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The relationship between proAKAP4 and sperm motility, viability, and morphology in mice

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Submitted by
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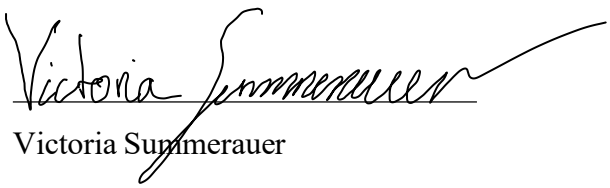

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Abstract

Estimating the quality of a frozen mouse sperm sample in advance and ensuring an in-vitro fertilization (IVF) will be successful is a challenging task. A biomarker for sperm fertility could greatly help improving the efficiency of reproductive techniques of animal experiments. The protein proAKAP4 is crucial for sperm motility and has been suggested as a diagnostic biomarker for male fertility. A recent study by Boersma et al. (2022) showed that proAKAP4 levels were related negatively to some sperm motility parameters. To better understand proAKAP4 in relation to sperm quality, we determined the relationship between proAKAP4 concentration and sperm morphology, viability, and motility parameters in mice. For the analysis, computer-assisted sperm motility analysis (CASA), sperm morphology analysis and viability staining were applied to determine sperm quality. Frozen sperm samples of 44 donor mice (SWISS, B6D2F1, C57BL/6N, and BALB/c) from a previous study were available. ProAKAP4 concentration as determined in the precursor project did not differ between mouse strains and was categorized into three groups, low (<25 ng/mL; N=7), medium (≥25 ng/mL, <50 ng/mL; N=29) and high (≥50 ng/mL; N=8). We found no significant relationship between proAKAP4 groups and sperm quality parameters. However, sperm quality parameters differed strongly between strains, with SWISS and BALB/c mice having a reduced sperm quality compared to the hybrid strain B6D2F1. Future studies should include *in vitro* fertilization assays to determine the significance of proAKAP4 as a biomarker in mouse fertility.

Die Qualität gefrorenen Mausspermaprobe im Voraus abzuschätzen und sicherzustellen, dass eine IVF erfolgreich verläuft, ist eine herausfordernde Aufgabe. Ein Biomarker für die Spermienfruchtbarkeit könnte wesentlich dazu beitragen, die Effizienz von Reproduktionstechniken im Tierversuch zu verbessern. Das Protein proAKAP4 ist entscheidend für die Beweglichkeit der Spermien und könnte als diagnostischer Biomarker für die männliche Fertilität dienen. Eine aktuelle Studie von Boersma et al. (2022) zeigte, dass die proAKAP4-Konzentration eine negative Beziehung zu einigen Spermienmotilitätsparametern hatten. Um proAKAP4 in Bezug auf die Spermienqualität besser zu verstehen, haben wir die Beziehung zwischen proAKAP4-Konzentration und Spermienmorphologie, Membranintegrität und Motilitätsparametern bei Mäusen bestimmt. Für die Analyse wurden computergestützte Analysen der Spermienmotilität (CASA), die Untersuchung der Spermienmorphologie und eine

Lebend-Tot-Färbung angewendet, um die Spermienqualität zu bestimmen. Die gefrorenen Spermienproben von 44 Spendermännchen (SWISS, B6D2F1, C57BL/6N und BALB/c) standen von der Vorgängerstudie zur Verfügung. Die ProAKAP4-Konzentration, die im Vorgängerprojekt bestimmt worden war, unterschied sich nicht zwischen den Mausstämmen und wurde in drei Gruppen eingeteilt: niedrig (<25 ng/mL; N=7), mittel (≥25 ng/mL, <50 ng/mL; N=29) und hoch (≥50 ng/mL; N=8). Wir fanden keinen Zusammenhang zwischen proAKAP4-Gruppen und Spermienqualitätsparametern. Jedoch unterschieden sich die Stämme deutlich hinsichtlich ihrer Spermienqualität, wobei insbesondere SWISS und BALB/c-Mäuse im Vergleich zum Hybridstamm B6D2F1 eine reduzierte Spermienqualität hatten. Zukünftige Studien sollten auch die In-vitro-Fertilisation anwenden, um die Bedeutung von proAKAP4 als Biomarker für die Fruchtbarkeit von Mäusen zu bestimmen

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1 Introduction and Question

The common laboratory mouse (*Mus musculus*) shares 99 % of its genes with humans; therefore, laboratory mice are the laboratory animal model of choice for many genetic research questions. Using modern genetic manipulation techniques, a considerable number of disease models can be generated. Breeding and maintaining mice in captivity are relatively easy and economic compared to other mammalian species. Assisted reproductive techniques (ART), like embryo transfer, sperm or embryo cryopreservation, and in vitro fertilization (IVF), are used to create, archive and distribute these mouse models. Although these methods are well established and widely used, some specific problems exist. Sperm quality, for example, is an only unsatisfactorily defined variable. It is especially difficult to estimate the quality of a frozen mouse sperm sample in advance and to ensure that an IVF will be successful. Of course, many factors come into play here. A biomarker for fertility in sperm could be of great help to improve the efficiency of reproductive techniques and thus primarily reduce the number of animals required per experiment, which is also in line with Russel and Burch's principles of 3R, i.e., the replacement, reduction, and refinement of animal experiments (1–3). In human fertility, approximately 15 % of couples trying to get pregnant are infertile worldwide. Infertility is defined as the absence of pregnancy after at least 12 months of regular unprotected sexual intercourse. It is declared as a disease of the male or female reproductive system, with approximately 20 % – 30 % of infertility cases being due to the male sex alone and 50% of males being involved. The most common problems causing infertility in males are the ejection of semen, absence or low sperm, sperm shape abnormalities (morphology), and movement of the sperm (4,5). Primarily reduced sperm motility (asthenozoospermia) seems to be one of the most common problems associated with male infertility. Therefore, it is essential to understand the functions and mechanisms that regulate flagellum motility (6). The most significant advances in human reproductive medicine have come from analyzing reproductive physiology and genetics in mice. To find a biomarker for fertility in mice would therefore serve many purposes at once.

1.1 A-kinase anchoring protein (AKAP), AKAP4, and ProAKAP4

A promising protein family in this regard is the A-kinase anchoring protein (AKAP) family, which builds the molecular basis of sperm motility and morphology. It represents a functionally conserved family of signal-organizing scaffolding proteins. AKAP4 especially plays an essential role in the fibrous sheath assembly during spermatogenesis and flagellar function in spermatozoa. It is a highly conserved sperm-specific protein for anchoring cAMP-dependent

protein kinases. It has been the first AKAP to be described as associated with the sperm flagellum (7,8). AKAP4 (predominant in mice) and AKAP3 (predominant in humans) are sperm-specific AKAP isoforms that mainly make up the fibrous sheath. As spermatozoa need to minimize their cellular components and develop an active propulsor machinery in the flagellum to reach and fertilize the oocyte, both proteins are already transcribed and synthesized during spermatogenesis. The proteins are localized in the principal piece of the sperm tail, where they are responsible for constructing the fibrous sheath, which in turn dictates the degree of flexibility, plane of motion, and beating shape of the flagellum (8,9). Further, AKAP4 is synthesized as a precursor (proAKAP4). In mature spermatozoa, where proAKAP4 is already processed to AKAP4, the precursor protein cannot be detected. This leads to the conclusion that it could play a key role in flagellar development. Furthermore, for spermatozoa to be able to give a rapid cell response to various stimuli, they are dependent on the compartmentalization of signaling by scaffold proteins such as AKAP4. This becomes a key mechanism to differentiate signaling cascades initiated by different stimuli but propagated by the same amount of second messengers presented in the cell (8). The link between the AKAP4 protein and infertility, flagellum development, and reduced sperm motility could be shown in studies using AKAP4 knockout mice (10,11). In a recent study, the influence of sperm cryopreservation on sperm motility and proAKAP4 concentration in mice was examined (Boersma et al. 2022). It could be shown that the proAKAP4 levels had a negative relationship with sperm direction parameters. In contrast, neither a positive nor a negative effect could be found in relation to sperm motility. The effect of the freezing and thawing procedure was also not significant in relation to proAKAP4 levels, which allows the analysis of proAKAP4 expression levels in already stored sperm samples.

1.2 Hypotheses and aim of the study

This study hypothesizes that the proAKAP4 concentrations correlate with sperm quality, measured as the percentage of morphological abnormalities, viability, and motility of spermatozoa. If this could be confirmed, the proAKAP4 concentration could be used as a biomarker for sperm quality. To test this, the frozen samples, collected and analyzed in the study of Boersma et al. (2022) were used (14). In these samples, the sperm viability, morphology, and motility were determined and related to proAKAP4 levels of the former study (Boersma et al. 2022). This study was performed with four laboratory mouse strains, SWISS, B6D2F1, C57BL/6N, and BALB/c, to be able to test additionally for strain-specific differences.

2 Material and Methods

2.1 Ethical statement

No animal experiments were carried out in the course of this work. The donor animals from this study were used in another study which was reported to, discussed and approved by the Ethics committee (Tierschutz- und Ethikkommission, ETK) of the University of Veterinary Medicine Vienna, and the Bundesministerium für Bildung, Wissenschaft und Forschung, Austria (filed number 2022-0.109.869).

2.2 Reagents and Media

A medium developed by Toyoda, Yokoyama, and Hosi (TYH), sperm cryoprotective agent (CPA), mineral oil, and eosin-nigrosin (EN) were used as reagents and media for this study. TYH and CPA were prepared in-house (Institute of *In vivo* and *In vitro* Models) using ingredients purchased from Sigma-Aldrich, Austria. For sperm cryopreservation, cryoprotective agent (CPA) was used containing 18 % raffinose pentahydrate, 3 % skim milk (BD BBL™ / Difco™, Fisher Scientific, Austria), and 100 mM L-glutamine (12). To dilute the sperm for sperm quality analysis, TYH medium was used, a modified Krebs-Ringer bicarbonate solution. TYH consists of 119.37 mM sodium chloride, 4.78 mM potassium chloride, 1.71 mM calcium chloride, 1.19 mM magnesium sulfate heptahydrate, 1.19 mM potassium dihydrogen phosphate, 25.07 mM sodium bicarbonate, 5.56 mM D+ glucose, 0.5 mM sodium pyruvate, Penicillin G (7.5 mg / 100 ml), Streptomycin (5.0 mg / 100 ml), and polyvinyl alcohol (100 mg / 100 mL) and was supplemented with 0.75 mM methyl-beta-cyclodextrin(12,13).
The eosin-nigrosin was purchased from Sanova, Austria (Cat.no. 12424.00250).
The Mineral Oil (BP2629-1) was purchased from Fisher Bioreagents (USA) and was used to cover small medium drops in a Petri dish to prevent evaporation and rapid pH and temperature change.

2.3 Study Design

This study aims to put proAKAP4 concentrations, as measured in Boersma et al. (2022), in relation to sperm quality parameters, measured as sperm motility (measured by computer-assisted sperm analysis, CASA), sperm morphology and sperm viability (using eosin-nigrosin staining). Sperm samples (collected from 46 male donor mice and cryopreserved, as described in Boersma et al. 2022) were stored in a nitrogen tank and thawed for the purpose of this study. Two straws could not be used for different reasons, leaving 44 samples for the purpose of this study.

2.4 Sperm thawing

For thawing, the straw, which contained a 10 µl and a 20 µl drop of sperm, was kept in air for 10 seconds and then put into a water bath (37 °C) for 10 minutes. Then the straw was cut open, and with the aid of a metal rod, the 10 µl drop was pushed on a slide for the morphology and viability analysis (chapter 2.5). The 20 µl drop was pushed into a 90 µl-drop of TYH under mineral oil in a 35 mm dish (labeled "PI" for preincubation dish) (Falcon Easy Grip Tissue culture dish, Corning Incorporated, USA). It was then incubated for another 30 minutes at 37 °C and 5 % CO_2. After the 30 minutes of incubation, the sperm motility analysis with the Sperm Class Analyzer® was performed (see chapter 2.6).

2.5 Sperm morphology and viability staining

After thawing, the sperm sample was put on a slide. A 20 µl drop of eosin-nigrosin staining solution (EN) was added and gently mixed for ~ 15 sec with the tip of the pipette (Fig.2). The drop was picked up with another slide. Two smears (duplicate) were made on two labeled slides and then left for drying (Fig.1,2).

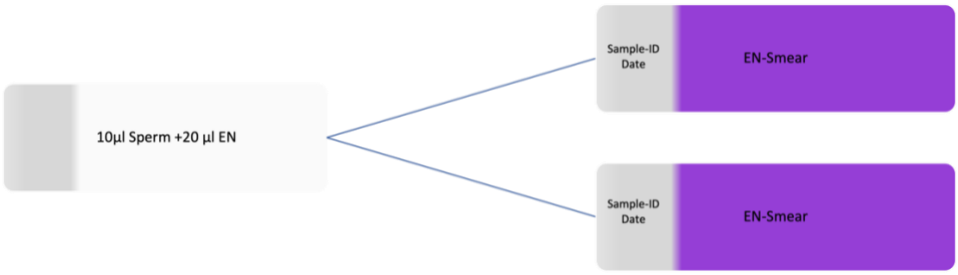

Figure 1. Staining procedure (1): *One slide (on the left) was used for mixing sperm suspension (10 µl) and eosin-nigrosin staining solution (20 µl), and two slides (on the right) were used for the smears in duplicates.*

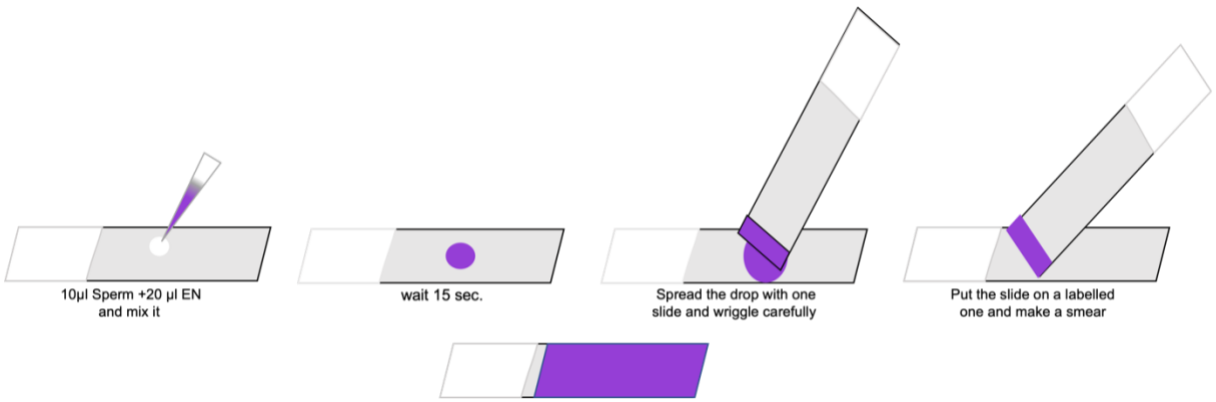

Figure 2. Staining procedure (2): *Mixture of sperm suspension and eosin-nigrosin staining solution and smear preparation*

For the sperm morphology analysis, from every slide 250 spermatozoa were evaluated. The spermatozoa were assessed in different morphological categories describing the three main parts of the sperm, head, midpiece and tail (11). The following 10 abnormalities were distinguished (see examples in Fig. 3): head: without acrosome ('no hook'), abnormal head (any deviation from the normal head size or form); midpiece: folded midpiece, looping midpiece, bent midpiece; tail: bent or folded tail, looping tail; head-neck connection: decapitated, abnormal head neck connection; both midpiece and tail affected: coiled midpiece and tail.

Any sperm having at least one abnormality, was assigned to the category 'abnormal', any sperm having more than one abnormality, was assigned to the category 'multiple abnormal'. Therefore, in total 12 categories were used. Because each abnormality was counted individually, the sum of normal and abnormal sperm could be more than 100%.

For the analysis of sperm viability, every sperm was assigned to the class 'live' (white) or 'dead' (from slightly pink to red or reddish), i.e., also slightly damaged or 'moribund' sperm were classified as 'dead'.

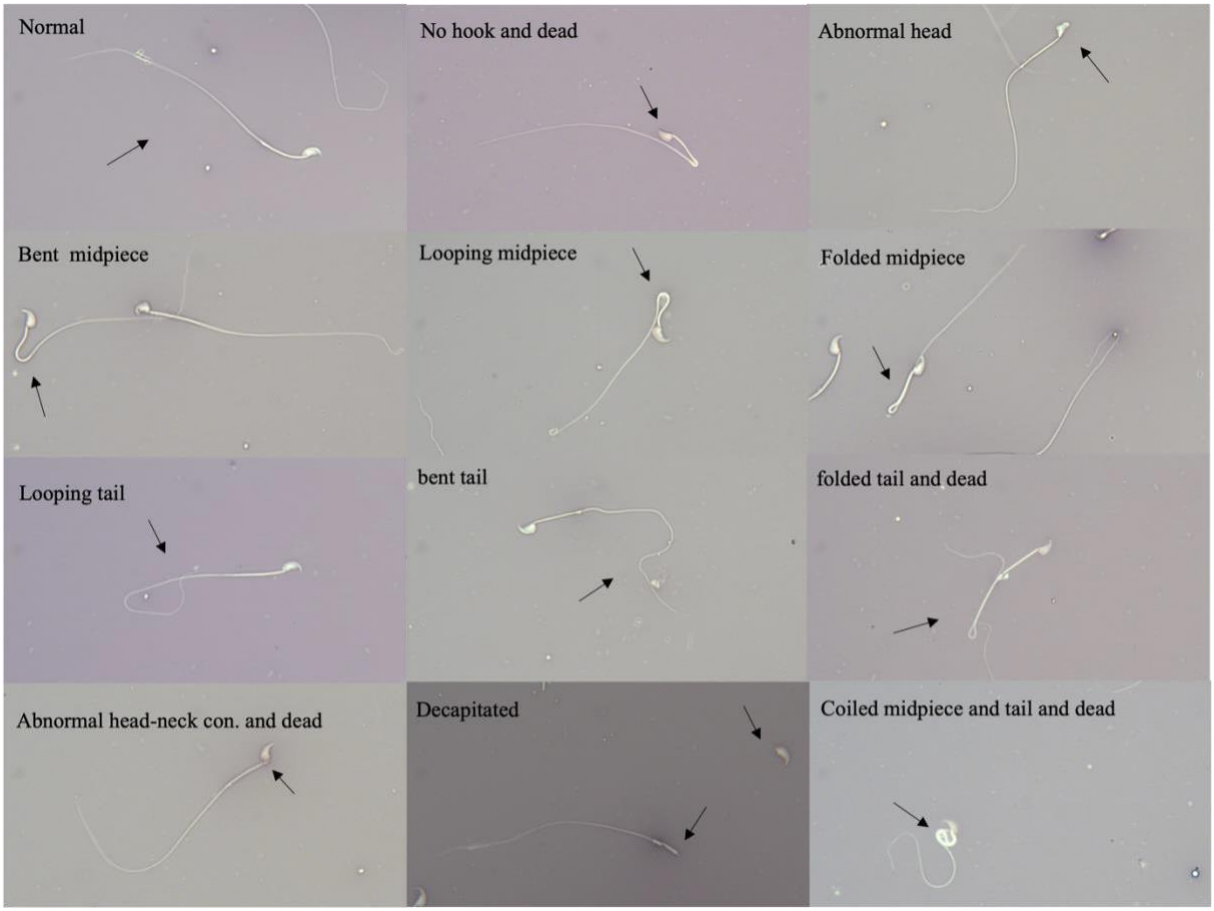

Figure 3. Examples for the abnormal morphology and viability categories

2.6 Computer-aided sperm analysis (CASA) with the Sperm Class Analyzer® (SCA)

After 30 minutes of incubation in the "PI-dish" where the dish was carefully shaken with a circling movement on a heating plate (OTS 40, Medite Medical GmbH, Germany) to have a homogenous suspension, 5 µl of the sperm suspension were pipetted carefully into a chamber of a pre-heated Leja® -Slide (20 µm deep; Leja Products BV, Netherlands). Excess media was removed by blotting it with Kimtech wipes to reduce the liquid flow in the chamber and prevent drift in the media during the analysis. The Leja-Slide was put on a USB-powered heating plate on the microscope stage. Spermatozoa were then analyzed through a microscope (Nikon Eclipse 2000, Nikon, Japan) with a 10 x objective (Nikon, Japan) using negative phase contrast and a high-speed digital camera (Basler acA1300-200uc, Basler, Germany). Using the Sperm Class Analyzer® (SCA®) software (Version 6.5.0.15), between ten and fifteen videos were recorded (frame rate 50 fps; 25 images per field) per sample, covering the whole slide, to analyze up to 500 spermatozoa (total magnification on the screen 494 x). All setup parameters were chosen according to Boersma et al. (2022) and are given in the appendix (Table A1 and A2). The following CASA parameters (Fig.4) were measured and calculated by the SCA® software: curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), path linearity (LIN, %, linearity of the actual sperm track), path wobble (WOB, %, departure of actual sperm track from average path), path straightness (STR, %, linearity of the average path), average lateral head displacement (ALH, µm) and beat-cross frequency (BCF, Hz). In addition, the motility of spermatozoa was determined, defined as the percentage of progressively motile spermatozoa (%, VCL > 10 µm/s). (14)

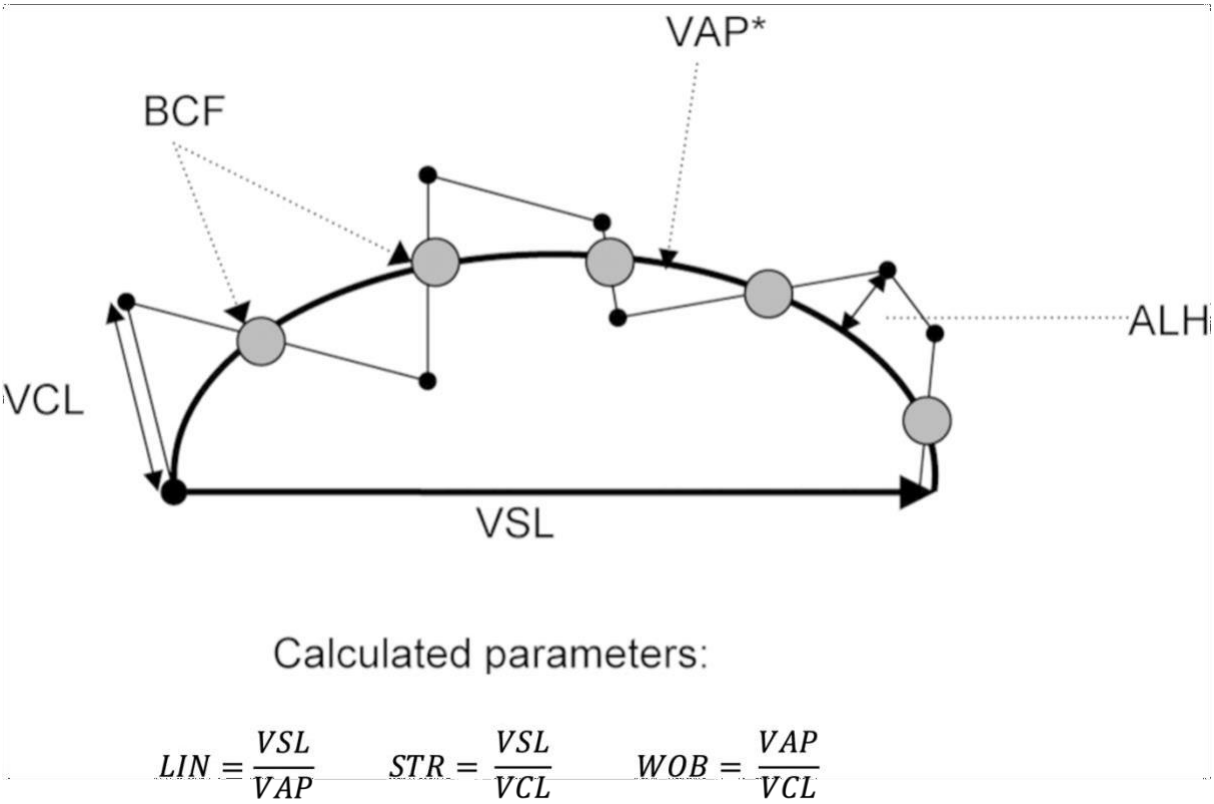

Figure 4. Sperm kinematic parameters measured by computer-assisted semen analysis (CASA). *Figure modified from Sloter et al. 2006* (15).

2.7 proAKAP4 Concentration

As described before, the proAKAP4 values (in ng/mL) as determined by Boersma et al. (2022) were used in this study. For details on the commercial sandwich ELISA (Mouse 4MID Kit, 4BioDx, France), refer to Boersma et al. (2022). For this study, the obtained concentration levels were classified into three groups: low (< 25 ng/mL), medium (≥ 25 ng/mL, < 50 ng/mL) and high (≥ 50 ng/mL). The total number of individuals in the sample differed in size for the three different proAKAP4 groups (low: N = 7, medium: N = 29, high: N = 8).

2.8 Statistical methods

The Statistical analysis was calculated with SPSS (Version 29.0.0.0). Boxplots were used to represent the data distribution graphically. According to SPSS convention, outliers are represented as small circles (distance to 1st or 3rd quartile greater than 1.5x interquartile range) or stars (distance to 1st or 3rd quartile greater than 3 x interquartile range). We ran a linear model where we included the different sperm morphology and motility viability parameter as the dependent variable and where we included mouse strain and proAKAP4 groups as fixed parameters. As a post-hoc test we used the Tukey-HSD test to test all possible group combinations.

3 Results

3.1 Sperm morphology

As described before (see 2.5), the morphological abnormalities were divided into 12 categories. The percentage of the different abnormalities for the four strains are given in Table 1. As can be seen there, roughly half (48.71 - 59.03 %) of the sperm of every strain was normal, and the other half had at least one abnormality. About 10.31 % of the sperm had multiple abnormalities, for the SWISS strain there were even 16.42 % in this category. Another frequent category was the 'bent & folded tail' with about 23.52 - 34.78 %.

Table 1: Percentage of sperm abnormalities for four different mouse strains and total amount.

	normal (%)	abnormal (%)	multiple abnorm (%)	no hook (%)
SWISS	48,71 ± 4,92	51,29 ± 4,92	16,42 ± 5,09	0,16 ± 0,21
B6D2F1	59,03 ± 6,43	40,97 ± 6,43	6,96 ± 2,89	0,39 ± 0,65
C57BL/6N	42,23 ± 9,28	57,77 ± 9,28	10,54 ± 1,85	0,42 ± 0,42
BALB/c	50,19 ± 6,58	49,81 ± 6,58	8,73 ± 2,52	1,05 ± 0,72
total	50,13 ± 9,46	49,87 ± 9,46	10,31 ± 4,63	0,51 ± 0,64
	abnormal head (%)	folded midpi. (%)	looping midpi. (%)	bent midpi. (%)
SWISS	2,8 ± 2,44	0,69 ± 0,37	6,33 ± 2,29	13,13 ± 3,99
B6D2F1	2,78 ± 5,46	0,88 ± 0,96	2,05 ± 1,02	6,55 ± 1,78
C57BL/6N	5,43 ± 1,75	3,57 ± 3,39	2,48 ± 0,62	6,17 ± 1,54
BALB/c	12,65 ± 9,05	1,37 ± 0,77	2,84 ± 1,69	4,88 ± 1,61
total	5,97 ± 6,84	1,7 ± 2,22	3,24 ± 2,17	7,38 ± 3,79
	bent & folded tail (%)	looping tail (%)	decapitated (%)	abnormal head con. (%)
SWISS	34,78 ± 4,02	3,49 ± 0,96	3,87 ± 1,12	2,44 ± 1,37
B6D2F1	26,02 ± 2,07	2,66 ± 1,04	4,32 ± 1,97	2,09 ± 1,44
C57BL/6N	24,62 ± 6,15	2,98 ± 1,29	12,35 ± 13,54	10,06 ± 3,45
BALB/c	23,52 ± 3,91	2,56 ± 1,33	7,28 ± 4,14	1,95 ± 1,1
total	26,8 ± 5,98	2,89 ± 1,22	7,16 ± 8,21	4,3 ± 4,12
	Coiled midpi. & tail (%)			
SWISS	0,02 ± 0,06			
B6D2F1	0,19 ± 0,37			
C57BL/6N	0,23 ± 0,29			
BALB/c	0,43 ± 0,4			
total	0,23 ± 0,35			

3.1.1 Relationship between morphological abnormalities and mouse strain

We found that the rate of morphological abnormalities ($F = 11.8$, $p < 0.001$, Fig.5A) and multiple abnormalities ($F = 13.797$, $p < 0.001$, Fig. 5B) were significantly affected by mouse strain. Similarly, we also found significant relationships between all other morphological

abnormalities ($F > 3.991$, $p < 0.014$) and mouse strains except the category looping tail ($F = 1.771$, $p = 0.169$). The percentage of morphological abnormalities and percentage of multiple abnormalities of B6D2F1 were significantly lower than SWISS ($p = 0.014$), C57BL/6N ($p < 0.001$), and BALB/c ($p = 0.029$). SWISS had a higher percentage of multiple morphological abnormalities compared to B6D2F1 ($p < 0.001$), C57BL/6N ($p = 0.002$), and BALB/c ($p < 0.001$). C57BL/6N tended to have more abnormalities than BALB/c ($p = 0.058$) and more multiple abnormalities than B6D2F1 ($p = 0.063$). The other strains did not differ from each other in these two categories (all $p > 0.198$).

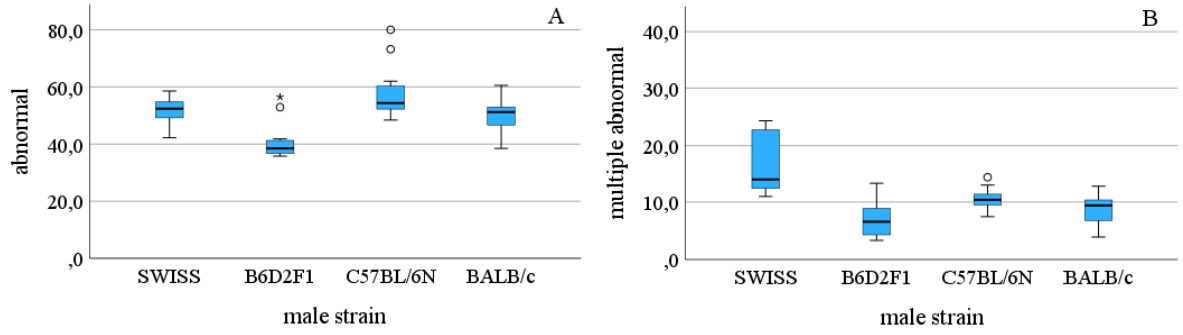

Figure 5. Relationship between male strain and percentage of abnormal spermatozoa (A) and percentage of multiple abnormal spermatozoa (B). *Note that the Y-axis range of the boxplots is truncated to 80 % and 40 %, respectively.*

3.1.1.1 Head abnormalities

The percentage of sperm head abnormalities over all four strains was 5.97 ± 6.84 %, whereas the spermatozoa without hook accounted only for 0.51 ± 0.64 %. Mouse strain had a significant influence on both parameters ($p < 0.001$, Fig.6). BALB/c had a higher percentage of spermatozoa without hook compared to SWISS ($p = 0.003$) and B6D2F1 ($p = 0.011$) and a higher percentage of head abnormalities compared to B6D2F1 hybrids ($p < 0.001$). B6D2F1 hybrids had a lower percentage of head abnormalities compared to C57BL/6N ($p = 0.004$).

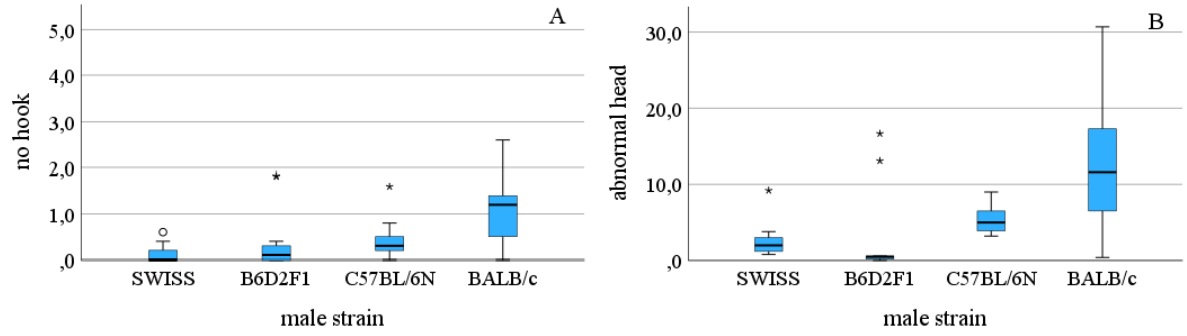

Figure 6. Relationship between male strain and percentage of spermatozoa with no hook (A) and percentage of spermatozoa with general head abnormalities (B). *Note that the Y-axis range of the boxplots is truncated to 5 % and 30 %, respectively*

3.1.1.2 Midpiece Abnormalities

The midpiece abnormalities were folded midpiece (1.7 ± 2.22 %), looping midpiece (03.24 ± 2.17 %), and bent midpiece (7.38 ± 3.79 %). All three subcategories were significantly influenced by mouse strain ($p < 0.001$; Fig.7). SWISS had more spermatozoa with a bent or looping midpiece than the three other strains ($p < 0.001$) but fewer spermatozoa with a folded midpiece than C57BL/6N ($p = 0.002$). The C57BL/6N strain shows a strikingly asymmetric distribution in the percentage of folded midpiece, as indicated by the high 3rd quartile and upper whisker (Fig. 7A). The percentage of spermatozoa with a bent midpiece in BALB/c males was below that of B6D2F1 hybrids ($p < 0.029$).

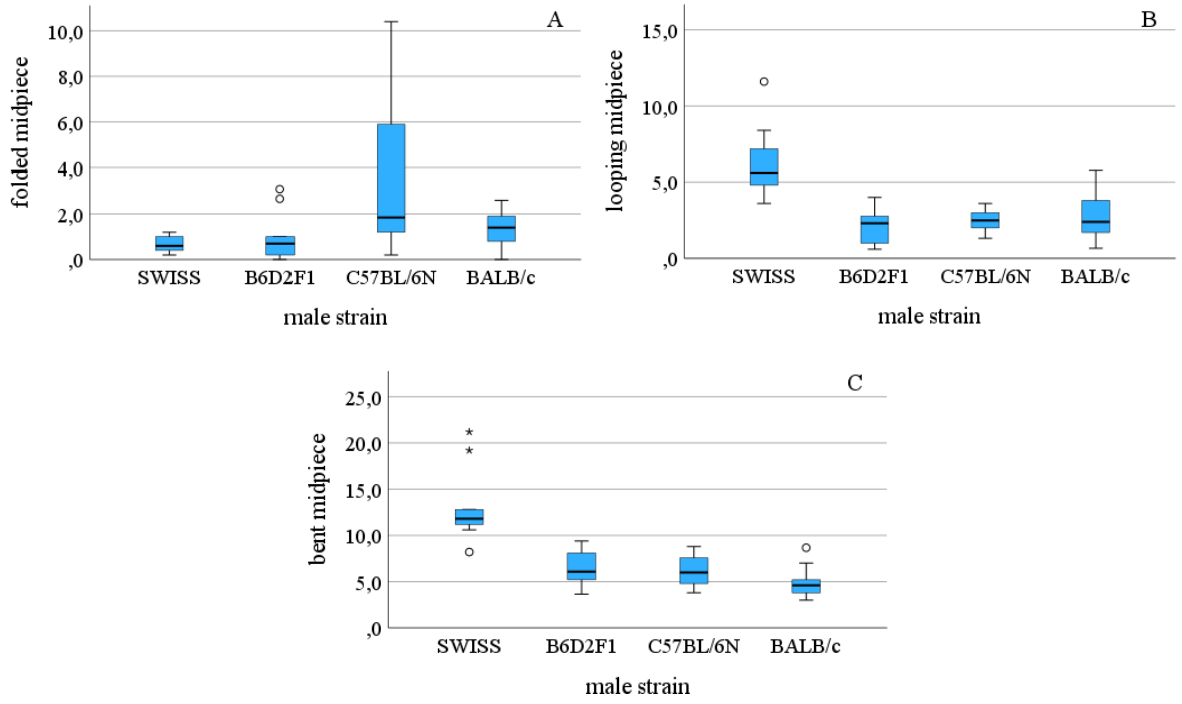

Figure 7. Relationship between male strain and percentage of spermatozoa with folded midpiece(A), percentage of spermatozoa with looping midpiece (B), and percentage of spermatozoa with bent midpiece (C). *Note that the Y-axis range of the boxplots is truncated to 10 %, 15 %, and 25 %, respectively.*

3.1.1.3 Tail abnormalities

Tail abnormalities accounted for 26.8 ± 5.98 % (bent or folded tail) and 2.89 ± 1.22 % (looping tail) of all abnormalities. Only the percentage of spermatozoa with a bent or folded tail was significantly influenced by mouse strain ($p < 0.001$, Fig.8). SWISS had a higher percentage of spermatozoa with a bent or folded tail compared to the three other strains (all $p < 0.001$), which did not differ from each other (all $p > 0.198$).

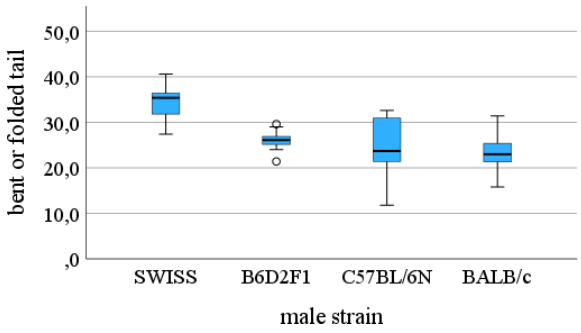

Figure 8. Relationship between male strain and percentage of spermatozoa with bent or folded tail. *Note that the Y-axis range of the boxplots is truncated to 50 %.*

3.1.1.4 Head-neck connection abnormalities

Among abnormalities pointing to a defective head-neck connection, we found 7.16 ± 8.21 % decapitated heads and 4.3 ± 4.12 % spermatozoa with an abnormal head-neck connection. We found a significant relationship between mouse strains and the percentage of spermatozoa with an abnormal head-neck connection ($p < 0.001$, Fig.9). C57BL/6N had a higher percentage of spermatozoa with an abnormal head-neck connection compared to the three other strains (all $p < 0.001$). A significant influence of mouse strain on the percentage of decapitated spermatozoa ($p < 0.014$) could not be confirmed in the paired comparisons.

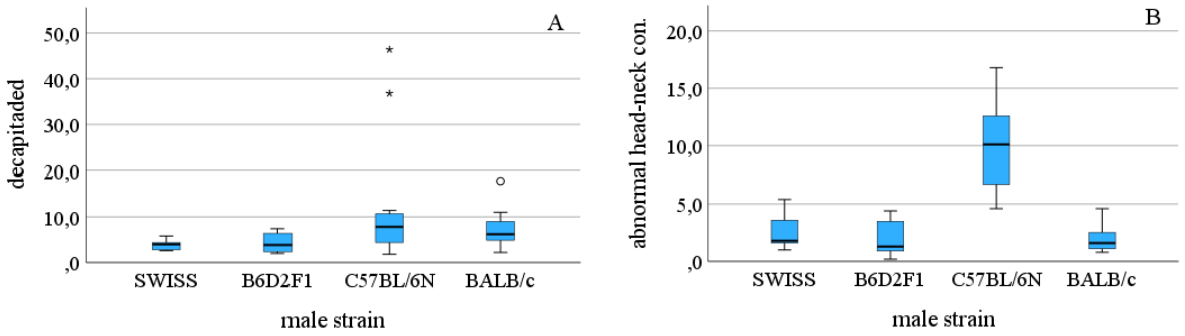

Figure 9. Relationship between male strain and percentage of decapitated spermatozoa (A), percentage of spermatozoa with abnormal head-neck connection(B). *Note that the Y-axis range of the boxplots is truncated to 50 % and 20 %, respectively.*

3.1.2 Relationship between morphological abnormalities and proAKAP4 groups

We did not find any significant relationships between proAKAP4 groups and the percentage of morphological abnormalities ($p = 0.149$, Fig.10) or multiple morphological abnormalities ($p = 0.691$, Fig.10) in frozen-thawed sperm samples across all strains. When testing for relationships between proAKAP4 groups and individual morphological abnormalities (see Appendix Table A3-A6), we found that folded midpiece ($p = 0.014$), bent midpiece ($p = 0.03$)

and bent or folded tail (p = 0.028) were significantly affected by proAKAP4 group in the ANOVA. The post-hoc-tests for these three parameters were not significant (folded midpiece: p > 0.415, bent midpiece: p > 0.6, bent and folded tail: p > 0.391). Therefore, there was only a tendency that the males in the low proAKAP4 group had a higher percentage of abnormal and multiple abnormal spermatozoa (Fig. 10).

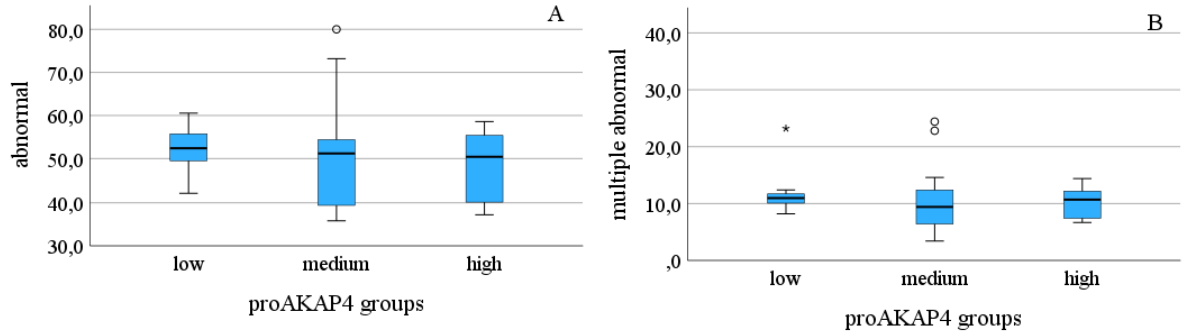

Figure 10. Relationship between proAKAP4 and percentage of abnormal spermatozoa (A) and percentage of spermatozoa with multiple abnormalities (B). *Note that the Y-axis range of the boxplots is truncated to 80 % and 40 %, respectively.*

Similarly, there was a tendency for the percentage of bent midpieces to increase from the low to the high proAKAP4 group (Fig.11B), and that the high proAKAP4 group had more spermatozoa with a bent or folded tail (Fig. 12).

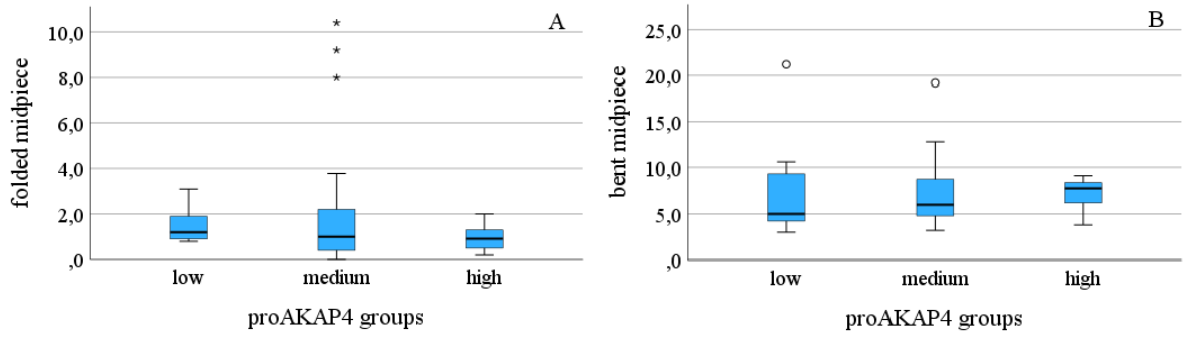

Figure 11. Relationship between proAKAP4 and percentage of spermatozoa with folded midpieces (A) and percentage of spermatozoa with bent midpieces (B). *Note that the Y-axis range of the boxplots is truncated to 10 % and 25 %, respectively.*

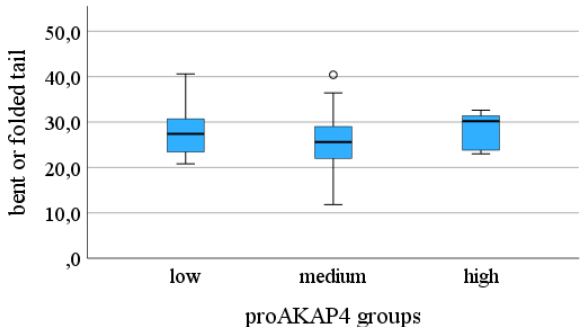

Figure 12. Relationship between proAKAP4 group and percentage of spermatozoa with bent or folded tail. *Note that the Y-axis range of the boxplots is truncated to 50 %.*

3.2 Viability

For viability analyses, it was differentiated between live (white = W) sperm and dead (pink or reddish = P/R) sperm. An overview of the relationship between the viability class and the morphological category (normal, abnormal, multiple abnormal) for the four mouse strains is given in Table 2. The percentage of 'dead' (P/R) sperm is increasing from normal to abnormal and further to multiple abnormal sperm.

Table 2: Percentage of live (W) or dead (P/R) sperm divided into four morphological groups (total, normal, abnormal, multiple) in relation to the total counted amount per mouse strain and total amount in addition to the standard deviation.

	total W (%)	total P/R (%)	normal W (%)	normal P/R (%)
SWISS	56,78 ± 5,43	43,22 ± 5,43	60,7 ± 5,89	39,3 ± 5,89
B6D2F1	56,41 ± 4,88	43,59 ± 4,88	61,13 ± 4,13	38,87 ± 4,13
C57BL/6N	48,2 ± 7,71	51,8 ± 7,71	58,38 ± 6,48	41,62 ± 6,48
BALB/c	53,52 ± 6,39	46,48 ± 6,39	58,3 ± 6,1	41,7 ± 6,1
total	53,52 ± 7,15	46,48 ± 7,15	59,58 ± 5,85	40,42 ± 5,85
	abnormal W (%)	abnormal P/R (%)	multiple W (%)	multiple P/R (%)
SWISS	51,53 ± 5,42	48,47 ± 5,42	46,15 ± 8,06	53,85 ± 8,06
B6D2F1	48,14 ± 7,62	51,86 ± 7,62	39,92 ± 13,54	60,08 ± 13,54
C57BL/6N	42,25 ± 9,3	57,75 ± 9,3	42,55 ± 12,76	57,45 ± 12,76
BALB/c	48,39 ± 7,31	51,61 ± 7,31	44,93 ± 13,87	55,07 ± 13,87
total	47,29 ± 8,36	52,71 ± 8,36	43,16 ± 12,7	56,84 ± 12,7

The percentage of dead spermatozoa was not significantly influenced by proAKAP4 groups ($p = 0.204$) (see Appendix Table A10), but by mouse strain ($p = 0.004$, Fig.13). C57BL/6N had a higher rate of dead spermatozoa compared to B6D2F1 ($p = 0.017$) and SWISS ($p = 0.022$).

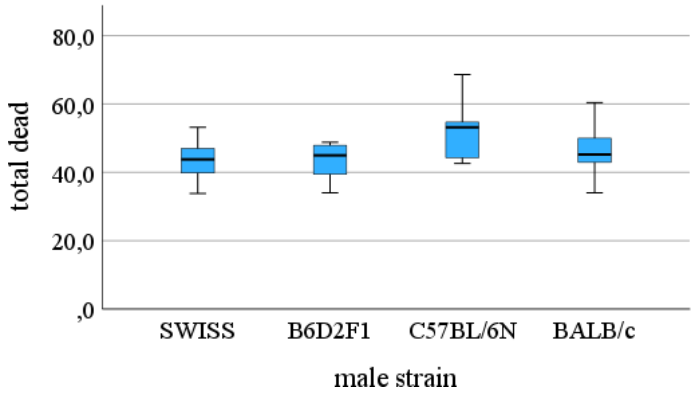

Figure 13. Relationship between male strain and percentage of dead spermatozoa. *Note that the Y-axis range of the boxplots is truncated to 80 %.*

3.3 Motility

As described before (see 2.6), the motility parameters were divided into 9 categories. The values for the different parameters for the four strains are given in Table 3. As can be seen there, less than 20 % were motile on average, only B6D2F1 hybrids had almost 25 % motile sperm.

Table 3: Sperm motility parameter for four different mouse strains and the total amount.

	motility (%)	VCL (µm/s)	VAP (µm/s)	VSL (µm/s)
SWISS	14,53 ± 1,99	148,3 ± 10,99	68,51 ± 6,74	48,97 ± 6,76
B6D2F1	24,46 ± 2,74	164,55 ± 12,56	83,59 ± 6,85	64,48 ± 7,59
C57BL/6N	19,42 ± 7,15	117,1 ± 10,9	58,9 ± 5,3	39,48 ± 5,54
BALB/c	13,03 ± 2,84	107,61 ± 9,13	54,14 ± 4,83	40,14 ± 3,84
total	18,2 ± 6,27	134,05 ± 25,87	66,41 ± 13,04	48,4 ± 12,1
	STR (%)	LIN (%)	WOB (%)	ALH (µm)
SWISS	65,89 ± 3,39	32,22 ± 2,55	46,48 ± 1,66	4,93 ± 0,31
B6D2F1	71,24 ± 3,59	38,16 ± 2,8	51,26 ± 2,22	5,23 ± 0,3
C57BL/6N	61,73 ± 3,97	32,68 ± 3,22	50,18 ± 2,37	4,17 ± 0,33
BALB/c	66,3 ± 3,93	35,78 ± 4,03	50,39 ± 3,07	3,72 ± 0,3
total	66,32 ± 5,14	34,86 ± 4,03	49,77 ± 2,96	4,5 ± 0,68
	BCF (Hz)			
SWISS	18,67 ± 0,85			
B6D2F1	18,59 ± 0,6			
C57BL/6N	17,89 ± 0,99			
BALB/c	19,18 ± 0,8			
total	18,56 ± 0,95			

There were no significant differences between proAKAP4 groups and the percentage of motile spermatozoa across all strains ($p = 0.993$) (see Appendix Table A7). However, the percentage of motile spermatozoa was significantly influenced by mouse strain ($p < 0.001$, Fig.14). B6D2F1 hybrids had a higher percentage of motile spermatozoa compared to SWISS and BALB/c (both $p < 0.001$).

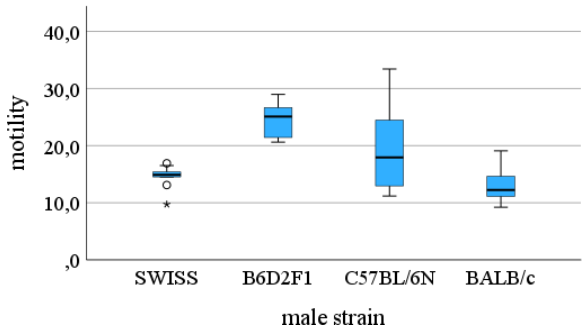

Figure 14. Relationship between male strain and percentage of motile spermatozoa. *Note that the Y-axis range of the boxplots is truncated to 40 %.*

3.3.1 Velocity Parameters

We did not find significant relationships between proAKAP4 groups and any of the two sperm velocity parameters straight line velocity (VSL) and average path velocity (VAP) in frozen-thawed sperm samples across all strains (see Appendix Table A7 and A8). Curvilinear velocity (VCL) appeared to be significantly (p=0.016) influenced by the proAKAP4 groups (Fig.15), which could not be confirmed in the post-hoc test (see Appendix Table A7).

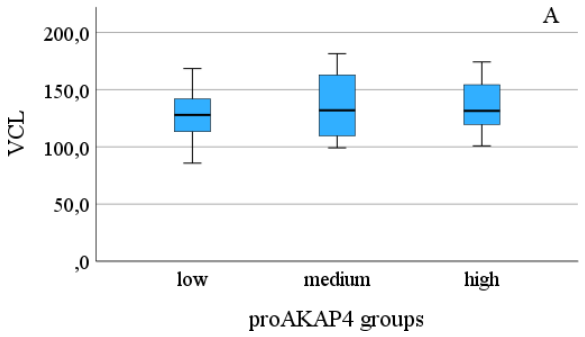

Figure 15. Relationship between proAKAP4 and the curvilinear velocity (VCL) of spermatozoa.

All three velocity parameters, VSL, VAP and VCL were significantly affected by mouse strain (all $p < 0.001$, Fig.16), with B6D2F1 hybrids having a higher VSL ($p < 0.001$), VAP ($p < 0.001$), and VCL ($p < 0.007$) than the other strains. Also, the sperm of SWISS mice were faster than those of the inbred strains, C57BL/6N and BALB/c (VSL, $p < 0.018$, VAP, $p < 0.001$, and VCL, $p < 0.001$).

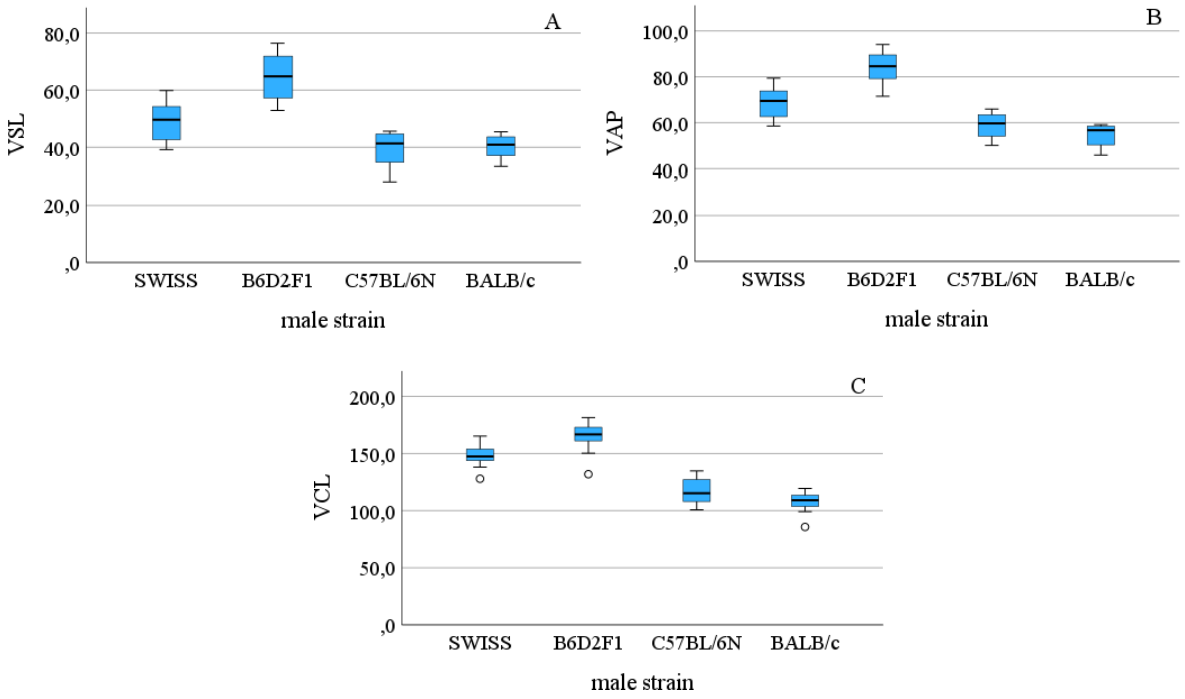

Figure 16. Relationship between mouse strain and the straight-line velocity (VSL; A), average path velocity (VAP; B), and the curvilinear velocity (VCL; C) of spermatozoa.

3.3.2 Direction and other parameters

We did not find significant relationships between proAKAP4 groups and the direction parameters linearity (LIN), wobble (WOB), straightness (STR), or beat-cross frequency (BCF) (all $p > 0.366$) of spermatozoa in frozen-thawed sperm samples across all strains (see Appendix Table A8 and A9). The significant relationship between proAKAP4 groups and the average lateral head displacement (ALH; $p = 0.041$, Fig.17) could not be confirmed in the post-hoc-test ($p > 0.284$).

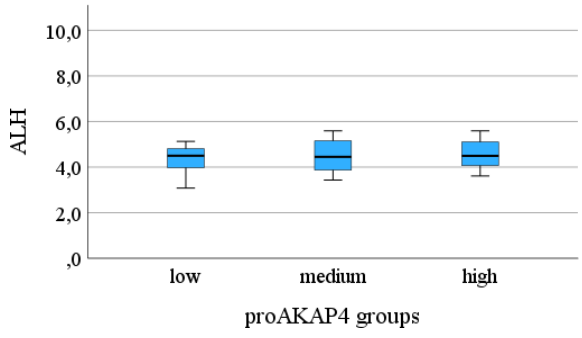

Figure 17. Relationship between proAKAP4 group and the average head displacement (ALH)

The parameters LIN, WOB, STR, ALH, and BCF were significantly affected by mouse strain (all $p < 0.001$, Fig.18). For these five motility parameters, no clear pattern existed: for linearity (LIN), B6D2F1 hybrids were above SWISS and C57BL/6N (both $p = 0.002$); for wobble (WOB), SWISS was lower than B6D2F1, BALB/c and C57BL/6N (all $p < 0.013$); for

straightness (STR), B6D2F1 were above the three other strains ($p < 0.023$), and C57BL/6N below BALB/c ($p = 0.039$). For the average lateral head displacement (ALH), BALB/c was below the three other strains ($p < 0.006$), and C57BL/6N again below B6D2F1 and SWISS (both $p < 0.001$). For the beat-cross frequency (BCF), C57BL/6N was below BALB/c ($p = 0.005$).

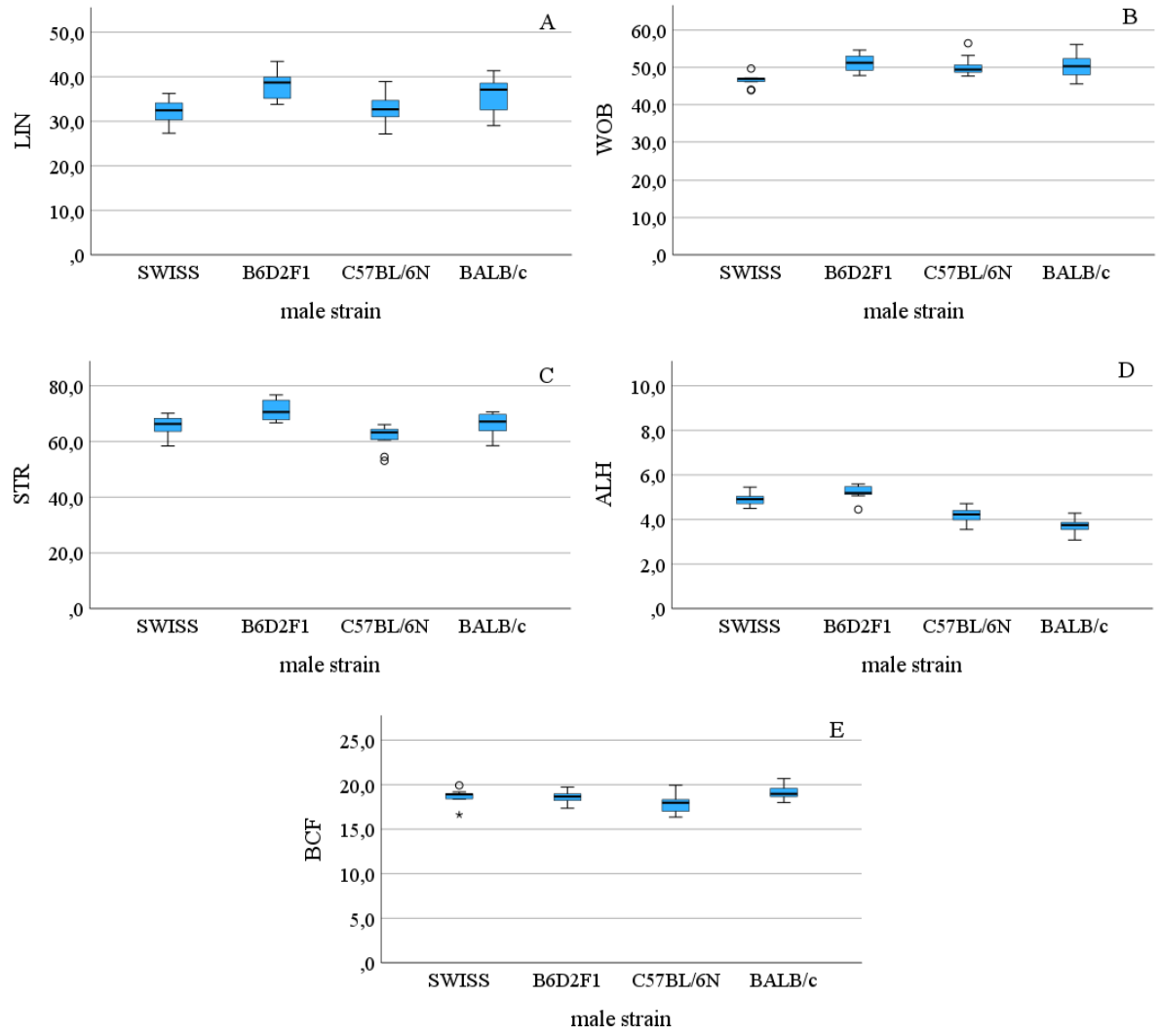

Figure 18. Relationship between mouse strain the LIN of spermatozoa (A), the WOB of spermatozoa (B), the STR of spermatozoa (C), the wide of ALH o spermatozoa (D) and the BCF of spermatozoa (E).

4 Discussion

As this is a follow-up study of Boersma et al. (2022), the frozen sperm samples were thawed and used for the measurements. The proAKAP4 data from this precursor study were related in the present study to sperm morphology, viability, and motility parameters. Boersma et al. (2022) detected significant negative correlations between proAKAP4 concentration and some sperm motility parameters. In the present study, we took an alternative approach to find potential relationships between proAKAP4 concentration and sperm traits by dividing the proAKAP4 values into three groups: low (< 25 ng/mL), medium (≥ 25 ng/mL, < 50 ng/mL) and high (≥ 50 ng/mL). In contrast to Boersma et al. (2022), we could not find any effect between proAKAP4 groups and the motility parameter. Further, no significant relationships, but some tendencies between proAKAP4 levels and morphology or viability parameters could be found. For example, the males in the low proAKAP4 groups had a higher percentage of abnormal and multiple abnormal spermatozoa, and a lower percentage of bent midpieces (Fig. 10 and 11). A possible explanation for the missing significance can be found in the specific data distribution of the data pool used in this study: the variance within the proAKAP4 groups was higher than the variance between the proAKAP4 groups. This led to the statistical finding that a significant effect of the variable 'proAKAP4 group' could be found in the ANOVA, but not in the post-hoc test. Due to the definition of the proAKAP4 groups the group size differed largely (low: N = 7, medium: N = 29, high: N = 8). It can be postulated that the effects would be more robust and might appear as significant in the post-hoc tests if there were more data points in each proAKAP4 group. This hypothesis should be tested in a follow-up study using more animals, including males, if possible with a known good fertility and a known subfertility phenotype. Until now, no studies have been conducted examining the sperm quality in relationship to proAKAP4 concentration in mice. It is known that mice lacking the AKAP4 protein completely, (Akap4 knock-out mice) did not develop the fibrous sheath and sperm motility is lost (16). Also, Fang et al. (2019) found that the loss of the Akap4 gene and consequently the AKAP4 protein led to an abnormal sperm morphology, highly reduced motility, and *in vivo* infertility. Based on our results, we cannot conclude that the differences in proAKAP4 concentration lead to similar differences in sperm quality. However, significant strain-specific differences could be found in morphology and viability parameters as well as in motility parameters. We observed significant strain differences in morphological abnormalities. The percentage of morphological abnormalities was significantly lower in B6D2F1 sperm compared to the other strains. This result is not surprising as hybrid strains are known for their higher vigor ('hybrid vigor') and fertility compared to inbred strains (17). On the other hand, SWISS had significantly more

multiple abnormalities than the other three strains. This is rather unexpected for an outbred strain which is used in many studies because of its good fertility compared to inbred strains. Several considerations can be made to explain this finding: first, morphological abnormalities do not automatically lead to a poorer fertilization; second, the population of SWISS mice used by the breeder of the strain might be genetically problematic, in spite of the outbred breeding scheme, and had, therefore, more morphological abnormalities. When analyzing the sperm motility data measured by CASA, the velocity parameters (VCL, VAP, VSL) and the values for the average head displacement (ALH) and straightness (STR) of SWISS males took an intermediate position between the significantly lower values of the inbred strains (C57BL/6N and BALB/c) and the higher values of the hybrid strain B6D2F1. Also, the other direction parameters of SWISS mice (LIN and WOB) were significantly lower than B6D2F and the inbred strains. This confirms the finding that the SWISS sperm samples used in this study have a reduced sperm quality. The mean percentage of motile sperm over all four strains was 18.2 ± 6.27 % (range of strain mean values 13.03 % - 24.46 %, Table 3), which is exactly in the range of 13.4 - 25.7 % given for the frozen-thawed motility of the same sample pool by Boersma et al. (2022). The hybrid strain B6D2F1 had the most motile sperm (24.46 ± 2.74 %) and BALB/c was at the lower end (13.03 ± 2.84 %). BALB/c, an inbred strain which is known from literature to have a lower motility rate, a higher rate of morphologically abnormal sperm, and consequently a reduced *in vivo* and *in vitro* fertility, have been used as a model for human infertility (18,19). Interestingly, in our data BALB/c had by far the highest percentage of spermatozoa without an acrosome (‘hook’) and an abnormal head shape (Fig. 6). Both are sperm traits well known to be important for successful capacitation and fertilization (6). The other relationships between mouse strain and morphological abnormalities were rather inconsistent. SWISS males had the most sperm with looping and bent midpieces (Fig. 7), and bent or folded tails (Fig. 8); C57BL/6N males had a strikingly high percentage of folded midpieces. Ohta et al. (2009) examined the sperm head abnormality rate (SAR) for the BALB/c substrain, also used in our study (ByJ). For 9-week-old males, they found a SAR of 28.5 ± 0.4 % (18). These rates exceed by far the percentage of abnormal sperm we found for BALB/c in our study, about 12 % (Fig. 6). To test whether there is a relationship between these morphological findings and fertility, *in vivo* and/or *in vitro* fertilization assays have to be set up. In conclusion, we did not find any clear relationships between proAKAP4 levels and sperm motility, morphology, or viability parameters. To clarify the significance and the postulated diagnostic value of proAKAP4 for the estimation of male fertility in mice, future studies using more data within one mouse strain and *in vitro* fertilization assays are required.

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6 Appendix

Table A3: Settings for Computer-assisted sperm analysis System

Parameters	
1. Area (µm2) (min)	23
2. Area (µm2) (max)	199
3. Drifting (µm/s)	20
4. Static (µm/s)	10
5. Slow-Medium (µm/s)	15
6. Rapid (µm/s)	25
7. Progressiv (µm/s)	50
8. Connectivlity (pixels)	18
9. VAP Points	15
10. VCL/VAP	VCL

Table A4: Capture Settings for Computer-assisted sperm analysis System

Capture	
Analysis timeout	15
Box size (pixels)	152
Frame rate (fps)	50
Number of images	25
Previem	One second
Resolution	Normal
Style	Automatic

Table A5: Statistic values of sperm abnormalities; abnormal, multiple abnormal, no hook: UNIANOVA and Post-Hoc tests.

		abnormal		multiple abnormal		no hook	
UNIANOVA		F	Sig	F	Sig	F	Sig
maleStrain		11,799	<,001*	13,797	<,001*	6,287	0,001*
proAKA4 group		1,999	0,149	0,373	0,691	2,843	0,071
POST-HOC			Sig		Sig		Sig
B6D2F1	BALB/c		0,029*		0,598		0,011*
	C57BL/6N		<,001*		0,063		0,751
	SWISS		0,014*		<,001*		0,888
BALB/c	B6D2F1		0,029*		0,598		0,002*
	C57BL/6N		0,058		0,582		0,109
	SWISS		0,969		<,001*		0,003*
C57BL/6N	B6D2F1		<,001*		0,063		0,751
	BALB/c		0,058		0,582		0,109
	SWISS		0,199		0,002*		0,366
SWISS	B6D2F1		0,014*		<,001*		0,888
	BALB/c		0,969		<,001*		0,003*
	C57BL/6N		0,199		0,002*		0,366
POST-HOC			Sig		Sig		Sig
high	low						
	medium						
low	high						
	medium						
medium	high						
	low						

Table A6: Statistic values of sperm abnormalities; abnormal head, folded midpiece, looping midpiece: UNIANOVA and Post-Hoc tests.

		abn. head		foldedmidp.		looping midp.	
UNIANOVA		F	Sig	F	Sig	F	Sig
maleStrain		9,209	<,001*	9,97	<,001*	10,759	<,001*
proAKA4 group		2,479	0,097	4,745	0,014	0,161	0,852
POST-HOC			Sig		Sig		Sig
B6D2F1	BALB/c		<,001*		0,253		0,606
	C57BL/6N		0,004*		<,001*		0,664
	SWISS		0,183		0,998		<,001*
BALB/c	B6D2F1		<,001*		0,253		0,606
	C57BL/6N		0,507		0,177		0,999
	SWISS		0,056		0,242		<,001*
C57BL/6N	B6D2F1		0,004*		<,001*		0,664
	BALB/c		0,507		0,177		0,999
	SWISS		0,53		0,002*		<,001*
SWISS	B6D2F1		0,183		0,998		<,001*
	BALB/c		0,056		0,242		<,001*
	C57BL/6N		0,53		0,002*		<,001*
POST-HOC			Sig		Sig		Sig
high	low				0,416		
	medium				0,673		
low	high				0,416		
	medium				0,728		
medium	high				0,673		
	low				0,728		

Table A7: Statistic values of sperm abnormalities; bent midpiece, bent or folded tail, looping tail: UNIANOVA and Post-Hoc tests.

		bentmidp.		bent or fold tail		looping tail	
UNIANOVA		F	Sig	F	Sig	F	Sig
maleStrain		21,758	<,001*	15,856	<,001*	1,771	0,169
proAKA4 group		3,869	0,03*	3,928	0,028*	2,711	0,079
POST-HOC			Sig		Sig		Sig
B6D2F1	BALB/c		0,029*		0,496		
	C57BL/6N		0,975		0,848		
	SWISS		<,001*		<,001*		
BALB/c	B6D2F1		0,029*		0,496		
	C57BL/6N		0,076		0,924		
	SWISS		<,001*		<,001*		
C57BL/6N	B6D2F1		0,975		0,848		
	BALB/c		0,076		0,924		
	SWISS		<,001*		<,001*		
SWISS	B6D2F1		<,001*		<,001*		
	BALB/c		<,001*		<,001*		
	C57BL/6N		<,001*		<,001*		
POST-HOC			Sig		Sig		Sig
high	low		0,601		0,997		
	medium		0,772		0,392		
low	high		0,601		0,997		
	medium		0,853		0,482		
medium	high		0,772		0,392		
	low		0,853		0,482		

Table A8: Statistic values of sperm abnormalities; decapitated, abnormal head-neck connection, coiled midpiece and tail: UNIANOVA and Post-Hoc tests.

		decapitated		abn. head neck con.		coiled midpi. & tail	
UNIANOVA		F	Sig	F	Sig	F	Sig
maleStrain		3,992	0,014*	33,749	<,001*	4,526	0,008*
proAKA4 group		3,008	0,061	2,548	0,092	2,314	0,113
POST-HOC			Sig		Sig		Sig
B6D2F1	BALB/c		0,231		0,999		0,182
	C57BL/6N		0,077		<,001*		0,913
	SWISS		1		0,982		0,513
BALB/c	B6D2F1		0,231		0,999		0,182
	C57BL/6N		0,958		<,001*		0,491
	SWISS		0,297		0,956		0,011*
C57BL/6N	B6D2F1		0,077		<,001*		0,913
	BALB/c		0,958		<,001*		0,491
	SWISS		0,117		<,001*		0,207
SWISS	B6D2F1		1		0,982		0,513
	BALB/c		0,297		0,956		0,011*
	C57BL/6N		0,117		<,001*		0,207
POST-HOC			Sig		Sig		Sig
high	low						
	medium						
low	high						
	medium						
medium	high						
	low						

Table A9: Statistic values of sperm motility parameters; motility, VCL, VAP: UNIANOVA and Post-Hoc tests.

		motility		VCL		VAP	
UNIANOVA		F	Sig	F	Sig	F	Sig
maleStrain		13,178	<,001*	73,325	<,001*	54,59	<,001*
proAKA4 group		0,007	0,993	4,607	0,016*	2,659	0,083
POST-HOC			Sig		Sig		Sig
B6D2F1	BALB/c		<,001*		<,001*		<,001*
	C57BL/6N		0,055		<,001*		<,001*
	SWISS		<,001*		0,007*		<,001*
BALB/c	B6D2F1		<,001*		<,001*		<,001*
	C57BL/6N		0,011*		0,159		0,247
	SWISS		0,891		<,001*		<,001*
C57BL/6N	B6D2F1		0,055		<,001*		<,001*
	BALB/c		0,011*		0,159		0,247
	SWISS		0,099		<,001*		0,004*
SWISS	B6D2F1		<,001*		0,007*		<,001*
	BALB/c		0,891		<,001*		<,001*
	C57BL/6N		0,099		<,001*		0,004*
POST-HOC			Sig		Sig		Sig
high	low				0,306		
	medium				0,99		
low	high				0,306		
	medium				0,215		
medium	high				0,99		
	low				0,215		

Table A10: Statistic values of sperm motility parameters; VSL, STR, LIN: UNIANOVA and Post-Hoc tests.

		VSL		STR		LIN	
UNIANOVA		F	Sig	F	Sig	F	Sig
maleStrain		41,012	<,001*	12,395	<,001*	7,359	<,001*
proAKA4 group		1,719	0,193	1,034	0,366	0,189	0,829
POST-HOC			Sig		Sig		Sig
B6D2F1	BALB/c		<,001*		0,023*		0,36*
	C57BL/6N		<,001*		<,001*		0,002*
	SWISS		<,001*		0,019*		0,002*
BALB/c	B6D2F1		<,001*		0,023*		0,36*
	C57BL/6N		0,994		0,039*		0,155
	SWISS		0,017*		0,996		0,118
C57BL/6N	B6D2F1		<,001*		<,001*		0,002*
	BALB/c		0,994		0,039*		0,155
	SWISS		0,008*		0,093		0,991
SWISS	B6D2F1		<,001*		0,019*		0,002*
	BALB/c		0,017*		0,996		0,118
	C57BL/6N		0,008*		0,093		0,991
POST-HOC			Sig		Sig		Sig
high	low						
	medium						
low	high						
	medium						
medium	high						
	low						

Table A11: Statistic values of sperm motility parameters; WOB, ALH, BCF: UNIANOVA and Post-Hoc tests.

		WOB		ALH		BCF	
UNIANOVA		F	Sig	F	Sig	F	Sig
maleStrain		6,731	<,001*	58,375	<,001*	4,581	0,008*
proAKA4 group		0,432	0,652	3,478	0,041*	0,53	0,593
POST-HOC			Sig		Sig		Sig
B6D2F1	BALB/c		0,849		<,001*		0,373
	C57BL/6N		0,732		<,001*		0,22
	SWISS		<,001*		0,151		0,997
BALB/c	B6D2F1		0,849		<,001*		0,373
	C57BL/6N		0,997		0,005*		0,005*
	SWISS		0,008		<,001*		0,562
C57BL/6N	B6D2F1		0,732		<,001*		0,22
	BALB/c		0,997		0,005*		0,005*
	SWISS		0,012		<,001*		0,197
SWISS	B6D2F1		<,001*		0,151		0,997
	BALB/c		0,008		<,001*		0,562
	C57BL/6N		0,012		<,001*		0,197
POST-HOC			Sig		Sig		Sig
high	low				0,295		
	medium				0,938		
low	high				0,295		
	medium				0,284		
medium	high				0,938		
	low				0,284		

Table A12: Statistic values of sperm viability parameter; dead spermatozoa (PR): UNIANOVA and Post-Hoc tests.

		P/R	
UNIANOVA		F	Sig
maleStrain		5,36	0,004*
proAKA4 group		1,656	0,204
POST-HOC		Sig	
B6D2F1	BALB/c	0,708	
	C57BL/6N	0,017*	
	SWISS	0,999	
BALB/c	B6D2F1	0,708	
	C57BL/6N	0,213	
	SWISS	0,677	
C57BL/6N	B6D2F1	0,017*	
	BALB/c	0,213	
	SWISS	0,022*	
SWISS	B6D2F1	0,999	
	BALB/c	0,677	
	C57BL/6N	0,022*	
POST-HOC		Sig	
high	low		
	medium		
low	high		
	medium		
medium	high		
	low		