains: Group 1 (BKA against *E. coli* and *S. aureus*, n = 38), Group 2 (BKA against *E. coli*, n = 13), Group 3 (BKA against *S. aureus*, n = 46), and Group 4 (no BKA, n = 22). Boars with BKA against *E. coli* (Group 1 plus 2) were older ($P < 0.001$) than boars with BKA against *S. aureus* only or without BKA. Thermo-resistance of spermatozoa was lowest in boars without BKA ($P = 0.002$). Lysozyme concentration was higher in boars with BKA against *S. aureus* only compared to boars with BKA against both bacterial species ($P = 0.005$) and boars with BKA against *E. coli* only ($P = 0.047$). In Group 2, the ratio of morphologically normal spermatozoa was lower than in all other groups ($P < 0.001$) and mitochondrial activity of spermatozoa was lower compared to Group 3 ($P = 0.023$). The results suggest an age related variance of BKA against *E. coli* in boar semen. BKA is related to different sperm quality characteristics. Further research is necessary to discover the molecular components, which are responsible for BKA of boar seminal plasma.

1. Introduction

Over the last decades, artificial insemination techniques in swine industry have been extensively used worldwide. The semen collection and preservation process is a non-sterile procedure and bacterial contaminations in semen can reduce male reproductive performance (Maroto Martin et al., 2010). To minimize the bacterial load during storage, antibiotic substances are usually included in extender formulations (Schulze et al., 2017). Bacterial contamination of fresh boar ejaculates ranges from 10^3 to 10^5 colony forming units per ml and many different contamination spots have been identified (Althouse et al., 2000). The major contaminants are gram-negative bacteria, and most of them belong to the Enterobacteriaceae family (Althouse and Lu, 2005). These microorganisms can be a part of the normal microflora, but also opportunistic or conditional pathogens that produce genital infections in susceptible swine (Maes et al., 2008).

Bacteria can deteriorate semen quality with far-reaching consequences. Often, sperm motility (Auroux et al., 1991), morphology (Ubeda et al., 2013), agglutination behavior (Wolff et al., 1993), viability (Sepulveda et al., 2014), and acrosome integrity (el-Mulla et al., 1996) during liquid storage are affected and result in reduced longevity and fertilizing ability of boar spermatozoa (Prieto-Martinez et al., 2014). Bacterial contamination of semen may also impair female health by inducing endometritis and causing embryonic or fetal death after artificial insemination (Kuster and Althouse, 2016).

Due to the detrimental effect of bacteriospermia and its fitness consequences, ejaculates contain several molecules with antibacterial properties (Poiani, 2006). Antibacterial activity of ejaculates has been observed in a variety of taxa and appears effective against a wide range of bacterial species (Chitnis et al., 1987; Otti et al., 2009; Rowe et al., 2011; Yenugu et al., 2006). Therefore, antibacterial properties of the seminal plasma could play an important role to protect spermatozoa both before and during insemination.

In recent years, several substances that may account for the antibacterial activity of semen have been identified (Poiani, 2006), including several enzymes such as the lysozyme (Otti et al., 2009; Rowe et al., 2013). Lysozymes are enzymes which damage bacterial cell walls by catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycans. It might have bacteriolytic functions in a number of secretions such as tears, saliva, mucus and semen of mammals (Mendeluk et al., 1997), birds (Sotirov et al., 2002; Rowe et al., 2013; Atikuzzaman et al., 2017) and different teleost species (Lahnsteiner and Radner, 2010).

The aim of the present study was to determine the lysozyme

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https://doi.org/10.1016/j.jri.2018.11.001
Received 6 July 2018; Received in revised form 18 October 2018; Accepted 1 November 2018

concentration and the overall bacterial killing activity (BKA) of seminal plasma against gram-negative and gram-positive bacteria represented by *Escherichia* (*E.*) *coli* and *Staphylococcus* (*S.*) *aureus*, respectively. The potential relevance of BKA and lysozyme concentration for functional characteristics of boar spermatozoa were examined and evaluated. Investigations were performed under conditions of commercial semen processing in artificial insemination centers, and methods proven for their predictive value with respect to the fertilizing potential of spermatozoa were used to detect subtle effects on sperm quality.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were of analytical grade. Unless stated otherwise, they were purchased from Merck (Darmstadt, Germany) and Roth (Karlsruhe, Germany). Propidium iodide (PI) and rhodamine 123 (R123) were obtained from Sigma-Aldrich (Steinheim, Germany), whereas fluorescein-isothiocyanate conjugated peanut agglutinin (FITC-PNA) and *Pisum sativum* agglutinin (FITC-PSA) were purchased from Axxora (Lörrach, Germany). Acridine orange was obtained from Polysciences Europe GmbH (Eppelheim, Germany).

2.2. Semen collection and processing

All boars in this study were routinely used for the production of artificial insemination doses in three boar studs in Germany (ejaculates: A: n = 28; B: n = 48; C: n = 43). The average age (mean ± SD) of the 119 fertile Pietrain boars included in this study was 18.1 ± 10.5 months. They received commercial feed (pellets) for artificial insemination boars and were housed in individual pens equipped with nipple drinkers according to the European Commission Directive for Pig Welfare. Semen production protocols were carried out according to the general guidelines for semen processing used in artificial insemination studs participating in a quality control audit of the Institute for the Reproduction of Farm Animals Schönow (Riesenbeck et al., 2015). Only ejaculates that passed minimum requirements for commercial use in artificial insemination were included. Criteria for the selection of ejaculates comprise a minimum of 75% morphologically normal spermatozoa, at least 70% total sperm motility, and a total number of $\geq 30 \times 10^9$ spermatozoa per ejaculate.

Ejaculates were collected randomly by the gloved-hand method. The pre-sperm phase of the ejaculate was discarded and the gel fraction of the semen was removed by gauze filtration during collection. The day of collection is specified as Day 0 (d0). After collection, ejaculate volume and sperm concentration were determined to produce the insemination doses of 85 ± 1 ml. Sperm concentration was measured using a NucleoCounter SP-100 (Chemometec, Denmark) and adjusted to 24×10^6 spermatozoa/ml. Dilution was performed with an isothermic (32 °C) Beltsville Thawing Solution extender (Minitüb, Tiefenbach, Germany). Finally, all extended samples were placed in a temperature-controlled box at 21 °C for 90 min and subsequently stored in a temperature-controlled cabinet at 17 °C for seven days. Immediately after collection, an aliquot of each ejaculate was treated by centrifugation (10 min, 800 × g) to separate sperm and seminal plasma which were frozen in liquid nitrogen for later analysis of lysozyme concentration and BKA.

2.3. Evaluation of boar sperm morphology

To assess sperm morphology on d0, spermatozoa were fixed by 1% formalin in phosphate-buffered saline at a concentration of $50–100 \times 10^6$ spermatozoa/ml. Two hundred spermatozoa per sample were evaluated using phase contrast microscopy (800 × total magnification, Jenaval, Carl Zeiss Jena, Germany). The percentage of morphologically normal spermatozoa and further subcategories were given according to Schulze et al. (2014).

2.4. Flow cytometric assessment of boar spermatozoa

Analyses were performed using an Accuri C6 flow cytometer (BD Biosciences, Erembodegem, Belgium) equipped with a 488 nm solid state laser and a 640 nm diode laser. Fluorescence signals of FITC-PNA, FITC-PSA and R123, gathered via 533/30 nm band-pass filter, and PI, gathered via 670 nm long-pass filter, were plotted on logarithmic scales. Acridine orange forward- and side-scatter signals were plotted on linear scales. The sperm population was gated referring to the expected forward- and side-scatter signals. A total of 10.000 events fitting this gate were counted. For incubation at 38 °C, a dry block heater was used (Techne Dri-Block® DB2.D, Techne AG, Burkhardtsdorf, Germany).

2.4.1. Evaluation of mitochondrial activity of boar spermatozoa

Sperm viability and mitochondrial activity were assessed on d2 of semen storage by double-staining with R123/PI and flow cytometry as described previously (Schulze et al., 2013b). In brief, aliquots of 250 µL were mixed with 1.0 µL R123 (final concentration 0.2 µg/mL) and 2.5 µL PI (final concentration 9.6 µg/mL) and incubated under light exclusion for 20 min. After incubation, 15 µL of each sample were mixed with 2.0 mL isotherm phosphate-buffered NaCl solution. The percentage of spermatozoa with active mitochondria and intact plasma membrane was determined.

2.4.2. Evaluation of boar sperm DNA integrity

For evaluation of the sperm DNA integrity, a sperm chromatin structure assay was performed on d2 in diluted semen samples according to Evenson and Jost (2000). Briefly, 200 µL TNE-buffered aliquots of semen samples were supplemented with 400 µL of acid solution, mixed on a vortex for 30 s and incubated with 1.2 mL of staining solution (containing acridine orange) on iced water for 3 min in the dark. DNA-fragmentation index and high DNA stainability were evaluated.

2.4.3. Evaluation of plasma and acrosome membrane integrity of boar spermatozoa

A triple-stain flow cytometric method applying PI, fluorescently labeled PNA and PSA was used on d3 to discriminate between viable and dead spermatozoa and to particularly characterize the membrane integrity in the acrosome region as described previously (Schulze et al., 2013a). First, 125 µL of a fixation solution (containing 0.5% formaldehyde) were supplemented to 375 µL of the sample in lightproof reaction vessels. Then 12.5 µL FITC-PNA and 2.5 µL FITC-PSA (final concentration 2.4 µg/mL each) and, after 5 min of incubation, 5.0 µL PI (final concentration 9.6 µg/mL) were added. Total incubation time at 38 °C was 10 min. Then, 15 µL of the samples were mixed with 2.0 mL isotherm phosphate-buffered NaCl solution. The percentage of sperm with intact plasma and acrosome membrane was determined.

2.5. Evaluation of thermo-resistance of boar spermatozoa

Additionally, on d7 of semen storage, a thermo-resistance test (TRT) was performed. Subsamples of 10 ml were incubated at 38 °C in a water bath under air access. At 30 and 300 min of incubation, motility characteristics and sperm kinematics were determined using the computer-assisted sperm analysis system AndroVision® (Minitüb, Germany) as described previously (Schulze et al., 2015).

2.6. Evaluation of the bacterial killing activity (BKA)

We assessed BKA of seminal plasma against *Escherichia* (*E.*) *coli* (ATCC no. 8739) and *Staphylococcus* (*S.*) *aureus* (ATCC no. 6538 P) using a liquid growth inhibition assay modified after Otti et al. (2009)

as described by Rowe et al. (2011). Briefly, seminal plasma samples were diluted 1:1 (v:v) with Tryptic Soy Broth (TSB; no. CM0129, Oxoid-Thermo Fischer, Germany). Then, 5 µl of diluted seminal plasma and 45 µl of bacteria (concentration corresponding to an optical density (OD) of 0.01) were added to a 96-well plate. Control groups were TSB with bacteria and TSB with seminal plasma. The plate was incubated 24 h at 37 °C, and OD at 595 nm was determined using a spectrophotometer (Biotek, Bad Friedrichshall, Germany). Next, BKA in seminal plasma for each microbial strain is defined as

$$\text{BKA}\ (\%) = (1-\text{R}) \times 100, \quad \text{where R}=\text{OD}_{\text{sample}}/\text{OD}_{\text{control bacterial solution}}$$

In case of R < 1, the OD of the samples was reduced compared to the control groups, hence, these cases show BKA. Cases of R ≥ 1 show no BKA or the negative BKA is due to the enhanced bacterial growth in the sample.

2.7. Evaluation of the lysozyme concentration

The lysozyme concentration in seminal plasma was determined by the lyso-plate method described by Osserman and Lawlor (1966). Briefly, 1% noble agar in phosphate-buffered saline (pH 6.3) was mixed with lysozyme-sensitive bacteria *Micrococcus lysodeikticus* (ATCC #4698, Sigma-Aldrich) to reach a final bacterial concentration of 50 mg per 100 ml agar. The agar was poured into preheated (50 °C) petri-dishes and was horizontally leveled with a water spirit to avoid quick and uneven cooling of the medium. In each cooled agar-plate, 24 holes of 4.5 mm diameter were engraved and filled with 25 µl sample (seminal plasma) or lysozyme standard solution (0.5, 1.0, 2.5, 5.0, 10.0, 12.5, 25.0, 50.0 and 100.0 µg/ml Hen White Egg Lysozyme, Sigma-Aldrich). Plates were incubated at room temperature for 18 h. During this period, a zone of clearing developed in the area of the gel surrounding the sample inoculation site as a result of bacterial lysis. The diameters of the cleared zones are proportional to the log of the lysozyme concentration. We photographed each plate in a photobox (Imaging system; peqlab) with a ruler next to it as a reference scale. The diameter of the cleared zone was measured digitally three times using the software ImageJ (version 1.48, http://imagej.nih.gov/ij/) and the mean value was converted on a semilogarithmic plot into lysozyme equivalents (expressed in µg/ml) according to the standard curve (Giraudeau et al., 2010; Heinrich et al., 2017).

2.8. Statistical analysis

Data analysis was performed using SPSS Statistics 23 (IBM, Armonk, USA). All descriptive data are expressed as mean ± standard deviation (SD). Some BKA analyses returned negative values, meaning the bacterial growth was not reduced but rather enhanced by the seminal plasma. Therefore, the data set was split into groups showing BKA and no BKA: Group 1 (BKA against *E. coli* and *S. aureus*, n = 38), Group 2 (BKA only against *E. coli*, n = 13), Group 3 (BKA only against *S. aureus*, n = 46), and Group 4 (no BKA, n = 22). Sperm quality characteristics were compared using one-way analysis of variance (ANOVA). When ANOVA revealed a significant effect between Groups 1–4, the values were compared using Tukey test (*post hoc*). Differences among means were considered significant at $P \leq 0.05$. Intra group correlations were tested by Spearman's rho and analysis of the corresponding scatterplots.

3. Results

Table 1 shows the investigated semen parameters for all preserved boar ejaculates according to their BKA characteristics. In Group 3, a higher lysozyme concentration than in Group 1 ($P = 0.005$) and Group 2 ($P = 0.047$) was found. No significant differences between the groups were revealed for initial parameters like ejaculate volume, sperm concentration, total sperm number, as well as DNA integrity on d2 and plasma membrane integrity on d3. The percentage of morphologically normal spermatozoa was significantly lower in Group 2 compared with Group 1 ($P = 0.008$), Group 3 ($P < 0.001$), and Group 4 ($P = 0.010$). A higher proportion of spermatozoa with mitochondrial activity on d2 was found in Group 3 compared with Group 2 ($P = 0.023$).

The age of boars in Group 3 and Group 4 was significantly lower ($P < 0.001$) compared with Group 1 and Group 2. On d7, a stress test was performed to assess sperm longevity at body temperature. The percentage of motile spermatozoa in thermo-resistance test after 30 min incubation at 38 °C was significantly lower in Group 4 compared with Group 1 ($P = 0.003$), Group 2 ($P = 0.017$), and Group 3 ($P = 0.007$). Similar trends were observed after 300 min incubation at 38 °C. Within Group 1, BKA against *E. coli* and *S. aureus* were not correlated ($r_s = -0.06$, $P = 0.722$, n = 38).

Tables 2 and 3 show the correlations coefficients r_s and significances *P* (Spearman's rho) within the groups with BKA against *E. coli* (Group 1 plus 2, Table 2) and BKA against *S. aureus* (Group 1 plus 3, Table 3). Although many different sperm quality parameters show significant correlations with each other, special interest was put into the following relations. The age of the boars correlated with BKA against *E. coli* ($r_s = 0.418$, $P = 0.002$, Group 1 plus 2, n = 51, Fig. 1a) but not with BKA against *S. aureus* ($r_s = 0.112$, $P = 0.309$, Group 1 plus 3, n = 84, Fig. 1b). Within the ejaculates with BKA against *S. aureus* (Group 1 plus 3) the initial proportion of morphologically normal spermatozoa correlated positively with lysozyme concentration ($r_s = 0.509$, $P < 0.001$, n = 84, Fig. 2) and negatively with the percentage of spermatozoa bearing cytoplasmic droplets ($r_s = -0.397$, $P < 0.001$, n = 84). For semen with BKA against *E. coli* (Group 1 plus 2), there was no correlation between morphologically normal spermatozoa and lysozyme concentration (Fig. 2) or percentage of spermatozoa bearing cytoplasmic droplets and lysozyme concentration ($r_s = 0.254$, $P = 0.078$, n = 51 and $r_s = -0.177$, $P = 0.222$, n = 51, respectively). Within the ejaculates of Group 1 plus 2, the BKA against *E. coli* correlated positively with the total motility in the thermo-resistance test after 300 min ($r_s = 0.317$, $P = 0.023$, n = 51).

4. Discussion

The present study was conducted to investigate the potential relation between antibacterial defense of seminal plasma and sperm quality, particularly upon storage, from Pietrain boar ejaculates. The BKA of ejaculates is manifested through a reduction or even inhibition of included bacteria species. When measured in plasma or serum samples, BKA is mediated by antibacterial proteins and enzymes (e.g. lactoferrin, lysozyme), opsonizing proteins (e.g., complement and acute phase proteins), and natural antibodies (i.e., immunoglobulins M and A) and therefore it is considered a functional measure of the humoral part of constitutive innate immune defense (Heinrich et al., 2016). Despite the potential involvement of the different innate immune molecules, BKA is mainly mediated by complement (Moore et al., 2011), although the main mediators depend also on the bacterial species and strain tested (Beechler et al., 2012).

In humans (Kuzmin et al., 1998) and in different teleost species (Lahnsteiner and Radner, 2010), a positive relationship between seminal plasma lysozyme levels and sperm motility was found. Contrary to this, the present study did not reveal a relation between lysozyme concentration of seminal plasma and sperm motility of the tested boars with BKA against *E.coli*. It should be noted that only preserved ejaculates were evaluated. Furthermore, the lack of relation is not surprising taking into account that most bacterial contaminants of boar sperm are gram-negative bacteria belonging to the Enterobacteriaceae family (Althouse et al., 2000) and generally, lysozymes are less effective against these bacteria compared with their effect on gram-positive bacteria (Hall et al., 2002). A similar pattern has been described in the case of the superb fairy-wren (*Malurus cyaneus*) by Rowe et al. (2013).

Table 1
Semen quality of 119 preserved boar ejaculates and lysozyme concentration as well as bacterial killing activity against *E. coli* and *S. aureus* in corresponding seminal plasma. Samples were classified in four groups according to their BKA (Group 1 = BKA against *E. coli* and *S. aureus*, Group 2 = BKA against *E. coli*, Group 3 = BKA against *S. aureus*, Group 4 = no BKA).

Parameter	Group 1 (n = 38 samples)	Group 2 (n = 13 samples)	Group 3 (n = 46 samples)	Group 4 (n = 22 samples)	*P*-value
Boar Age (month)	21.7 ± 11.9[a]	26.9 ± 17.5[a]	15.5 ± 5.1[b]	11.5 ± 3.8[b]	< 0.001
Ejaculate volume (ml)	258.1 ± 77.3[a]	272.2 ± 61.8[a]	252.8 ± 69.1[a]	210.2 ± 74.9[a]	0.043
Sperm concentration (10^9/ml)	0.318 ± 0.123[a]	0.311 ± 0.103[a]	0.337 ± 0.179[a]	0.387 ± 0.143[a]	0.336
Total sperm number (10^9)	77.2 ± 24.4[a]	82.9 ± 34.4[a]	79.1 ± 31.9[a]	76.3 ± 25.2[a]	0.915
Normal sperm cells (%)	80.8 ± 10.3[a]	69.2 ± 17.6[b]	87.0 ± 8.4[a]	81.7 ± 13.0[a]	< 0.001
Sperm with mitochondrial activity (%)	79.2 ± 5.0[a,b]	75.3 ± 9.8[a]	81.4 ± 7.0[b]	79.0 ± 6.3[a,b]	0.034
Sperm with impaired DNA integrity (%)	2.0 ± 1.4[a]	4.1 ± 6.6[a]	2.2 ± 2.0[a]	2.4 ± 1.4[a]	0.105
Sperm with intact membrane/acrosome (%)	78.0 ± 9.5[a]	76.9 ± 10.6[a]	80.4 ± 7.4[a]	82.6 ± 10.1[a]	0.129
Total Motility in TRT 30 (%)	69.6 ± 21.0[a]	72.3 ± 20.2[a]	67.3 ± 27.4[a]	43.7 ± 37.2[b]	0.002
Total Motility in TRT 300 (%)	56.8 ± 25.6[a]	62.3 ± 28.4[a]	48.7 ± 27.5[a]	38.0 ± 32.3[a]	0.068
Lysozyme concentration (µg/ml)	2.04 ± 1.10[a]	1.96 ± 0.67[a]	2.93 ± 1.34[b]	2.22 ± 1.10[a,b]	0.003
BKA *E. coli* (%)	22.6 ± 18.5	39.5 ± 13.8	no BKA	no BKA	*n.a.*
BKA *S. aureus* (%)	35.9 ± 20.5	no BKA	24.8 ± 13.6	no BKA	*n.a.*

BKA = bacterial killing activity.
TRT 30 = thermo-resistance test at d7 of storage (30 min incubation at 38 °C).
TRT 300 = thermo-resistance test at d7 of storage (300 min incubation at 38 °C).
[a,b]Values with different superscripts within a row differ significantly ($P < 0.05$; ANOVA; post hoc Tukey test; exact *P-values* see in results sections).

However, a positive relation between the lysozyme levels in seminal plasma and boar semen quality was shown for the group of boars with BKA against *S. aureus*. Ejaculates with higher BKA against *S. aureus* had more morphologically normal spermatozoa and less spermatozoa with cytoplasmatic droplets. Lysozyme was higher in the group of samples with BKA only against *S. aureus* and no BKA against *E. coli*. This suggests a bactericidal effect of the lysozyme especially against *S. aureus*, which belongs to the gram-positive bacteria. In seminal plasma with BKA against *S. aureus*, lysozyme had a positive effect on the ratio of morphologically intact spermatozoa. In seminal plasma with BKA against *E. coli* the lysozyme did not have any effect on morphology of spermatozoa. This indicates that different substances may operate for the BKA against the different bacterial strains and explains why the absolute BKA values against *E. coli* and *S. aureus* do not correlate within Group 1 with BKA against both bacteria. Moreover, boars might invest specifically in the antibacterial defenses directed against the species infecting their reproductive tract. In boar semen, up to now, zinc-dependent proteins with antibacterial, membrane stabilizing and motility regulating characteristics could be isolated (Strzeżek et al., 1987). Most of the proteins in boar semen belong to the group of spermadhesins (Manaskova and Jonakova, 2008). They are mainly synthesized in vesicular glands and in the epididymis (Calvete et al., 1993). Of outstanding importance are the porcine seminal plasma proteins I and II, which account for almost half of the total protein content in boar ejaculates. Additionally, these protein ligands also exert immunomodulatory, antibacterial and antioxidant functions in the female reproductive tract (Yang et al., 1998) and could account for the measured BKA of seminal plasma in this study. Further research should be conducted on the bacterial flora and the investment in different antibacterial defenses in boars.

Already in the 1970s, a peptide named seminalplasmin was isolated from bull ejaculates. In a non-enzymatic way, it affects different microbial agents (Chitnis et al., 1987). Previous studies showed that seminalplasmin is bactericidal at high concentrations and bacteriostatic in low concentrations. In this connection, it is intriguing that the net antibacterial activity of bovine seminal plasma varies 30-fold between batches of semen, and between breeds and species of bovine animals (Shivaji, 1984). In 1981, 1068 samples of bovine seminal plasma from 125 bulls of eight different breeds were scored for antibacterial activity against *E. coli*, as well as for their fertilizing capacity by artificial insemination of 2200 cows (Bhargava, 1981). The authors found no correlation between the antibacterial activity against *E. coli* and fertility in bulls. The connections of BKA to boar sperm quality characteristics found in this study, especially for thermo-resistance of boar spermatozoa which are related to pregnancy rate and litter size in sows (Schulze et al., 2013b), indicate a link between BKA and fertilizing capacity of boar ejaculates, which remains to be investigated. Recently, antibacterial competence in ejaculates of *Anas platyrhynchos* could even be related with the phenotype of these drakes (Rowe et al., 2011). The beak of males with high bacterial killing activity against *E. coli* showed a high carotenoid content, this way allowing the females to choose the males with higher fertility (Peters et al., 2004; Rowe et al., 2011).

Interestingly, BKA against *E. coli* seemed to be increased with age. The boars of Group 1 and 2 with BKA against *E. coli* were significant older than the boars of Group 3 and 4 with no BKA against *E. coli*. This was already described in birds: stonechat (*Saxicola torquata*) blood had a rising bactericidal capacity against *E. coli* with increased age (Tieleman et al., 2010). BKA against at least one bacterial strain seems to be beneficial for the functional survival of sperm during storage. The thermo-resistance at day seven after 30 min incubation at 38 °C was significantly lower in Group 4 (no BKA). The test showed the same tendency after 300 min incubation at 38 °C. If the negative BKA values are caused by contamination of the fresh ejaculates these contaminating bacteria are supposed to be gentamicin-resistant and presumably grew during the seven-day storage period. On the other hand, if BKA of seminal plasma suppressed this potential growth of antibiotic-resistant bacteria the substances responsible for this bacterial growth inhibition could be potential new ingredients for semen extenders. Facing the growing risk of antibiotic-resistant bacteria, further investigation of the bacterial flora in semen with no BKA and elucidation of the BKA responsible substances would be of great interest. The first focus of these investigations should be on the substances for BKA against *E. coli* as within this group BKA against *E. coli* correlates positively with total motility after 300 min at 38 °C.

In this context, it should be considered that gram-negative bacteria release lipopolysaccharides, which act as an endotoxin and exerts spermicidal activity (Hakimi et al., 2006). A high bacterial preload in fresh boar ejaculates based on a poor hygiene management during semen collection and associated with no or low BKA leads to the observed poor semen quality. Nevertheless, we can only speculate which mechanisms are responsible for the deterioration of sperm quality in this multifactorial interaction between environmental and boar-specific effects.

Table 2
Correlation coefficients r_s and significance *P* (Spearman's rho) of investigated sperm quality parameters, lysozyme concentration in corresponding seminal plasma and boar age within the groups with bacterial killing activity against *E. coli* (Group 1 plus 2, n = 51). Significant correlations are marked in bold numbers. Differences between the groups, which seemed of special interest to the authors, are highlighted with grey squares and are shown as scatter-plots in Fig. 1 and 2.

BKA against *E. coli* (Group 1 plus 2, n = 51) Spearman's rho		Ejaculate volume (ml)	Sperm concentration (10^9/ml)	Normal sperm cells (%)	Sperm with cytoplasmatic droplets (%)	Sperm with mitochondrial activity (%)	Sperm with impaired DNA integrity (%)	Sperm with intact membrane/ acrosome (%)	Total Motility in TRT 30 (%)	Total Motility in TRT 300 (%)	Lysozyme concentration (µg/ ml)	BKA *E. coli* (%)
Boar Age (month)	r_s	0.151	0.128	−0.035	−0.025	0.294	−0.402	−0.044	0.297	0.370	−0.248	0.418
	P	*0.289*	*0.370*	*0.807*	*0.864*	*0.036*	*0.003*	*0.761*	*0.034*	*0.008*	*0.085*	*0.002*
Ejaculate volume (ml)	r_s		−0.507	0.199	−0.179	0.182	0.024	−0.069	0.085	0.091	0.186	0.110
	P		*0.000*	*0.163*	*0.209*	*0.202*	*0.870*	*0.632*	*0.555*	*0.525*	*0.202*	*0.442*
Sperm concentration (10^9/ ml)	r_s			−0.326	0.315	−0.016	0.002	0.041	0.025	0.204	−0.150	−0.118
	P			*0.020*	*0.025*	*0.909*	*0.991*	*0.775*	*0.863*	*0.152*	*0.305*	*0.411*
Normal sperm cells (%)	r_s				−0.948	0.497	−0.337	0.070	0.279	0.028	0.254	−0.167
	P				*0.000*	*0.000*	*0.016*	*0.626*	*0.048*	*0.844*	*0.078*	*0.241*
Sperm with cytoplasmatic droplets (%)	r_s					−0.490	0.314	−0.096	−0.342	−0.077	−0.177	0.124
	P					*0.000*	*0.025*	*0.504*	*0.014*	*0.589*	*0.222*	*0.387*
Sperm with mitochondrial activity (%)	r_s						−0.332	0.217	0.541	0.276	0.124	0.121
	P						*0.017*	*0.126*	*0.000*	*0.050*	*0.396*	*0.397*
Sperm with impaired DNA integrity (%)	r_s							−0.216	−0.234	−0.089	−0.059	−0.221
	P							*0.127*	*0.098*	*0.532*	*0.688*	*0.120*
Sperm with intact membrane/acrosome (%)	r_s								0.309	0.277	−0.073	0.153
	P								*0.027*	*0.049*	*0.618*	*0.285*
Total Motility in TRT 30 (%)	r_s									0.679	0.017	0.083
	P									*0.000*	*0.906*	*0.565*
Total Motility in TRT 300 (%)	r_s										−0.168	0.317
	P										*0.248*	*0.023*
Lysozyme concentration (µg/ml)	r_s											−0.185
	P											0.203

Table 3
Correlation coefficients r_s and significance *P* (Spearman's rho) of investigated sperm quality parameters, lysozyme concentration in corresponding seminal plasma and boar age within the groups with bacterial killing activity against *S. aureus* (Group 1 plus 3, n = 84). Significant correlations are marked in bold numbers. Differences between the groups, which seemed of special interest to the authors, are highlighted with grey squares and are shown as scatter-plots in Figs. 1 and 2.

BKA against *S. aureus* (Group 1 plus 3, n = 84) Spearman's rho		Ejaculate volume (ml)	Sperm concentration (10^9/ml)	Normal sperm cells (%)	Sperm with cytoplasmatic droplets (%)	Sperm with mitochondrial activity (%)	Sperm with impaired DNA integrity (%)	Sperm with intact membrane/ acrosome (%)	Total Motility in TRT 30 (%)	Total Motility in TRT 300 (%)	Lysozyme concentration (µg/ ml)	BKA *S. aureus* (%)
Boar Age (month)	r_s	0.165	0.090	−0.021	−0.054	0.260	−0.191	−0.167	0.286	0.284	−0.143	0.112
	P	*0.133*	*0.415*	*0.851*	*0.629*	*0.018*	*0.084*	*0.132*	*0.009*	*0.009*	*0.199*	*0.309*
Ejaculate volume (ml)	r_s		−0.529	0.104	−0.166	0.078	−0.008	−0.182	−0.019	0.019	0.214	−0.197
	P		*0.000*	*0.349*	*0.133*	*0.482*	*0.941*	*0.100*	*0.866*	*0.862*	*0.054*	*0.072*
Sperm concentration (10^9/ ml)	r_s			−0.180	0.182	−0.008	0.065	0.098	0.158	0.283	−0.175	0.179
	P			*0.103*	*0.100*	*0.946*	*0.560*	*0.380*	*0.154*	*0.010*	*0.116*	*0.104*
Normal sperm cells (%)	r_s				−0.893	0.418	−0.253	−0.067	0.187	−0.058	0.509	−0.109
	P				*0.000*	*0.000*	*0.021*	*0.547*	*0.091*	*0.602*	*0.000*	*0.327*
Sperm with cytoplasmatic droplets (%)	r_s					−0.402	0.313	0.042	−0.146	0.019	−0.397	0.117
	P					*0.000*	*0.004*	*0.703*	*0.189*	*0.864*	*0.000*	*0.293*
Sperm with mitochondrial activity (%)	r_s						−0.318	0.183	0.451	0.302	0.173	0.075
	P						*0.003*	*0.097*	*0.000*	*0.005*	*0.123*	*0.501*
Sperm with impaired DNA integrity (%)	r_s							−0.066	−0.154	−0.154	0.023	−0.115
	P							*0.551*	*0.165*	*0.164*	*0.838*	*0.302*
Sperm with intact membrane/acrosome (%)	r_s								0.134	0.157	0.034	0.075
	P								*0.228*	*0.157*	*0.760*	*0.499*
Total Motility in TRT 30 (%)	r_s									0.688	0.108	0.113
	P									*0.000*	*0.337*	*0.308*
Total Motility in TRT 300 (%)	r_s										−0.172	0.061
	P										*0.124*	*0.585*
Lysozyme concentration (µg/ml)	r_s											−0.209
	P											*0.060*

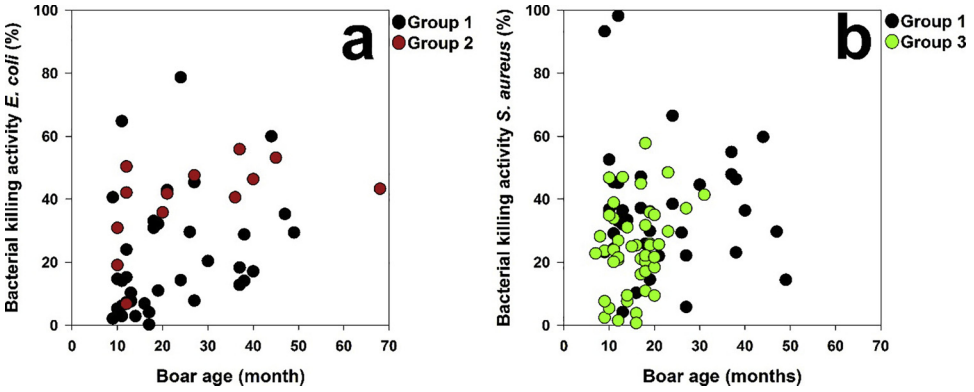

Fig. 1. Scatter-plots of bacterial killing activity (BKA) in relation to boar age. Samples were classified in groups according to their BKA characteristics (Group 1 = BKA against *E. coli* and *S. aureus*, Group 2 = BKA against *E. coli* and Group 3 = BKA against *S. aureus*).
BKA against *E. coli* (**a**) correlates positively with boar age (r_s = 0.418, P = 0.002, n = 51) and BKA against *S. aureus* (**b**) shows no correlation with boar age (r_s = 0.112, P = 0.309, n = 84).

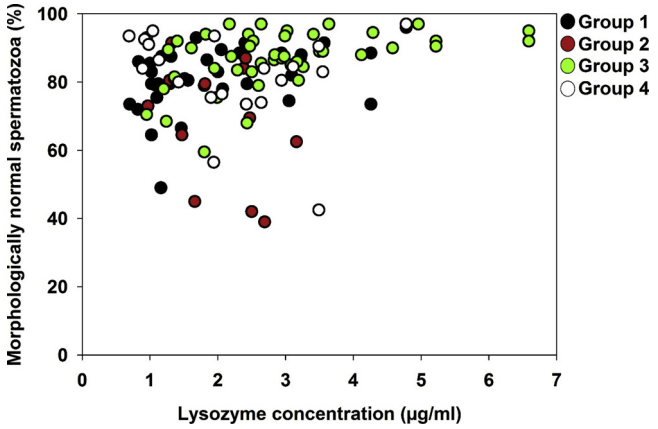

Fig. 2. Proportion of morphologically normal spermatozoa in dependence of lysozyme concentration in corresponding seminal plasma. Samples were classified in groups according to their BKA characteristics (Group 1 = BKA against *E. coli* and *S. aureus*, Group 2 = BKA against *E. coli*, Group 3 = BKA against *S. aureus*, Group 4 = no BKA).
The proportion of morphologically normal spermatozoa correlates positively with lysozyme concentration only in samples with BKA against *S. aureus* (Group 1 plus 3, r_s = 0.509, $P <$ 0.001, n = 81).

5. Conclusions

In conclusion, all these examples emphasize the importance of protecting the genital tract of both males and females from non-commensal bacteria. Due to the enormous impact bacteria can have on the morphology and the metabolism of spermatozoa, males with an effective immunological defense in their ejaculates gain an evolutionary advantage in reproduction. Based on our result, the exclusion of young boars with missing or low BKA against *E. coli* from use in artificial insemination could be advantageous. Further investigations need to answer if there is an immediate relation between bacterial killing activity and fertility in practice. Moreover, biochemical analysis is required for further characterization of specific mediators of antimicrobial defense in boar semen.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

This research was partially supported by the AiF Inc. (ZF4276702SK6). G.Á.C. was supported by funds from the Leibniz Institute for Zoo and Wildlife Research. We are grateful to all donors who consented to participate in this study. We thank Katja Pohle for her help with the immunological measurements.

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