From the Department of Biomedical Sciences
(Head: O.Univ.-Prof. Dr.med.vet. Mathias Müller)
the Institute of Laboratory Animal Science
at the University of Veterinary Medicine, Vienna
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Computer-assisted sperm analysis in mice – a literature review

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Doris Bittenecker

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Supervisor:

Dr. med. vet. Auke Boersma

Institute of Laboratory Animal Science

Department of Biomedical Sciences

University of Veterinary Medicine, Vienna

Reviewer:

Ao.Univ.-Prof. Dr.med.vet. Sabine Schäfer-Somi Dipl.ECAR

Insemination and Embryo Transfer Platform

Department/University Clinic for Small Animals and Horses

University of Veterinary Medicine, Vienna

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1. Introduction and research question

Sperm characteristics, as their kinematics, morphology and metabolism have been one focus of scientists in the field of reproduction since the 1940s (Amann and Katz 2004). More recently, numerous scientists all over the world are working to explore the function of genes related to sperm production (Nayernia et al. 2003, Bray et al. 2005, van der Horst and Maree 2013). Initially, semen analysis and motility parameters were assessed by manual and visual-subjective methods, leading to a large amount of inter- and intra-observer variability and very limited objectivity. Research groups in France (Prof George David, Le Kremlin-Bicêtre) or the United Kingdom (Hector Dott) used cine- and photo-micrography in the 1970s to measure basic motility parameters. Methods applied were for example number fluctuation counting (to estimate the average speed of the sperm in a sample, using a formula that takes into account the measured area as well as time and number of cells counted) or photomicrographic tracking (Katz and Dott 1975). Their methodology is based on earlier findings, such as the probability-after-effect, in which a circle is drawn around a sperm, the radius being the distance it can have covered until the next picture is taken. Inserted into a formula, the speed can be computed (Rothschild 1953). David Katz and Jim Overstreet developed a simple and inexpensive method to videotape sperm under the microscope, already aware that the possibility of repeatable analysis of a video sequence is advantageous. Some of the basic characteristics of describing sperm kinematics that are still used today go back to this research group (Katz and Overstreet 1981). After the objectivity could be increased in this way, the next step was to perform the analysis largely automatically. As this field of research gained more and more interest, a uniform nomenclature to ensure good scientific practise had to be developed to ensure comparability and standardisation. According to that, the three-letter terminology for sperm characterisation was developed (explained in chapter 4.b.) and it was defined how many decimal places numeral values should have (velocity parameters with one decimal place, numerical proportions as a whole number) (Mortimer et al. 2015).

Due to technological advancement, computer-assisted sperm analysis (CASA), also called computer-aided sperm analysis, novel systems were developed in the 1980s and established in the field of research. One of the first commercial CASA systems was "*CellSoft"* in 1985, Hamilton Thorne *"HTM-2000"* following one year later. Whereas the former used a free-standing phase contrast microscope, the HTM-2000 combined all parts in one housing (Amann and Katz 2004, Amann and Waberski 2014). Capturing pictures at a frequency of 50 Hz was

first possible with the "*Sperm Motility Quantifier"* (SMQ) in 1992. By that time these systems had a lot of inaccuracies and sources of error that had to be ruled out. Restricted resolution of the digital images and the lack of ability to differentiate dead from alive spermatozoa set limits at that time. Another constraint was found in the contamination of the ejaculate, as there are always some other cells or debris within a sample. Owing to technological advancement for both hardware and software it was possible to improve the accuracy and reliability of the measurements. Until 2000, there have been three consensus conferences about the utility of these computer-based systems, and several guidelines have been published (Miki et al. 2004, Mortimer 2000, Mortimer et al. 2015, Tablado et al. 1998). Most of them derive from the field of human research, but they nevertheless provide a guide and serve as orientation (ESHRE Guidelines on the application of CASA technology in the analysis of spermatozoa, 1998). By now, CASA has developed to a level where it can be used in multiple settings, in research laboratories as well as in clinical andrology (e.g. for prognostic evaluations concerning fertility or resorting sperm populations to improve fertilisation success). In veterinary medicine it has been used in a great number of animal species, in animal breeding and in laboratory settings. Especially in reproductive toxicology CASA is a frequently used technical device to evaluate the effects of a possibly harmful drug (Miki et al. 2004, Mortimer 2000, Mortimer et al. 2015, Tablado et al. 1998).

Making use of the computer-assisted sperm analysis in modern research is contributing to the implementation of the 3R principles of Russel and Burch. These two well-known scientists were aware at an early stage that while animal testing is often necessary, there are certain principles that need to be followed. All scientists should strive to implement the 3Rs: To replace animal experiments when there are alternatives, to further reduce them where it can be done and to improve the conditions for the laboratory animals in a favourable way – in short: replace, reduce, refine (Russell and Burch 1959). As an example, applied in combination with the cryopreservation of sperm of laboratory animals, primarily mice and rats, CASA reduces the number of individuals needed by predicting the quality of frozen sperm samples and therefore reducing the necessary number of superovulated females for *in vitro* fertilisation (IVF). These predictions are only possible based on the accurately and objectively measured results of CASA systems. At the same time, modern CASA devices allow a faster processing of samples and subsequently lead to reduced personnel costs in comparison with manual methods which in turn represents an economic advantage (Li et al. 2016, Nakagata 2000, Raspa et al. 2018).

The examination of human male infertility is one of the main applications for CASA. It relies often on research performed on mice. Using gene knockouts, more than 200 genes which influence spermatogenesis have been identified until now (Harris et al. 2007). There are still restrictions, technically or of biological origin, as in animals sperm concentration is much higher than in humans and human sperm samples show considerably greater viscosity, that limit the use in human clinical application. However, this also illustrates the importance of more research in the field of male reproduction and sperm analysis to understand all biological processes in a sperm cell (Mortimer et al. 2015).

The aim of this diploma thesis is to survey literature on computer-assisted sperm analysis in the mouse, to describe and evaluate different methods for sperm collection in mice and to summarise the current state of CASA in this species. The focus is on the laboratory mouse because this is the species mainly used for CASA at the Institute of Laboratory Animal Science in the context of routine cryopreservation of mouse spermatozoa and in several research projects. The research question is if and to what extent CASA is suited for the evaluation of sperm quality in mice, and to identify possible applications for CASA in future research projects using laboratory mice.

2. Material and methods

Key publications given by the supervisor of this diploma thesis served as a starting point for efficient literature search. Furthermore, the companies producing and selling computer-assisted sperm analysis systems, e.g. Hamilton Thorne, Inc. (Beverly, Massachusetts; www.hamiltonthorne.com) and Microptic, S.L. (Barcelona, Spain; www.micropticsl.com) provide lists of scientific studies based on their respective systems.

For a more thorough systematic literature search the search engines PubMed (www.ncbi.nlm.nih.gov/pubmed), Google Scholar (scholar.google.com) and Scopus (www.scopus.com), as well as the catalogue of the library of the Vetmeduni Vienna were used. Results in German and English language are included.

The search terms were:

"automated sperm motility analysis mouse"
"computer-assisted sperm analysis mouse/mice"
"computer-aided sperm analysis mouse/mice"

For chapter 3 additionally:
"sperm collection mouse/mice"
"electroejaculation mouse/mice"

For chapter 4 additionally:
"Sperm Class Analyzer mouse"
"Integrated Visual Optical System mouse"
"CEROS mouse"

Concerning the different chapters, the papers had to meet varying requirements, therefore no general inclusion or exclusion criteria can be listed. References from other species than the mouse were included only when necessary for the respective topic. For example, for general information in some paragraphs (chapter 1, 4 a and b, 5), studies from the human field are added. Based on the bibliographies of the articles read, more references could be found and were added if relevant.

Although the most extensive search was prior at the beginning of writing, while the work was in progress, new sources still came up. Thus, intensive literature search did not end until the thesis was almost finished.

3. Options for sperm collection in mice

Sperm collection is a prerequisite for any assessment of sperm quality; therefore different options, used in literature, are described and discussed in this chapter. Besides this, there are several other applications for sperm collection: For the reduction of husbandry costs, as well as decreasing numbers of live animals on shelf and to facilitate the distribution of mouse mutant strains, collecting sperm for cryopreservation is a significant advance in breeding mice (Nakagata 2000). The limitation of many sperm collection methods is the fact that they are terminal for the male mouse. Alternatives for repeated use of males would support the implementation of the 3R principles of Russel and Burch (Yamauchi and Ward 2007). As *in vitro* fertilisation is daily routine in many laboratories that use mice, it is substantial to have a reliable method of collecting mouse spermatozoa. The sperm is either used directly to perform fertilisation or cryopreserved for later use. In genetically altered mouse strains, it is possible to test the sperm prior usage if the wanted mutation in the genome will be transmitted to the offspring. Depending on the field of application a different concentration of sperm is needed. This has to be taken into account when deciding which will be the method of choice for a study. Nonterminal techniques (see points b–e below) allow repeated collection and would probably offer a good alternative, especially in genetically modified mouse strains that cannot breed naturally due to the phenotype caused by their mutation (Tecirlioglu et al. 2002).

a. Epididymal sperm from sacrificed mice

Beyond all doubt the most commonly used method for collecting sperm is to euthanise the male mouse by cervical dislocation or another method (e.g. lethal inhalation of carbone dioxide or injection of pentobarbital) and dissect the epididymides with the attached vasa deferentia. They are transferred into a Petri dish containing a medium which is, depending on the application, a cryoprotective agent, sperm preincubation medium or IVF medium (Nakagata 2000). After removing remaining tissues like blood or fat that might contaminate the sample (ideally using a spring scissors), a few cuts are made into the epididymides to enable the mature sperm to swim out for a period of 3–15 minutes (varies within different protocols) (Pérez-Crespo et al. 2008). Following the incubation period, the organs are removed and the sperm suspension is cryopreserved or more medium is added, depending on the application, to reach the desired target concentration. For example, if a computer assisted sperm analysis

should be performed, 2–4 x 10^5 sperm/ml appeared to be appropriate (Goodson et al. 2011). Comparing the different possibilities of isolating sperms, this is the one with the highest output of mature spermatozoa, but with the restriction that it can only be performed once as it is terminal (Boersma et al. 2015, Foxworth et al. 1996, Nakagata 2000, Papp S. 2014, Tecirlioglu et al. 2002).

b. Microsurgical epididymal sperm aspiration (MESA) and percutaneous epididymal sperm aspiration (PESA)

With sperm aspiration out of the epididymis from anaesthetised mice, a minimal invasive and repeatable method of collecting sperm is given, even compromising the male's *in vivo* fertility and thus keeping the option of natural mating at a later time (Boersma et al. 2015, Del Val and Robledano 2013). Del Val and Robledano (2013) tested different surgical protocols (varying surgical access, needle and capillary to collect the sample) in a pilot study to ensure that the least harmful method is performed. The anaesthetised male mouse's scrotum was shaved, disinfected and an incision was made, similar to the one that is routinely performed for vasectomies in mice. After locating and completely pulling out the testis, the tail of the epididymis was identified and positioned in order to perform a successful procedure. Keeping it cautiously in place with forceps, the puncture was set with a 32G needle, the sperm were allowed to form a drop and were aspirated into a capillary, guided via a mouth pipette. The testis then was replaced into the scrotum, and the skin incision was sutured. After ten days of recovery the males were mated naturally each with two females and the surgery was performed a second time to proof its repeatability. The outcome was a 100 % success in receiving a sample, the IVF rate was about 57 % and all males were able to produce offspring naturally afterwards. There were no significant differences when the procedure was performed a second time (Del Val and Robledano 2013).

A similar, less invasive approach, called microsurgical epididymal sperm aspiration (MESA), was developed by Boersma et al. (2015), as well as a further refined method, called percutaneous epididymal sperm aspiration (PESA) in which the sampling is performed through the skin. The testes have to be gently secured in the scrotum with forceps, the skin gets disinfected and the cauda epididymidis which is visible through the very thin skin is punctured using a 30G needle on an insulin syringe prefilled with 50µl of TYH medium. When negative

pressure was applied, a successful aspiration was visible as small clouds in the medium. The content of the syringe was ejected into a culture dish. All the samples collected via this method showed sufficient motility and the ability to fertilise oocytes (success rates greater than 80 % in the B6D2F1 strain). As the procedure is quite easy to perform and the sperm is fertile it should be considered as alternative, especially if no large quantity of spermatozoa is required. PESA-treated male mice were able to mate naturally again after the procedure and no histological changes of the tissue were seen (Boersma et al. 2015).

In human assisted reproduction, the cryopreservation of sperm collected by MESA or PESA has been successfully performed (Glina et al. 2003, Patrizio 2000). This possibility could up to now not be shown in mice.

c. Separation of mating mice during ejaculation

This method is a non-invasive and repeatable alternative although it has not made its way into routine yet. There are some characteristics in the mating of mice that must be taken into account in order to carry out the process of separating the mating partners successfully and repeatedly. The ejaculation announces itself with the so-called ejaculatory reflex, which lasts several seconds and always brings with it a certain sequence of events: At first, the speed of the male's thrusting increases, then the male shudders and clutches the female with all four limbs. Most often the male afterwards falls to one side of the female. The penis itself also shows changes during the ejaculatory shudder: It becomes wider, redder and the tip forms a cup-like shape, especially towards the distal end. About a second later the plug is ejected, on which the sperm-rich fraction of the ejaculate is placed in the form of a drop. Following this, the male mouse is retracting the penis and the copulation ends. These details have to be known in order to separate the animals at the right time point, namely precisely when the ejaculatory reflex already started, but prior to the ejection of the plug with sperm into the female's genital tract (McGill and Coughlin 1970, Sutter et al. 2015).

Papp (2014) performed the procedure with B6D2F1 mice of both sexes (Charles River Laboratories, Germany), for oocyte collection C57BL/6N were used as well. The experiment was performed between 6.00 to 11.00 am, to be as close as possible to the natural mating time and feasible at the same time. A female mouse in oestrus was placed into a male's cage and the pair was observed permanently. When intromission was detected the tails of both

individuals were grabbed and as soon as the ejaculatory shudder was noticed they were gently separated. The male mouse then was put on a cage lid and the ejaculate, consisting of the plug with a sperm drop, was carefully taken off the penis tip with forceps. After the procedure the animal got single housed and used again only after two days of rest. The semen was separated from the plug immediately after the collection to prevent debris contamination and was transferred into a drop of human tubal fluid (HTF) medium. On average the mice had to be separated after 8.1 minutes, time measured from placing the female in the cage until the ejaculatory shudder, with an 80 % success rate for collecting the sperm. In the remaining 20 % the separation took place too late, probably mainly due to inexperience as the majority occurred at the beginning of the experiment. The number of motile spermatozoa was sufficient to perform IVF successfully. The advantages of this method are clearly its minimal invasive nature and that the unharmed male mice can be used repeatedly, which is clearly a refinement in line with the 3R principle. Further pros are the little costs as no special equipment is needed and the simplicity of the application once the experimenter has mastered it. If this method is transferable to other strains though still has to be investigated (Papp S. 2014). As several research groups have already found out, the genotype has an influence on the sex drive, therefore it would have to be evaluated for each strain individually whether the method is applicable (Dominguez-Salazar et al. 2004, McGill and Blight 1963, Osadchuck LV, Salomacheva IN, Bragin AV, Osadchuk AV 2008).

d. Collection of spermatozoa from the uterus of mated females

For genetically very valuable males or in case repeated sperm collection is intended (e.g. for genetic analysis), flushing the uterus from recently mated females can be considered as the method of choice. The opportunity to receive multiple sperm samples from one male also enables to assess whether the quality of the spermatozoa changes with time and if certain drugs cause mutations (King et al. 1994).

In the study of King et al. (1994), the sperm samples were checked for genetic mutations and not for motility as it was not part of the research question. Females with a copulatory plug (checked twice daily) were euthanised and the uterus was taken out and flushed with 1.5 ml phosphate-buffered saline (PBS). Another indication for successful mating beside the plug was the up to fivefold extended uterine volume. The researchers were successful in 93.8 % of the

cases, noticing that the sample showed a lot more contamination when the mating took place more than 24 h before (King et al. 1994).

As a refinement, Foxworth et al. (1996) performed a repeatable collection from oviduct-ligated females by flushing the uterus under general anaesthesia with good results. The sperm collected in this manner were not further examined regarding motility or viability; just a genetic analysis was performed. An important finding was that the female mice had to give birth at least once prior to the sperm flushing to dilate the birth canal and thereby to allow the cannulation and sperm collection. The authors stressed furthermore that it was important to flush the uterus within about seventeen hours after mating to successfully obtain sperm (Foxworth et al. 1996).

Songsasen and Leibo (1998) showed that it is even possible to cryopreserve the spermatozoa collected within 30 min after the observation of a vaginal plug with a modified method as described by King et al. (1994). After IVF and embryo transfer, live pups could be obtained at similar rates as with epididymal spermatozoa (Songsasen and Leibo 1998).

In a more recent study performed by Yamauchi and Ward (2007), the killing of the females took place only about one hour after mating, in order to receive the sperm as fresh as possible since spermatozoa exhibit weak motility and show poor quality when the mice are mated in the evening and the uterus flushing is performed only the following day. To ensure a positive study outcome the male mice were trained for mating at an unusual time, contrary to their natural behaviour: After mating a few times during night, once the males were able to mate efficiently, the females were placed into the males' cages only in the morning. The female mice were sacrificed as soon the check for a vaginal plug was positive. An incision was made in the dissected uterus at the caudal end and the ejaculated spermatozoa were flushed out into a petri dish. Sperm collected with this method show capacitation characteristics in more than 70 %, which implicates a higher chance for DNA damage as the nuclease activity increases during capacitation. However, the spermatozoa were able to fertilise oocytes and to generate live foetuses (day 15) when intracytoplasmic sperm injection (ICSI) and embryo transfer were performed (Yamauchi and Ward 2007).

e. Electroejaculation

Electroejaculation has been used repeatedly with good results in a broad variety of mammals, but in rodents there are still unsolved issues. One of the first attempts was made by Scott and Dziuk (1959), using a single bipolar rectal probe with 10–15 V in mice from various genetic backgrounds and 3–24 month of age. All trials resulted in clumps of ejaculated spermatozoa that showed poor motility and urine contamination (Scott and Dziuk 1959). Snyder (1966) performed his experiment with 51 brown house mice, applying an electronic potential of 50–100 V in square wave form. He was able to obtain up to 1.7 x 10^6 spermatozoa per ejaculate. Nevertheless he had to record 6 % immediate mortality, within 14 days further 16 % deaths have been added and in 12 % of the procedures there was no ejaculation at all (Snyder 1966). In both studies there were no detailed measurements of sperm viability, motility or fertilising capacity.

Seventeen years later Anderson et al. (1983) tried to improve the technique of electroejaculation in C57BL/6J mice. His group used sine waves on a bipolar rectal probe with a comparably low voltage range from 1–5 V at 60 Hz. They were able to collect ejaculates from three experimental groups (“fertile, infertile and non-mating” males defined based on previous mating experiments). For the “normal fertility” group they gave minimum values of 2.5 x 10^6 spermatozoa/ml and 2.3 x 10^3 motile spermatozoa per ejaculate. Furthermore, they showed that the evaluation of the collected sperm based on four criteria allowed to distinguish between fertile and infertile males to a certain extent. Those so called “deficiencies” were ejaculate volume, coagulum weight, sperm concentration and total motility (Anderson et al. 1983).

All three research groups performed the experiments on unanaesthetised, i.e. fully conscious animals and observed mortality rates related to uremic poisoning as it occurs when the coagulation clot is not fully ejaculated and blocks the urethra of the male mouse, although this was only 1 out of 192 ejaculations (0.5 %) in the last-mentioned study. A restriction of this technique is the contamination of the ejaculate, coming either from the male’s accessory sex glands or from the urinary bladder. It was shown that the shape of the probe, as well as the form of the electric wave had effects on impurity of the sample and mortality rate of the animals.

More in line with present-day animal welfare standards, Tecirlioglu et al. (2002) used different injection anaesthetics and list the effects of waveform, electronic device and rectal probe when performing three repeated electroejaculations in three groups of C57BL/6J mice. They

observed the best results with automated computer-generated waveforms delivered through a sound card. A detailed computer-assisted motility analysis of the collected sperm was performed using the Hamilton-Thorne Motility Analyser HTM-2047. Independent from the mentioned variables, all sperm samples collected via electroejaculation showed significantly lower progressive and rapid motility scores compared to sperm that was collected directly from the epididymides, matching the results from earlier attempts in this field. Sine and triangle waves of 0.5–3 V showed the best effects as they provide a smoother flow and in consequence allow an equally distributed stimulation of the surrounded nerve fibres. The probe shape most probably shows differences due to the expansion of the electric area in the different forms (e.g. strip, ring). In spite of optimising and adapting the new findings, the subsequent mortality rates still varied between 11 % and 16.6 %. The formation of a coagulation clot still constitutes a barrier in the use of electroejaculation in mice. Overcoming this issue could probably allow to apply this method of nonterminal sperm collection more in routine practice (Tecirlioglu et al. 2002).

4. Current status of computer-assisted sperm analysis (CASA)

Sperm cells can be distinguished in many aspects when compared to other cells: They are smaller, highly differentiated and most importantly they are able to generate motion by themselves. These characteristics and their different motility status within one population require to gain information as precise as possible if we want to understand their function in detail. Furthermore spermatozoa show complex swimming patterns and not only straight movement (Bray et al. 2005) and in their limited life span they even undergo changes in their motility. Turner (2003) and Buffone et al. (2012) review the structural and molecular basis of different types of sperm motility and acrosomal exocytosis. Locomotion which is required for a successful fertilisation is one of the determining parameters to describe sperm quality. With the aid of modern technology for sperm analysis it is possible to track each individual sperm and simultaneously the motility of a large number of spermatozoa as representative for the entire sample (Grzmil et al. 2007).

a. Operating principle of CASA systems

In comparison with questions and applications in human research, sperm samples from eutherian animals bring other challenges: The natural selection is not present in humans anymore; therefore human sperm samples often show poor motility and fertility (Davis and Siemers 1995). Additionally, the sperm concentration is much lower in humans, the reason why samples from animals often need to be diluted to make the application of CASA possible. In order to conduct good research, one must be aware of both the similarities and differences (Mortimer et al. 2015).

For understanding the analytic procedure, it is important to know the elements that are necessary to carry out a computer assisted sperm analysis: In any case, a microscope with a 10x or 20x objective with an integrated or separate warming plate forms the basis. Furthermore, a video camera, a frame grabber card for film and a data processor (i.e. a computer) is needed (Mortimer 2000). To rule out sources of error it is recommended to use dark-field or negative-phase contrast techniques, as this makes it easier for the camera to detect a sperms head (will appear white on a darker background). This knowledge has existed

for a long time, as we can see from the mentioning in Rothschild's research (Rothschild 1953). Once recorded the software processes the data and carries out the calculations. As computer only can work with digitalised pictures, this translation is the first step in analysis (Amann and Waberski 2014).

Depending on the research question and the used system, up to 12 different parameters can be gathered. Many of them are calculated and therefore depending on others. A principle of distinction also needs to be made if only motility or morphology or both are from interest; this will be dealt with in chapter 6 (Amann and Waberski 2014). Of course, the variance decreases as the number of cells analysed increases and the precision also goes up as more fields and sperm are examined. Therefore, it should be carefully considered in advance how much data are required for a significant outcome (Verstegen et al. 2001).

Technology for digital image analysis differs fundamentally from subjective evaluation by a human observer. Whereas a person is detecting the beating flagellum of a sperm and change of location, today's technique is not yet so far advanced and the parameters have to be calculated indirectly (Mortimer et al. 2015). This circumstance also represents a limitation in morphological analysis (Yániz et al. 2015). The flagellum of a mammalian sperm makes more than 90 % of the total length of the cell and is generating the motion (Miki et al. 2004). The reason why direct detecting its movement is not possible is the frequency of the beats. It is moving up to 80 times per second (number applies to washed human spermatozoa) (Mortimer 2000). If an accurately reconstructed path wants to be achieved, the number of measurements must be at least twice as high as the highest frequency from the object of interest. This particular rate is called Nyquist frequency (Davis and Siemers 1995). The most commonly used 60 images per second would lead to blurred photos and imprecise results as the tail moves faster. Fortunately, the frequency of the head is lower what makes it the more practicable part of the sperm to be followed. Caused by the fact that the flagellum is the sole part of the spermatozoon responsible for the movement it is possible to draw conclusions about it through detecting the head of the sperm (Mortimer 2000). Most commonly about 30 frames at 60 Hz are recorded for detailed analysis. It has been shown that this period of time holds enough details to reconstruct a sperms path (Amann and Katz 2004, Amann and Waberski 2014, Goodson et al. 2011).

To make an automated detection possible, the minimum and maximum size limits must be defined for an object to be recognised as sperm. This is again device-dependent, as they differ

in resolution capacity (how many µm/pixel they register). Then the computer will count the pixels that are filled by the object of interest on the captured image and classify it as a sperm head or not (Mortimer 2000). One example is a particle area from > 20 to < 70 µm² to give an order of magnitude (van der Horst et al. 2018b). An alternative to the conventional CASA-systems using only phase contrast objectives was developed by adding a DNA-specific fluorescent dye (based on Hoechst 33342), reducing the error rate significantly as no debris and cells with similar size to a sperm head, but without containing DNA, are counted. Since software version 10, the Hamilton Thorne IVOS system is capable of analysing samples with this staining method (Zinaman et al. 1996).

The computer counts and differentiates all heads by allocating them to a certain place in the picture (assigns x and y coordinates to them), the autofocus of the focal plane (z axis) is technician independent. In the next step the following picture gets worked up the same way and the computer proceeds until the whole video sequence is digitalised. Around each sperm head a so-called zone of probability is raised to connect the single detections of the pictures by checking where a certain head is placed in the frame that is subsequent in time. Considering the way a sperm would be able to move within that short period the circle is set, the positions are linked and the track can be reproduced (Amann and Waberski 2014, Mortimer 2000). Not only can the trajectory be reconstructed, but also special motion patterns which are only shown in a certain activity status of a sperm (e.g. hyperactivity) can be recognised. Thus subpopulations can be determined and classified. Defining these boarders often is difficult and depends on the sperm samples and the experimental setup. Results are not reliable if the intervals are set to too wide or too narrow. Another divergence within the publications is how many of the motility parameters are considered for the classification. Maree L. and van der Horst G. (2013) found out that in their setup it was sufficient to divide the groups only by their curvilinear velocity (VCL) across all six species. This single parameter made it possible to specify three subpopulations regarding the speed of the sperms and interestingly most of the other parameters differed significantly as well (Maree and van der Horst 2013).

Another factor of a CASA system is the measurement chamber as different types lead to varying results: It has been found that a 20 µm deep chamber in combination with a 10x objective is preferable in terms of validity, as it enables the technical device to follow a single sperm (Amann and Katz 2004, Mortimer et al. 2015). To estimate the reliability and to validate different methods of determining the sperm concentration of human sperm samples, the manual method using a “Improved Neubauer” haemocytometer as “gold standard” was

compared to the Leja chamber (depth 20 μm) and the Makler chamber (depth 10 μm). The study pointed out that the Leja chamber represents an appropriate alternative to the haemocytometry, preferably if diluted sperm samples are used as the probability of inaccuracies at high concentrations was shown to be increased. In contrast the Makler chamber led to an overestimation of the sperm concentration of about 32 % (Bailey et al. 2007). Regardless of the chamber type, a correction factor for laminar flow artefacts, such as the Segre-Silberberg effect (Segré and Silberberg 1961), and depending on the viscosity of the used medium must be incorporated, as sperm tend to aline with surfaces and the capillary filling causes enhanced movement in one direction. Further, measurements should preferably be taken along the central long axis of the chamber to reduce inaccuracies and obtain reliable and representative results (Amann and Waberski 2014).

There is the need of a distinction between truly and only apparently motile spermatozoa. An actively moving sperm cell can bump into an immotile one, creating the effect that both show movement. This would falsely increase the percentage of motility. By collision spermatozoa sometimes form an agglutination with themselves or other cellular impurities of the sample and will no longer be detected as a “cell of interest” due to the size the new object has increased. That would lead to a decreased overall sperm count, but on the other hand to an incorrect measurement concerning progressive ones as especially immobile spermatozoa tend to agglutinate (Mortimer et al. 2015). Naturally occurring and non-avoidable collisions of sperm tracks can cause a mix up of the tracks of the individual cells. This aspect can be reduced by dilution to a certain extent or the application of a collision correcting algorithm if it is available for the used CASA system (Mortimer et al. 2015).

There are several methods for the statistical processing of CASA data: multivariate cluster analysis, regression analysis, principal component analysis (PCA) or the one-way repeated-measures analysis of variance (ANOVA) (Martínez-Pastor et al. 2011, Pérez-Crespo et al. 2008). The choice of the approach depends on the number of variables, the underlying research question, but also the preference of the scientist. Often huge data sets are generated in CASA settings. To make them more manageable and to facilitate an interpretation, a PCA is a useful statistical tool since many velocity parameters are interdependent. This form of analysis serves to reproduce a lot of information from individual numbers in fewer variables. Most of the times two clusters are divided, one including all parameters concerning speed (VAP, VSL, VCL) and the others shape-related (ALH, BCF, STR, LIN, WOB). Usually a PCA (values log10 transformed) is performed for both groups separately (Tourmente et al. 2015a,

Tourmente et al. 2013). When cluster analysis is performed, a preceding variable reduction, dependent on the study design, through an analysis of correlations is useful. Thus, nonrelevant values can be diminished, which represents a reduction of a possible source of error. In case of a non-normal distribution of the measured values a transformation of the data may be necessary (Martínez-Pastor et al. 2011).

b. Common measured CASA parameters

By measuring kinematic parameters, motion and certain locomotion patterns can be described. Whereas the speed is a direct measurement, some values (e.g. WOB) must be calculated based on others. Using these measurands, single spermatozoa can then be divided into subpopulations (Davis and Siemers 1995).

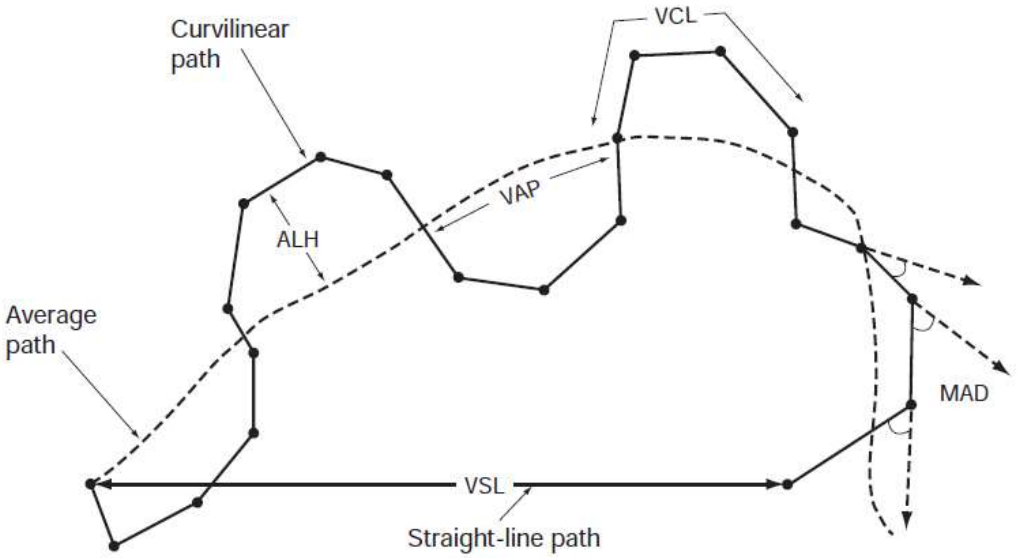


Figure 1: Illustration of the calculation basis of the individual parameters (World Health Organization 2010)

Parameters that can be gathered simultaneously are listed in the order proposed by the WHO (World Health Organization 2010):

I. Curvilinear velocity (VCL): Value averaged over time in µm per second representing the true track the sperm head is taking and therefore always the highest of the three velocity parameters (Mortimer 2000; Bone et al. 2001)

II. Straight line velocity (VSL): The mean velocity, again given in µm per second, the sperm is swimming in the recorded time on the shortest route between its first and last point of the track; it is always the lowest velocity value (Bone et al. 2001)

III. Average path velocity (VAP): The µm per second the spermatozoon covers in the tracked time period and mean direction of locomotion

The distance is detected by the optical system and is converted into a filtered and as a result simplified trace. All recorded positions respectively their coordinates (x, y) get averaged and yield to a smoothed average path. The VAP is nearly identic with the VSL if the sperm is moving straight in one direction with only little sidewise motion of the head. The resemblance diverges with a high lateral head displacement of the head and nonlinear forward movement is shown (Mortimer 2000).

IV. Amplitude of lateral head displacement (ALH): This value is given in µm and describing the lateral extend of the sperm head during motion

The flagellum is the part where the motion is generated, the head only moves dependently: If a flagellar beat starts near the head, it generates smaller amplitudes and in consequence smaller ALH values with a more distinct motion in a certain direction. On contrast flagellar beats generated more backwards create higher amplitudes. Not to lose any details it is important that the calculation base is the entire width of the headway. Diverted from the VAP computation the ALH is depending on the smoothed path of the sperm. The value is either given as a maximum or a mean one: ALHmax is the highest measured value of head displacement in the analysed distance whereas ALHmean is the average value of all ALH measurements taken along the track. For correct interpretation it is advantageous to know which type of measurement the used CASA instrument is calculating. All Hamilton Thorne instruments as well as the Sperm Class Analyzer rely on ALHmax (Mortimer 2000, van der Horst et al. 2018b).

V. Linearity (LIN): Calculated as VSL divided by VCL and expressed as a percentage; tells about the divergence of the actual path from the straight line between start and end point of the track (Grzmil et al. 2007)

VI. Wobble (WOB): VAP divided by VCL with the result given in percent again, expressing the undulations of the sperm heads' trajectory along the mean path (Davis and Siemers 1995)

VII. Straightness (STR): One of the three ratios derived from the velocity parameters; VSL divided by VAP with the quotient value given as percentage; it is the classification number of the progressiveness of the locomotion (Grzmil et al. 2007)

VIII. Beat cross frequency (BCF): This value is given in hertz (Hz) and generated by the number of times the curved track crosses the straight one per second, so it expresses how often the sperm head intersects the direction of the trajectory and therefore is related to the number of flagellar beats (Khatun et al. 2018). It is used to detect and assess significant changes in whip pattern of the flagellum but with technical limitations. An underestimation takes place if the actual beat frequency is greater than the images per second the used CASA system can take (Mortimer 2000).

IX. Mean angular displacement (MAD): given in degrees; angle of rotation of the sperm head along the sperm's curvilinear lane, averaged over time (Khatun et al. 2018)

As some parameters are calculated, they are algorithm-dependent and can differ among the several CASA devices. An example with detailed explanations can be found at Davis and Siemers (1995) or in the WHO laboratory manual for the Examination and processing of human semen (2010).

Table 1: Literature overview about the CASA parameters examined and CASA systems used for the analysis of mouse sperm (for the explanation of parameter numbers I–IX see text above)

Reference	**Categories**	**Used system**
(Bertoldo et al. 2014)	total and rapid motility, I–III	IVOS
(Bray et al. 2005)	I–V, VII, VIII	CEROS version 10.8
(Bone et al. 2001)	I, II, V	IVOS version 10.6
(Cao et al. 2009)	I–V, VII	IVOS version 12.2L
(Cheng et al. 2007)	total motility, I–V, VII, VIII	IVOS version 12.2L
(Cordero-Martínez et al. 2014)	total and progressive motility, I–IV, VIII	TOX IVOS version 12.3
(Danshina et al. 2010)	I–V, VII, VIII	IVOS version 12

Reference	Categories	Used system
(Dardmeh et al. 2017)	total motility, I–VIII	SCA version 5.4.0.0
(Elangovan et al. 2006)	total motility	IVOS version 10
(Firman and Simmons 2010a)	motile, progressive and rapid sperm, III	CEROS version 10
(Firman and Simmons 2010b)	motile sperm, I–V, VII, VIII	CEROS version 10
(Firman et al. 2013)	motile, progressive and rapid sperm, I–III	CEROS version 10
(Gómez Montoto et al. 2011)	I–V, VIII	SCA version 4.0
(Goodson et al. 2011)	I–V, VII, VIII	CEROS
(Grover et al. 2005)	I–V, VII, VIII	IVOS
(Grzmil et al. 2007)	motile and progressive sperm, I–V, VII, VIII	CEROS
(Harris et al. 2007)	I–V	IVOS version 10
(Khatun et al. 2018)	total and hyperactive motility, I–IX	SAIS Plus version 10.1
(Li et al. 2016)	total and progressive motility	IVOS II
(Maree and van der Horst 2013)	I–VIII	SCA version 4.0.0.5 or 4.1.0.1
(Miki et al. 2004)	I–V, VII, VIII (in supplementary data)	IVOS version 12
(Nayernia et al. 2002)	I–IV, VII, VIII	CEROS version 10
(Nayernia et al. 2003)	I–IV, VII, VIII	CEROS version 10
(Odet et al. 2008)	I–V, VII, VIII	CEROS version 12
(Pérez-Crespo et al. 2008)	total and progressive motility, VII	SCA 2002
(Pitetti et al. 2013)	I–VIII	SCA
(Raspa et al. 2018)	total and progressive motility	IVOS
(Shao et al. 2008)	total, progressive and hyperactivated motility, I–III, V, VII	IVOS version 10.8 s
(Stenz et al. 2017)	motile sperm, I, IV	CEROS II

Reference	Categories	Used system
(Sztein et al. 2000)	total and progressive motility	IVOS
(Takeo et al. 2014)	total and progressive motility	IVOS
(Tourmente et al. 2013)	I–III	SCA
(Tourmente et al. 2015a)	I–VIII	SCA version 4.0
(Tourmente et al. 2015b)	I–III	SCA
(Varea-Sánchez et al. 2016)	I–III	SCA
(Vicens et al. 2014)	I–V, VII, VIII	SCA version.4.0
(Vosoughi et al. 2013)	immotile, non-progressive and progressive sperm	SCA version 5.1
(Wang et al. 2007)	total and progressive motility, I–V, VII, VIII	IVOS

This table shows that, regardless of the used system, a varying quantity of parameters are collected: Depending on the research question, often only certain values are of interest.

The accuracy and precision of the measured and calculated values is influenced by numerous factors, as listed in Table 2. Considering the enormous amount of data generated, several authors emphasise the importance of a correct use of the device, a strict standardisation of all listed factors and of a quality control and careful and correct interpretation of the data (Amann and Waberski 2014).

Table 2: Factors influencing accuracy and precision in kinematic sperm measurements according to Davis and Siemers (1995)

Diluent or extender
Video framing (or digitisation) rate
Physiological state of the sperm
Specimen temperature
Specimen concentration
Presence of debris
Instrument parameter settings
Instrument precision
Instrument accuracy
Microscope optics and method of illumination
Counting chamber type, depth, and counting method
Digitisation threshold (grey scale)
Number of points tracked
Number of fields (or sperm) analysed
Computation algorithms
Statistical methods
Laboratory supplies
Videotaping (inter-technician variation)
Between aliquot variation

c. Applications of CASA

CASA is utilised in a wide spectrum of research questions concerning male germ cells: It starts with basics such as analysing the role of the cytoplasmatic droplet (Yuan et al. 2013). Since mice are polygamous animals, research into the influence of sperm competition is also of interest, as this reproduction strategy affects morphology and leads to increased velocity parameters (Gómez Montoto et al. 2011, Tourmente et al. 2015b, Varea-Sánchez et al. 2016). Furthermore, the influence of altered physical conditions is investigated, such as heat stress: A one-time 30 min exposure of the testes to mild heat stress (42 °C) already induces DNA-damage, decreases sperm concentration and velocity parameters and increases the

probability for female offspring. This adverse effect is depending on the stage of the male germ cells when the heat exposure takes place (Pérez-Crespo et al. 2008).
Apart from this, CASA also provides the possibility of investigating the influence of various mutations on the sperm: Whereas some genes apparently do not influence sperm motility or fertility (Nayernia et al. 2003), detrimental effects in motile and morphological appearance are seen when others are knocked out, such as the autophagy related 7 gene (ATG7) (Shang et al. 2016). Some mutations cause age-related dysfunctions, with the decrease in motility and DNA defects increasing with age (Ozkosem et al. 2015). Nayernia et al. (2002) was able to show that different genetic backgrounds can influence the severity of a gene knockout. This means that it can even be decisive upon a mouse strain being still fertile or not (Nayernia et al. 2002).

Furthermore, cryopreservation is a key point if the storage of a certain mouse strain is important, but animals are no longer used directly in an experiment: By now, it is common knowledge that it is depending on the genetic background of the mice how well their sperm are suitable for being frozen (Sztein et al. 2000). The underlying protocol and used cryoprotective agents play a role, as well as the temperature influences the outcome: Rapid thawing at 37 °C showed the best results, whereas the temperature when collecting the sample did not make a difference (Sztein et al. 1997). A broad-based study with 735 genetically modified mouse lines with 12 different genetic backgrounds showed that thawed sperm benefit from pre-incubation before being used for IVF, whereas fresh samples did not show a positive effect (Ostermeier et al. 2008). If the quantity of progressive motile spermatozoa is less than 10 % in a sample, the IVF rate drops drastically lower than 30 % (Li et al. 2016). These results were reaffirmed by the observation that sperm without any sign of motility are more likely to show deviations from the norm in their karyotype (Yamauchi and Ward 2007). This in turn brings up the perception that the presence of a high percentage of immotile or dead sperms does have a negative effect on the fertilisation rate (sperm were capacitated in MBCD medium). A possible reason can be the increase of oxygen radical production of these spermatozoa, which inhibits fertilisation. However, the process of capacitation in the medium was not influenced by the radicals. These results underline the importance and value of a detailed analysis of sperm motility and points out further areas of application in the sector of cryopreservation and cryo-recovery of sperms (Li et al. 2016). As cryopreservation is the most advantageous way of storing sperm for a long time, it is important to investigate substances that positively influence motility and IVF success of thawed sperm as well (Bertoldo et al. 2014). In the field of assisted

reproduction, other questions arise, such as whether different IVF methods have an influence on early embryonic development (Peters et al. 2009). Researchers now use CASA as well to predict *in vitro* fertilisation rates based on motility parameters of spermatozoa. (Amann and Katz 2004, Verstegen et al. 2001). Concerning humans, the prediction even goes one further step by defining the group of sperm that will be able to penetrate the mucus in a woman's uterus for fertility prognosis (Mortimer 2000, Verstegen et al. 2001).
In addition, it is also possible to investigate the effects of toxic or pharmacologically active substances in the mouse model: Negative influences of certain chemicals such as formaldehyde vapour (Vosoughi et al. 2013) or cyclophosphamide (Elangovan et al. 2006) can be tested, as well as positive effects from substances such as D-aspartate (Raspa et al. 2018).

Goodson et al. (2011) developed an automated, quantitative method that objectively classified five distinct motility patterns of mouse sperm using Support Vector Machines (SVM), a commonly used method in supervised machine learning: From the visual classification into the five classes (hyperactivated, intermediate, progressive, slow and weakly motile), four equations with mathematically derived borders were constructed to make the division possible. For being as precise as possible, all CASA values were included in every single equation. To consider the factor of relative importance correctly as well, each parameter was multiplied by a calculated factor, depending on its significance in the applied equation. As an example, in SVM1 VAP was the most relevant and therefore had the greatest factor to be multiplied with. Mouse sperm tracks derived from CEROS (videotaped at 60 Hz) were used as a base for creating this software, called CASAnova. With the use of this system it is possible to distinguish motility pattern more complexly. A lot of tests on the reliability of the software have already been performed, showing promising results, but further assessments are needed to test it for other CASA applications (Goodson et al. 2011). Since the development, the software was used by different authors, e.g. Stenz et al. (2017).

d. Used strains

Table 3: Overview about the used mouse strains, sperm collection technique and the number and age of animals used in mouse CASA studies (not all details were mentioned in the corresponding paper)

Reference	Mouse strain			Collection technique	Number and age of mice
Wild derived muroid rodents					
(Firman and Simmons 2010a)	*Mus domesticus* wild-derived house mice as source of the Uwa:MDD colony divided into different selection lines			a	n = 154 ≥ 8 weeks old
(Firman and Simmons 2010b)	*Mus domesticus* wild-derived house mice after 5 generations of selection and different mating regimes			a	n = 68 (from 7 different lines) 8 weeks old
(Firman et al. 2013)	wild house mice from islands off the coast of Western Australia			a	n = 34 (4 groups) 15 weeks old
(Gómez Montoto et al. 2011)	11 species of muroid rodents incl. *Mus musculus*			a	n = 3–5/species adult males
(Maree and van der Horst 2013)	*Mus musculus*			a	n = 10
(Tourmente et al. 2013)	adult males from 9 different mouse species			a	n = 4–6/strain
(Tourmente et al. 2015a)	*Mus musculus* strain MPB (Poland)	*Mus spretus* strain SEB (Spain)	*Mus spicilegus* strain ZRU (Ukraine)	a	n = 5/strain 4–6 months old
(Tourmente et al. 2015b)	18 different species of muroid rodents notable: *M. musculus castaneus*, *M. musculus domestic*, *M. musculus musculus*			a	n = 4–18 (species-dependant) adult males

Reference	Mouse strain	Collection technique	Number and age of mice
(Varea-Sánchez et al. 2016)	17 muroid rodents	a	n = 5/species
(Vicens et al. 2014)	16 rodent species belonging to the subfamily *Murinae* (five different genera)	a	n = 5/species
Laboratory strains			
1) inbred			
(Dardmeh et al. 2017)	C57BL/6NTac	a	n = 24 16 weeks old
(Peters et al. 2009)	C3HeB/FeJ	a	n = not given 12 weeks old
(Raspa et al. 2018)	C57BL/6NTacCnrm	a	n = 27 (6/group)
(Stenz et al. 2017)	C57BL/6J	a	n = 10 100 days old
(Takeo et al. 2014)	C57BL/6J	a	n = 48 12–16 weeks old
(van der Horst et al. 2018b)	C57 strain	a	n = 10
2) outbred			
(Bertoldo et al. 2014)	SWISS	a	n = 12 3–4 months old
(Cao et al. 2009)	CD1	not mentioned	
(Cordero-Martínez et al. 2014)	CD1 (20–25g)	a	not mentioned
(Elangovan et al. 2006)	ICR	a	n = 4/group (5 groups)

Reference	Mouse strain		Collection technique	Number and age of mice
(Khatun et al. 2018)	ICR		a	n = not given 12 weeks old
(Pérez-Crespo et al. 2008)	CD1		a	n = 60 (2 groups) 2–5 months old
(Sztein et al. 2000)	C57BL/6J, DBA/2J, BALB/cJ, 129S3/SvImJ, FVB/NJ		a	n = 8/strain 3–5 months old
(Vosoughi et al. 2013)	NMRI		a	n = 36 (3 groups) 9.5–14.5 weeks old
3) hybrid				
(Bone et al. 2001)	CB6F1/Crl BR hybrid (female BALB/c and male C57BL/6)		a	n = 3/experiment 5 weeks old
(Sztein et al. 2000)	B6D2F1/J (C57BL/6J x DBA/2J) and CB6F1/J (BALB/cBy x C57BL/6By)		a	n = 8/strain 3–5 months old
4) mutant strains, including genetic background				
(Bray et al. 2005)	C57BL/6J knockout Chrna $7^{-/-}$ ($Chrna7^{tm1Bay}$ allele)	C57BL/6J wild-type ($Chrna7^{+/+}$) littermates	a	n = 4–8/strain 8–12 weeks old
	strain of origin: 129S7/SvEvBrd-$Hprt^{b-m2}$			
(Cheng et al. 2007)	$Taf7l^{-/Y}$ and wild-type mice		a	n = 3/genotype 8 weeks old
	with B6 or 129 genetic background			
(Danshina et al. 2010)	$Pgk2^{-/-}$, $Pgk2^{+/-}$ and WT ($Pgk2^{+/+}$)		a	n = 5/genotype
	with a mixed genetic background (129S6/SvEvTac and C57BL/6NCrl)			

Reference	Mouse strain		Collection technique	Number and age of mice
(Grover et al. 2005)	FORKO (FSH-R knock-out)	wild type mice	a	n = 6/strain 12 months old
	with a SV129 background			
(Grzmil et al. 2007)	B10. BR-Y^{del} (back-crossing B10.BR-Y^{del}/Ms males to B10.BR/SgSn females)	B10. BR (congenic control strain)	a	n = 3/strain 40–60 days old
(Harris et al. 2007)	Ste5Jcs1/Rw Ste5Jcs1/Ste5Jcs1		a	n = 3
	with a mixed genetic background (C57BL/6J and CAST/EiJ)			
(Li et al. 2016)	unique knockout mouse lines, all heterozygous	wild-type C57BL/6N	a	n = 113 cryopreserved sperm samples from 46 knockout lines 3–4 months old
	with the C57BL/6N background			
(Miki et al. 2004)	TC-1 embryonic stem cells > chimeric males mated with C57BL/6N females	heterozygous animals were mated to produce $Gapds^{-/-}$ and WT ($Gapds^{+/+}$)	a	$Gapds^{-/-}$ n = 4 WT n = 3 4–6 months old
	the genetic background cannot be inferred from the paper			
(Nayernia et al. 2002)	$Smcp^{-/-}$	$Smcp^{+/+}$	a	n = 4/genotype sexually mature
	on a mixed background (C57BL/6J x 129/Sv) and on a 129/Sv genetic background			

Reference	Mouse strain			Collection technique	Number and age of mice
(Nayernia et al. 2003)	two triple knock out lines	wild type		a	n = 3/line
	with mixed genetic background (CD-1 x C57BL/6J x 129/Sv)				
(Odet et al. 2008)	$Ldhc^{-/-}$ mice	$Ldhc^{+/-}$ mice	wild type mice C57BL/6N	a	n = 3/line
	129SvEv embryonic stem cells x C57BL/6N blastocysts				
(Pitetti et al. 2013)	single and double knockout mice lacking the insulin receptor ($Insr^{fx/fx}$) and/or the IGF1 receptor ($Igf1r^{fx/fx}$)			a	not mentioned
	the genetic background cannot be inferred from the paper				
(Shao et al. 2008)	$Jam\text{-}A^{-/-}$	$Jam\text{-}A^{+/+}$ as control group (WT)		a	n = 6/line sexually mature
	on a C57BL/6 background				
(Wang et al. 2007)	$Aldh2^{-/-}$	$Aldh2^{+/+}$		a (under anaesthesia)	n = 5–6/group 12 weeks old
	with an C57BL/6 background				
inbred, outbred and mutant mice used in one paper					
(Goodson et al. 2011)	CD1, C57BL/6J, 129S1/SvImJ (12), PWK/PhJ (PWK), $Gapdhs^{-/-}$, wild type mice			a	$n \geq 3$ of each strain > 8 weeks of age

e. Used media

There is a range of media that can be used for different scientific questions: A common one is HTF, applied for sperm motility assays in mice and in humans (Goodson et al. 2011, Nakagata 2000). The medium influences the swimming speed, depending on its composition, as it is proven that hyperactivation is only shown when capacitating conditions are provided (Amann and Katz 2004, Mortimer 2000). This is given through open cation channels (CatSper

channels), a raise of the intracellular pH-value and release of Calcium (Ca^{2+}) ions. The induction of hyperactivity is species specific and depending on the mobilisation of Ca^{2+} (van der Horst et al. 2018b). Capacitation of mouse spermatozoa is enabled if the medium contains an energy source, bicarbonate and calcium (Buffone et al. 2012).

Table 4: Used media in mouse CASA studies

Reference	**Medium**		**Reason for use**
(Bertoldo et al. 2014)	HTF medium		
(Bone et al. 2001)	equilibrated medium G		
(Bray et al. 2005)	Whittingham medium containing 30 mg/ml of bovine serum albumin (BSA)		
(Cao et al. 2009)	PBS solution for non-capacitation	modified Whitten medium with additives for capacitation	
(Cheng et al. 2007)	Krebs-Ringer bicarbonate medium (HM) without Ca^{2+}, BSA and $NaHCO_3$		
(Cordero-Martínez et al. 2014)	Tyrode medium		for noncapacitating conditions
(Danshina et al. 2010)	M16 medium		
(Dardmeh et al. 2017)	Dulbecco's Modified Eagles Medium (DMEM)		
(Elangovan et al. 2006)	modified HEPES medium		
(Firman and Simmons 2010a)	modified Tyrode's solution with 5.56 mM glucose and 4 mg/ml BSA		
(Firman and Simmons 2010b)	modified Tyrode's solution with 5.56 mM glucose and 4 mg/ml BSA		
(Firman et al. 2013)	HTF medium		
(Gómez Montoto et al. 2011)	Hepes-buffered modified Tyrode's medium		

Reference	Medium		Reason for use
(Goodson et al. 2011)	HTF medium		has been used extensively for both mouse and human IVF
(Grover et al. 2005)	Hank's medium M199, supplemented with 0.5 % BSA		
(Grzmil et al. 2007)	IVF Medium under liquid paraffin		
(Harris et al. 2007)	not given; referred to previous work of the group		
(Khatun et al. 2018)	modified Tyrode's medium		
(Li et al. 2016)	M2 Medium		
(Maree and van der Horst 2013)	Ham's F10 medium supplemented with 3 % BSA		sustains sperm functions of various species for long periods
(Miki et al. 2004)	M16 medium		
(Nayernia et al. 2002)	Medi-Cult (IVF medium)		
(Nayernia et al. 2003)	Medi-Cult (IVF medium)		
(Odet et al. 2008)	modified Krebs-Ringer bicarbonate solution with 4 mg/ml BSA	HTF medium with 4 mg/ml BSA	
(Pérez-Crespo et al. 2008)	M2 medium		
(Peters et al. 2009)	HTF medium		
(Pitetti et al. 2013)	M2 medium		
(Raspa et al. 2018)	HTF medium		
(Shao et al. 2008)	PBS medium	HTF medium	to detect differences between uncapacitated and capacitated conditions of sperm
(Stenz et al. 2017)	M2 medium		

Reference	Medium	Reason for use
(Sztein et al. 2000)	collected, frozen and thawed in cryoprotectant agent (CPA); counted in HTF medium	(CPA components: 18 % raffinose and 3 % skim milk in culture-grade water)
(Takeo et al. 2014)	Modified Krebs-Ringer bicarbonate solution (TYH medium) with 1.0 mg/ml polyvinyl alcohol and 0.75 mM methyl-β-cyclo-dextrin (MBCD) for preincubation	
(Tourmente et al. 2013)	Hepes-buffered modified Tyrode's medium	
(Tourmente et al. 2015a)	Hepes-buffered modified Tyrode's medium with 4mg/ml fatty acid-free BSA	
(Tourmente et al. 2015b)	Hepes-buffered modified Tyrode medium	mimics the composition of the mouse oviductal fluid and supports sperm survival, but not capacitation
(van der Horst et al. 2018b)	Ham's F10 medium	
(Varea-Sánchez et al. 2016)	Hepes-buffered modified Tyrode medium	
(Vicens et al. 2014)	no data given	
(Vosoughi et al. 2013)	Ham's F-10 (nutrient mixture-Ham-X1, Gibco, UK) culture medium with 10 % BSA	
(Wang et al. 2007)	Medium 199	

5. Different CASA systems

More than twelve commercially available CASA systems have been used for the analysis for animal sperm (Amann and Waberski 2014), including the *CellTrak-S* (MotionAnalysis, Santa Rosa, CA) (Davis and Siemers 1995), the *Sperm Vision HR* (Minitube, Ingersoll, ON, Canada) (Ozkosem et al. 2015), the *SAIS Plus* (Medical Supply, Seoul, Korea) or the *SM-CMA system* (MTG GmbH), which is unique in terms of using the sperm midpiece for differentiation (Amann and Katz 2004). Although many of these devices were used on mice, the focus of this thesis is based on the three systems described below because these are the most frequently used.

a. Sperm Class Analyzer

The Sperm Class Analyzer (SCA) is manufactured by Microptic Automatic Diagnostic Systems (Barcelona, Spain) and was launched in 1997. In four modules, the system allows a measurement of concentration and motility, morphology, vitality and DNA fragmentation. The microscope is using negative phase contrast or brightfield to detect the sperm heads (Maree and van der Horst 2013). The modules motility and morphology are available for more than 80 different species, including invertebrates (e.g. mussels), fish, birds, wild and domesticated mammals as mouse, rat, lion, horse, bull, different primates and humans. A module called “RatTox” is focussing on the test of potential effects of chemicals, pesticides, radiation on mouse and rat sperm. Morphometry is challenging in rodents due to their hook-shaped acrosome at the sperm’s head (see chapter 6.b.) (Mortimer et al. 2015). Most settings including cut-off values for all categories of motility, average path smoothing intensity (relevant for detection of hyperactivity), number of pictures that are taken and parameters like adjusting to different chamber types, digital cameras or animal species can be adapted. Maree and van der Horst (2003) examined only the motile sperm population of six species including *Mus musculus* and adapted the default VCL cut-off values to include 80 % rapid, 15 % medium and 5 % slow swimming spermatozoa (Maree and van der Horst 2013). In contrast, a study examining the effect of ablation of insulin/IGF signalling in mutant mice used exactly the manufacturer’s definitions of non-, slow, and fast progressive sperm classes (Pitetti et al. 2013).

To minimise artefact susceptibility intelligent filters can be picked where the images get minimised to a black and white mode. Due to the less computationally intensive image

processing sperm detection and error correction through sperm collisions get enhanced. This is of advantage if a sample is severely contaminated, for instance with debris. With the special fluorescence mode, it is possible to evaluate the vitality of the sperm concurrent to the motility or morphology analysis. The Halosperm® Test (Halotech DNA SL, Madrid, Spain) can also be evaluated using this system as well as with the IVOS-II software. This allows the reliable exclusion of debris or fat droplets, as they occur in egg yolk-based cryopreservation bulking agent. After image acquisition, the pictures can be overlaid with different colours to simplify, for instance, the tracking of individual sperm. This is also used to identify certain motility patterns in order to categorise the sperm. Massive clumping constitutes a limitation of accurate measurements here, as in all other CASA systems (Mortimer et al. 2015).

b. IVOS (Integrated Visual Optical System)

This CASA-System (Hamilton-Thorne Biosciences, Beverly, Massachusetts, USA), produced since 1992, is able to analyse 15 different variables (Grover et al. 2005). As indicated by the name (Integrated Visual Optical System), the main feature of the system is the integration of the microscope with video camera and PC into one solid box, to enhance user-friendliness. The IVOS has individual defaults for different species (e.g. Hz/frame rate, minimum contrast, magnification, minimum size in pixels, VAP and VCL) (Bone et al. 2001, Cao et al. 2009). Additional improvements in this system were a stroboscopic light diode and the possibility for fluorescent staining and detection (Amann and Katz 2004). Further features are a special tail detection software tool, the enabling to trace back the average path by adaptive smoothing of the trajectory from a single cell (required for hyperactivation analysis) and a smart tracking through collisions (Mortimer et al. 2015).

The sort function of the IVOS system makes it possible to classify subpopulations within the sperm sample based on a number of kinematic parameters (Mortimer 2000). Experts consider this to be one of the most decisive utilisations and of great value to differentiate and to be able to make a forecast of *in vitro* and *in vivo* fertility. As well as with the SCA it is possible to differentiate sperm from other cells or debris via fluorescence imaging options (LED-based or xenon strobe) by staining the compacted DNA in the nucleus. Therefore the spermatozoa can either be still alive and mobile or already dead (Mortimer et al. 2015).

c. CEROS

In contrast to the IVOS, the CEROS (Hamilton-Thorne Biosciences, Beverly, USA) can be used with an existing microscope, which greatly reduces the investment costs, whereas the software is practically the same. The software CASAnova was developed with a CEROS setup (using mouse sperm and a frame rate of 60 Hz) and validated by comparison with results gained from classifying via experienced human operators (as described in chapter 4.c.). The accordance was impressively at 88.2 % (Goodson et al. 2011). Restrictions in the CEROS setting compared to the IVOS are the absence of a stroboscopic illumination system and consequential software-based illumination control feature and the IDENT fluorescence capability (Mortimer et al. 2015). The lower acquisition costs in turn facilitate to achieve a high standard even in laboratories with low budget (Amann and Katz 2004).

d. Parameters and settings for measurements

Analyses should be carried out at approximately body temperature. The most commonly used 37 °C are, like some other settings, taken from the field of human research (ESHRE Guidelines on the application of CASA technology in the analysis of spermatozoa, 1998). Although the different settings of each CASA device can be changed with every examined sample, a detailed list is rarely given.

Table 5: Operational settings of CASA systems used for analysing the motility of mouse spermatozoa

Reference	**Concentration**	**Amount and Chamber depth**	**Records**	**Frequency**	**1. measurement**	**2. measurement**	**3. measurement**	**Further measurements**
(Bray et al. 2005)	-	10 μl in a 20 μm deep counting chamber	≥ 400 cells/sample	60 Hz	after 0 min of incubation	after 2 h of incubation (capacitated)	-	
(Bone et al. 2001)	12 x 10^6 sperm/ml	-	a 4 min video	12.5 Hz	after 2 h of incubation	4–5 measurements with different concentrations of the 3 evaluating chlorinated antifertility compounds		
(Cao et al. 2009)	2.0 x 10^6 sperm/ml	5 μl in a 20 μm Leja chamber	30 frames	60 Hz	after 0 min of incubation	after 60 min	after 120 min	2 further measurements (180 & 240min)
(Cheng et al. 2007)	no details given							
(Danshina et al. 2010)	-	in an 80 μm-deep 2X-CEL chamber	1 sec videos	-	-			

Reference	Concentration	Amount and Chamber depth	Records	Frequency	1. measurement	2. measurement	3. measurement	Further measurements
			≥ 30 points per path					
(Firman and Simmons 2010a)	-	10 μl loaded into a haemocytometer	5 fields of view were scanned	-	-			
(Firman and Simmons 2010b)	-	10 μl loaded into a haemocytometer	5 fields of view were scanned	-	-			
(Firman et al. 2013)	-	-	5 replicate scans	-	-			
(Goodson et al. 2011)	2–4 x 10^5 sperm/ml (= 50–120	25 μl in a Leja chamber	1.5 sec videos of 10 fields	60 Hz	after 2 min of dilution into medium	30 min intervals between measurements (up to 2 h after incubation start)		

Reference	Concentration	Amount and Chamber depth	Records	Frequency	1. measurement	2. measurement	3. measurement	Further measurements
	sperm/microscope field)							
(Grover et al. 2005)	-	an aliquot placed into 80 μm deep glass chamber	30 frames	60 Hz	after 5 min of incubation	-		
(Grzmil et al. 2007)	-	-	20 frames	60 Hz	after 10 min of incubation	after 1.5 h of incubation	after 3.5 h of incubation	-
(Harris et al. 2007)	5 x 10^6 sperm/ml	-	30 frames	60 Hz	after 0 min of incubation	after 90 min of incubation	-	
(Khatun et al. 2018)	no details given							
(Li et al. 2016)	-	in an 80 μm depth 2X-CEL chamber	8–10 fields (2000–3000 sperm)	-	pre-freeze and post-thaw motility measured, time points not given		-	
(Maree and van der Horst 2013)	different between species	5 μl of preparation in a 20 μm deep Leja chamber	50 images	50/75 Hz	after 10 min	-		

Reference	Concentration	Amount and Chamber depth	Records	Frequency	1. measurement	2. measurement	3. measurement	Further measurements
			200 motile spermatozoa					
(Miki et al. 2004)	-	-	90 frames	60 Hz	immediately after collection (within 60 min after cauda epididymides removal)	-		
(Nayernia et al. 2002)	-	-	6000 – 11.000 spermatozoa	60 Hz	after 1.5 h of incubation	after 3.5 h	after 5.5 h	-
(Nayernia et al. 2003)	-	-	6000 – 10.000	60 Hz	after 1.5 h of incubation	after 3.5 h	after 5.5 h	-

Reference	Concentration	Amount and Chamber depth	Records	Frequency	1. measurement	2. measurement	3. measurement	Further measurements
			spermatozoa					
(Odet et al. 2008)	-	-	≥ 200 sperm were recorded	-	after 30 min of incubation	after 1.5 h of incubation	after 4 h of incubation	-
(Pitetti et al. 2013)	-	5 μl in a 20 μm Leja chamber	5–10 video sequences	25 Hz	no data given			
(Raspa et al. 2018)	-	30 μl in a Leja chamber	10 fields/sample	-	no data given			
(Shao et al. 2008)	-	an aliquot into a 100 μm deep Microcell chamber	30 frames ≥ 300 spermatozoa in 5 fields	60 Hz	after 1 h of incubation	-		
(Tourmente et al. 2013)	~20 x 10^6 sperm/ml	in a 20 μm deep Leja chamber	-	-	after 0 min of incubation	-		

Reference	Concentration	Amount and Chamber depth	Records	Frequency	1. measurement	2. measurement	3. measurement	Further measurements
(Tourmente et al. 2015a)	~5 x 10^6 sperm/ml	~10 μl in a 20 μm Leja chamber	150 sperms	-	after 56 min of incubation	after 107 min of incubation	-	
(Vicens et al. 2014)	4–6 x 10^6 sperm/ml	5 μl in a 20 μm deep Leja chamber	up to eight videos of 4 s each (min. 150 tracks/sample)	-	within 5 min of sample collection	-		
(Vosoughi et al. 2013)	-	4 μl in a Leja chamber	≥ 400 spermatozoa	50 Hz	30 min incubation after sample collection + 3 min on the warming plate before analysis	-		

The Leja chamber is produced by Leja Products B.V. (Nieuw-Vennep, The Netherlands) and the 2X-CEL chamber from Hamilton Thorne Biosciences (Beverly, Massachusetts).

e. Differences between the CASA systems

As mentioned above, the optical systems differ within the single CASA devices. Due to this, a validation needs to be done for any system on its own and even in case components of a system (e.g. video camera or objective) or setup parameters affecting the frame rate are changed. The fact that it is utilised in toxicology studies and in animal production as well shows that the range of application is versatile. Before someone can make inferences out of the gained results it must be considered that only a clinical tendency, but no reliable prediction based on computer-assisted sperm analysis can be made (Amann and Katz 2004, Mortimer 2000). Furthermore it needs to be kept in mind that the outcome is different in each system and no direct comparison can be made, because hardware as well as image-sampling frequencies and calculation algorithm differ within the devices (Amann and Waberski 2014, Schleh and Leoni 2013).

Another difference is found within the parameter ALH: It is computed by the deviation of the average path velocity, which in turn already results from a calculation. VAP is created by averaging all measuring points of the curvilinear path. In order to get to these values, an algorithm is used, which is different between the CASA instruments. When the frequently used five-point smoothing is applied, the amplitude of lateral head displacement can vary widely and is not entirely reliable due to inaccuracy (Mortimer et al. 2015).

Measurement errors that still occur come about on the basis of imprecise instrument thresholds, erroneous processing of the digital images or too little spatial resolution (Davis and Siemers 1995). When compared with manual evaluation, CASA provides the clear advantages of objective, fast and detailed analysis and high precision and reproducibility within one group of settings. Considerable limitations are found in the sensitivity for small changes, e.g. whether slow sperm cells still count as motile or static. This little difference can have a huge impact on the results, depending on the percentage of this group in a present sample. Furthermore different microscopes set each other apart in their sensitivity for light or contrast (Schleh and Leoni 2013).

A validation and general guidelines for application would be beneficial, but are not realistic due to the differences between the systems and the different requirements in various application areas. As a correct interpretation can only be made if all generated data are included, it would

be desirable if in each publication the exact settings were given which were used on the respective CASA device (Schleh and Leoni 2013).

It is important to know that due to different requirements for production settings and for research, numerous commercial providers offer adapted software version, as for quality check in animal production most commonly only summarised data is important, whereas in research usually a detailed report and export of all data for further statistical analysis is crucial. Therefore, sufficient quality must be ensured in both settings, which can be achieved by regular controls and validations (Amann and Waberski 2014).

6. Aspects of computer-assisted analysis of sperm motility and morphology

a. CASA for the evaluation of mouse sperm motility

The parameters most commonly used for CASA (besides concentration) are the total and progressive motility of a sperm sample. Both values are given in percentages, the former expressing any type of active movement (more precisely, above a defined threshold value to distinguish it from immotile and passively motile sperm cells), whereas in the second one sperm must meet further author-dependent cut-off values (e.g. VAP $\geq$ 50 μm/s) (Li et al. 2016, Raspa et al. 2018). Verstegen et al. (2001) used a classification based on a medium and a low VAP cut-off (MVV resp. LVV). They distinguished between rapid sperm if VAP > MVV, medium if MVV > VAP > LVV, slow if LVV > VAP and static if cells do not show any movement at all. Additionally, a threshold value for the parameter straightness can be used to define progressive sperm and to limit the variation (Verstegen et al. 2001).

Due to the sensibility of germ cells to chilling, a constant temperature must be ensured during handling the sample in order to obtain reliable results. To perform a statistically based measurement, at least 200 spermatozoa need to be observed, as recommended by the World Health Organization (World Health Organization 2010). Although the differentiation between motile and static spermatozoa offers the possibility of a rough estimation of the quality of the sperm sample, it is important to divide into more subclasses of motile spermatozoa (Amann and Waberski 2014). A standardised and precise classification of sperm subpopulations is necessary and of a higher value than evaluating the population in total, because through the subdivision the statements made are more likely to be significant (Amann and Katz 2004, Davis and Siemers 1995). The cut-off points for the definition of subclasses are often set species-specific and are based on the analysis of a sample considered as “good” for the relevant species. The goal is e.g. to detect already minor changes caused by different pharmaceuticals or to select spermatozoa for a subsequent treatment (IVF, motility assessment, etc.). The differentiation is important, as subclasses respond differently to certain treatments (Maree and van der Horst 2013).

I. Motility categories of spermatozoa:

The movement pattern of a sperm cell depends on internal factors such as the stage of maturity or the ATP content of the cell, as well as the impact of the environment (external influencing factors are listed in Table 2). Total motility usually remains quite stable during the capacitation period, which is typically about 120 min in *in vitro* conditions, yet the different groups themselves change: Fewer sperms show progressive motility, but the number of hyperactive as well as slow sperms rises (Goodson et al. 2011). The description and definition of the groups vary between different authors and studies.

The following subpopulations have been distinguished in literature:

- Immotile: VCL approaching zero, hardly showing any flagellar motion (Amann and Waberski 2014)

- Slowly or weakly motile: Only a minor progressive motion can be detected, a low ALH value and no vigorousness (Goodson et al. 2011); this group can be excluded from the group of motile spermatozoa to avoid sources of error, because especially the slow sperms often get mixed-up with immotile, but passively moved ones that are just pushed by motile cells (Nayernia et al. 2003)

- Progressive: These sperms show high velocity parameters and symmetrical flagella bends with a low amplitude of the head's derivation from the average path, also the angle between the successive measurement points must be < 90° almost exclusively (Goodson et al. 2011); if only one or two cut-offs must be met for this category is author- or device-dependent and listed in Table 6; Bone et al. (2001) does not distinguish and only classifies as motile if any movement is seen (VAP ≥ 3 μm/sec)

- Intermediate: Rarely recorded as a separate group and defined as the transition phase to the hyperactivated state, showing more energetic movement than simple progressivity and starting to become more asymmetric in action, with angles about 90° between consecutive points on the trajectory (Goodson et al. 2011); apparently it is commonly seen only in humans and most other vertebra species do not show it excessively (Mortimer et al. 2015)

- Hyperactive: This category only is found after sperm underwent capacitation and rises during the incubation period up to 35 %; cells show changes in direction of > 90° along their path (Goodson et al. 2011)

  Other characteristics are magnified amplitudes of the flagellar beat with an increase in frequency and alteration of the wave form, leading to rising VCL and ALH values, whereas LIN drops. The moves are described as “whiplash” or “starspin” pattern (Bray et al. 2005, van der Horst et al. 2018b). For enabling sperm to start the process of capacitation, a medium containing calcium, bicarbonate ions and proteins (usually bovine serum albumin) is necessary (Mortimer 2000). Calcium is particularly important, as it is the main stimulating signal for initiation of hyperactivation. In contrast, the other motility categories are regulated through cAMP signals (Buffone et al. 2012, Harris et al. 2007). This salient locomotion pattern is mandatory for fertilisation: It could either be essential in preventing entrapment, upholding the sperms microenvironment, increase the opportunity of oocyte contact and being important for penetrating the zona pellucida (Mortimer 2000).

  The application of thresholds for VCL combined with STR to clearly define this group which were valid for human sperm samples, led to false results in mice, as sperms were defined as hyperactive when capacitation had not yet taken place. When the process of capacitation had finally been accomplished, the CASA system still was underrating the actual number of hyperactive spermatozoa which made it impossible to use it for reliable results. With the aid of a support vector machine, a software called CASAnova could be developed that enabled to clearly distinguish hyperactive sperm (see chapter 4.c.) (Goodson et al. 2011).

  Another option for the recognition has been provided with receiver operating characteristics (ROC) curve analysis: Swimming pattern and certain cut-offs for kinematic parameters of the hyperactivate sperm are compared with simple progressive ones. If specificity and sensitivity of those values exceed 90 % it can be categorised as truly hyperactive. In many species this statistic method has been effectively utilised in combination with the sort function of the SCA (Mortimer et al. 2015).

II. Factors affecting sperm motility

It could be shown that sperm motility is dependent on the size of the testes. High sperm competition, as it is the case in polyandrous species, is accompanied by an increase of testicle weight and total number of sperm (Firman et al. 2013, Tourmente et al. 2013). Hence it is important to look at the testes to body weight ratio too, as obesity in fact increases the weight of the reproductive organs, the ratio still diminishes. Adiposity leads to a deterioration of sperm quality in terms of decreased motility and a higher percentage of DNA damages (Dardmeh et al. 2017). Motility as well as velocity parameters and ATP-levels are increasing when there is competitive pressure, as it is the case in the promiscuously living species *Mus musculus* (Firman et al. 2013, Gómez Montoto et al. 2011, van der Horst and Maree 2013, Vicens et al. 2014).

In mouse sperm, glycolysis is used as the main pathway for ATP production in basal sperm motility. During hyperactivation a shift towards oxidative phosphorylation happens. The first process takes place in the fibrous sheath of the principal piece, while the second one occurs in the mitochondria of the midpiece (Buffone et al. 2012, Turner R. 2003). The necessary energy for locomotion can be drawn from glucose, fructose or sorbitol. All three substances led to an increased longevity and can therefore be considered as additives to the medium to provide an energy supply (Cao et al. 2009).

Cryopreservation of sperm negatively affects all sperm motility parameters. The quantification of this effect is obviously an important application of CASA, as mentioned in chapter 4. c..

Postcopulatory selection, which can differ among populations, increases both, total amount of ATP and sperm concentration. This directly affects sperm velocity and results in faster sperm (Tourmente et al. 2013). Another important factor on the sperm motility is the midpiece of a spermatozoon, where the mitochondria are located. Studies across different vertebrate species have shown that there is a linkage of sperm size and velocity parameters: Firman and Simmons (2010b) detected that in mice the midpiece size was a predictor of sperm swimming velocity, i.e. sperm with longer midpieces had faster swimming velocities. On the other hand, a negative correlation between swimming speed and longevity of a sperm was discovered (Firman and Simmons 2010b).

Many pharmacologically active substances have an impact on sperm motility. Exemplary D-aspartate increases progressive motility after an oral intake over four weeks (Raspa et al.

2018), whereas the aminopeptidase N needs to be inhibited to positively influence the same category of sperm (Khatun et al. 2018, Raspa et al. 2018). A negative effect of different enzymes on mouse sperm parameters was shown in the context of evaluating potential male contraceptives, for instance with oxalate derivatives. They are selective inhibitors of lactate dehydrogenase-C4, a sperm-specific enzyme that plays an important role in ATP production for maintaining progressive motility as well as to induce capacitation and hyperactivation (Cordero-Martínez et al. 2014). Moreover, it was discovered that inhibition of glycolytic enzymes of sperm, including those of human and mice (namely glyceraldehyde 3-phosphate dehydrogenase (GAPDH)), can be effectively accomplished by a variety of chloro-compounds and could be developed into contraceptive agents for men (Bone et al. 2001). In another study examining the positive effect of probiotics on sperm quality it could be demonstrated that *Lactobacillus rhamnosus PB01* could affect weight and some male fertility potential biomarkers including sperm motility parameters and hormones in a diet-induced obesity mouse model. The change in the sperm velocity and motion path parameters demonstrated in this study, may be associated with the direct effect of probiotics on spermatogenesis and maturation process or indirectly by removing the adverse effects of obesity and increasing the level of total antioxidant capacity (Dardmeh et al. 2017).

III. Different classification options

The following table shows that different parameters can be used for the allocation into subclasses, and that it depends on both, the device and the author, how the cut-offs are determined.

Table 6: Cut-off values used for classification of the different motility classes in mouse sperm analysed by CASA

CASA device	**Hyperactivated**	**Progressive or rapid**	**Medium**	**Slow or weakly motile**	**Comment**	**Reference**
CEROS	VCL ≥ 180 μm/sec ALH ≥ 9.5 μm LIN ≤ 38 %	VAP ≥ 25 μm/sec VCL ≥30 μm/sec	25 > VAP > 10 μm/sec	VAP ≤ 10 μm/sec OR VSL ≤ 10 μm/sec	-	(Bray et al. 2005)
CEROS	"standard mouse parameters" as given by the manufacturer were used as in (Nayernia et al. 2003)					(Firman and Simmons 2010a)
CEROS	"standard mouse parameters" as given by the manufacturer were used as in (Nayernia et al. 2003)					(Firman and Simmons 2010b)
CEROS	vigorous if VCL > 279 μm/sec		-	nonvigorous if VCL < 176μm/sec defined as slow AND weakly motile if no significant forward motion was detected	"mouse default settings" as given by the manufacturer	(Goodson et al. 2011)

CASA device	Hyperactivated	Progressive or rapid	Medium	Slow or weakly motile	Comment	Reference
CEROS	no details given					(Grzmil et al. 2007)
CEROS	-	VAP ≥ 75 μm/sec STR ≥ 50 %	-	were excluded from analysis	motile if VAP ≥ 25 μm/sec VSL ≥ 30 μm/sec	(Nayernia et al. 2003)
CEROS	VCL > 240 μm/sec ALH > 18 μm BCF < 40 Hz	VAP > 50 μm/sec STR > 50 %	-		counted as motile, if any type of movement was identified	(Odet et al. 2008)
IVOS	motile if VAP 3–500 μm/sec no distinct settings for VCL				“mouse default settings” as given by the manufacturer	(Bone et al. 2001)
IVOS	VCL > 180 μm/sec	VAP 50.0 μm/sec	motile if VAP ≥ 10 μm/sec no cut-off value for VSL		“mouse default settings” as	(Cao et al. 2009)

CASA device	Hyperactivated	Progressive or rapid	Medium	Slow or weakly motile	Comment	Reference
	ALH > 9.5 μm LIN < 38 %	STR 50 %			given by the manufacturer	
IVOS	VAP > 50 μm/sec STR > 50 %	-	motile if VAP ≥ 10 μm/sec no cut-off value for VSL		-	(Cordero-Martínez et al. 2014)
IVOS	VAP > 80 μm/sec STR > 50 %		-		-	(Danshina et al. 2010)
IVOS	no details given					(Elangovan et al. 2006)
IVOS	-	VAP ≥ 50 μm/s	-		-	(Li et al. 2016)
IVOS	-	VAP > 50 μm/s STR > 50 %	-		-	(Miki et al. 2004)

CASA device	Hyperactivated	Progressive or rapid	Medium	Slow or weakly motile	Comment	Reference
IVOS	VCL > 150 μm/sec LIN < 50 %	-	-		-	(Shao et al. 2008)
IVOS	-	VAP > 50 μm/sec STR > 80 %	motile if VAP > 7.4 μm/sec		-	(Raspa et al. 2018)
SAIS	no details given					(Khatun et al. 2018)
SCA	-	VCL > 45 μm/s	45 > VCL > 35 μm/s	VCL < 35 μm/s	-	(Dardmeh et al. 2017)
SCA	-	VCL > 120 μm/sec STR > 80 %	120 > VCL > 80 μm/s	80 > VCL > 50 μm/s	all cut-off values were adjusted and set higher than defaulted ones	(Maree and van der Horst 2013)
SCA	-	STR > 85 %	-		-	(Pérez-Crespo et al. 2008)

CASA device	Hyperactivated	Progressive or rapid	Medium	Slow or weakly motile	Comment	Reference
SCA	-	VAP > 45 μm/sec	45 > VAP > 15 μm/sec	VAP < 15 μm/sec	-	(Pitetti et al. 2013)
SCA	no details given					(Tourmente et al. 2013)
SCA	no details given					(Tourmente et al. 2015a)
SCA	no details given					(Tourmente et al. 2015b)
SCA	-	VCL > 242 μm/sec STR > 70 %	242 > VCL > 146 μm/sec	146 > VCL > 50 μm/sec	-	(van der Horst et al. 2018b)
SCA	no details given					(Vicens et al. 2014)

The restriction of all these different settings is that the limits are often set author-dependent, why a meaningful comparison cannot be made. Exemplary, the data for one very detailed normospermic mouse sperm sample from van der Horst et al. (2018) is given here:

Table 7: Values of a normospermic mouse sperm sample; measurements performed with the SCA (version not specified) and the C57 mouse strain (van der Horst et al. 2018b)

Measured parameter		**Numeric value**
Motility (%)		89.7 ± 8.5
VCL (µm/s)	Mean	293.3 ± 30.3
	Rapid	320.7 ± 32.4
	Medium	193.3 ± 41.1
	Slow	80.1 ± 12.4
VSL (µm/s)	Mean	119.7 ± 12.0
	Rapid	128.8 ± 14.0
VAP (%)	Mean	147.4 ± 13.4
	Rapid	158.1 ± 15.5
LIN (%)	Mean	41.3 ± 6.2
	Rapid	40.5 ± 6.0
STR (%)	Mean	81.3 ± 4.1
	Rapid	81.5 ± 4.1
WOB (%)	Mean	50.6 ± 5.6
	Rapid	49.6 ± 5.4
ALH (µm)	Mean	9.1 ± 1.2
	Rapid	9.8 ± 1.3
BCF (Hz)	Mean	10.5 ± 1.4
	Rapid	10.3 ± 1.4

b. CASA for examination of mouse sperm morphology

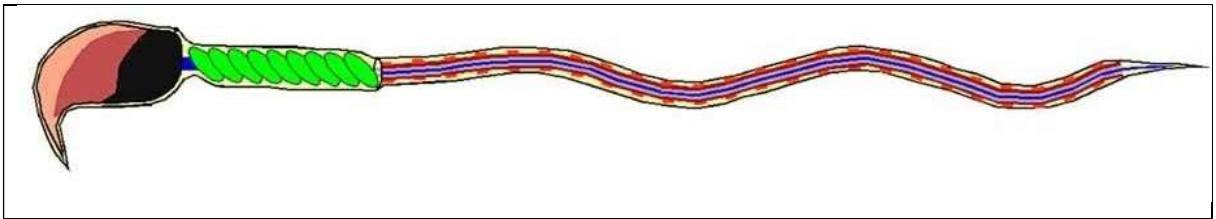
The utilisation of CASA in this area took place only after the analysis of sperm motility: In the early 1990s there were the first publications in which CASA was used to evaluate the morphology of sperm in different laboratory animal species, such as rats (Davis et al. 1994) and rabbits (Amann and Waberski 2014, Gravance and Davis 1995). The continuous development of technology made it now possible to detect low-grade divergences from normal sperm morphology that could not be recognised by human operators, using automated sperm morphology analysis (ASMA) instruments, often integrated as an additional module in CASA devices. Sensitivity increases, whereas variance is decreasing, making it a valuable and objective tool, especially in reproductive toxicology (Tablado et al. 1998). In recent studies the term computer assisted sperm morphometric analysis (CASA-Morph resp. CASMA) is more commonly used (Maroto-Morales et al. 2016, Yániz et al. 2015). Sample staining to increase contrast has often been applied in combination with bright field microscopy. Single and multiple stain methods were used, varying among protocols and being species-specific. Some stains however lead to a loss of the cell membrane, thereby influencing the measured head size itself. Phase contrast microscopy is an alternative, because here an analysis is possible without staining. A recent alternative is also offered by fluorescence microscopy, which has less error potential as a fluorescence probe binds specifically to DNA (Yániz et al. 2015). The analysis can either be performed with the software of the CASA device, or with free available alternatives as the software ImageJ (Firman and Simmons 2010a). In some publications a commercial CASA system for motility assessment and the morphological analysis with a software for microscopic image recording is combined (Gómez Montoto et al. 2011, Varea-Sánchez et al. 2016). A limiting factor is again that the different methods and specimen preparation techniques from each laboratory make a comparison difficult or even impossible (Verstegen et al. 2001).

I. Measurable sperm morphometric parameters

Five basic variables have been used to quantify the sperm morphology: total sperm length, head width, head length, midpiece length and flagellum length. The presence of a cytoplasmic drop has been evaluated for specific questions. (Firman and Simmons 2010b, Raspa et al. 2018). These parameters possess different applications and diagnostic values, as it could be

shown for example that sperm midpiece length predicts sperm swimming velocity in mice (Firman and Simmons 2010b).

The schematic structure of a mouse spermatozoon is shown in Fig. 2.



head	flagellum		
acrosome and nucleus	midpiece	principal piece	end piece

Figure 2: Schematic illustration of a mouse spermatozoon (Buffone et al. 2012)

Most morphometric studies focus on the sperm head and occasionally the midpiece, due to the technical difficulties to visualise and analyse the flagellum (Yániz et al. 2015). The evaluation included the measurement of the area and the perimeter of the sperm head, the nucleus and acrosome on its own, as well as various resulting shape calculations (Amann and Waberski 2014, Yániz et al. 2015). Most commonly the morphologic analysis is performed in brightfield with an objective magnification of 600x–1000x (van der Horst et al. 2018b, Varea-Sánchez et al. 2016). For meaningful data that can be processed further, a classification into subpopulations is favourable, like in motility assessment (Maroto-Morales et al. 2016).

In most rodents the sperm head has a characteristic apical extension, the so-called sperm hook. This morphological singularity makes analysis difficult, but also offers the possibility of measuring different angles (Varea-Sánchez et al. 2016) and further variables like the total curve shaped head length (Arc) (Mortimer et al. 2015). The so-called RatTox Module of the Sperm Class Analyzer is overcoming this speciality and has been used to distinguish sperm morphometric characteristics of Sprague-Dawley and Wistar rats, including eight different parameters of the sperm head and four of the sperm midpiece. They developed cut-off values for evaluating the percentage of normal sperm in these two rat strains using the automatic analysis mode (van der Horst et al. 2018a). Similar studies have not been performed for the mouse yet.

II. Diagnostic value of morphometric measurements

In species with high sperm competition, as free-living mice, the percentage of abnormal sperm shapes is minimal (van der Horst et al. 2018b). Polygamy does not lead to longer, but faster sperm, at least short-termed. This finding reveals that selection first influences motility and that effects on morphology need longer in evolutionary length of time (Firman and Simmons 2010b). Nevertheless, Varea-Sánchez et al. (2016) found a direct proportionality between hook length and relative testicular weight and detected a positive correlation between swimming velocity and degree of retraction of the hook. These results were obtained with a study design including many different species within the family of muroid rodents and also led to the realisation that a high competitive pressure leads to a smaller range of variation of the hook length (Varea-Sánchez et al. 2016). An increase in the mutation rate is most likely caused by changed conditions, resulting in a defect of the sperm (Tablado et al. 1998). With high inbreeding coefficients in laboratory settings and the production of inbred strains, an increase in morphologically altered spermatozoa is associated (Verstegen et al. 2001).

Being an issue in motility evaluation, also in morphological analysis the used software and the statistical method as well as sample preparation and used optical system are variables that make comparison difficult or have to be taken into account (Maroto-Morales et al. 2016, Yániz et al. 2015). Therefore, the PCA (explained in chapter 4.a.) is also used for morphological analysis in order to simplify the exposition of large amounts of data (Varea-Sánchez et al. 2016, Yániz et al. 2015). For more extensive studies, kinematic and morphological data should be used complementary that more reliable statements about sperm function can be made (van der Horst et al. 2018b), since it has been shown that including only morphometric data is not sufficient to evaluate fertilisation potential and results sometimes are contrary (Maroto-Morales et al. 2016).

c. Important findings using CASA in mice

The chapter summarises the key results of CASA studies examining mouse sperm, comprising motility and morphology studies, listing them in tabular form.

Table 8: Main findings of mouse CASA studies

Reference	**Fundamental result of the study**
(Bertoldo et al. 2014)	Sperm motility after thawing was three to six times lower when compared to fresh samples. This adverse effect could be reduced if metformin was added to the cryopreservation medium, which also operated beneficial when IVF was performed.
(Bray et al. 2005)	Mice with a knockout in the CHRNA7 subunit of the nicotinic acetylcholine receptor showed lower values of the percentage of progressive and hyperactivated sperms, as well as VAP, VSL, VCL and ALH in comparison to wildtype mice.
(Cheng et al. 2007)	Sperm from Taf7l $^{-/Y}$ mice showed significantly lower total motility and lower VAP, VSL and VCL values in comparison to wildtype mice. BCF in contrast was higher in the mutant mice.
(Dardmeh et al. 2017)	The oral administration of the probiotic *Lactobacillus rhamnosus PB01 (DSM 14870)* showed a stabilising effect on consistent body weight. Obese mice showed poorer results in sperm kinematic analysis when compared to normal weight animals. After four weeks of probiotic supplementation there was a significant increase in VCL, VSL, VAP and ALH in the sperm of the obese mice.
(Elangovan et al. 2006)	Cyclophosphamide treatment in male mice caused decrease in body and testes weight, sperm count and motility, testosterone and luteinising hormone level, as well as histological chances in the testes. The extent of the change was growing with increasing dosage and when treatment stopped regeneration was detected. Irreversible effects as well as infertility only occur with high dosage treatments (200 mg/kg of body weight).
(Goodson et al. 2011)	Although all three are inbred strains, mice from the 129S1/SvImJ and PWK/PhJ strain showed primary more

Reference	Fundamental result of the study
	energetic sperm than animals from the C57BL/6J strain. Incubation of 90 min caused an increase in slow sperms, greater in BL6 and 129 mice. These two lines also showed significant lower values of hyperactivated cells.
(Grover et al. 2005)	FSH-receptor knockout mice showed decreased total sperm count and lower values of VAP, VSL, VCL and ALH compared to wild type mice. Further, the proportion of morphologically abnormal sperm was increased to 20–30 % in the knockout mice, showing that the mutation also has an impact on morphological characteristics.
(Grzmil et al. 2007)	Sperm of mutant males with partial deletion on the long arm of the Y chromosome (B10.BR-Y^{del}) showed reduced velocity values throughout all measured parameters (VAP, VCL, VSL, ALH, BCF, LIN) in comparison to the congenic control strain B10. The interrelationships of these results due to a head shape variation must be further investigated.
(Harris et al. 2007)	Homozygous Ste5Jcs1 male mice (an infertility ENU mutant) only showed low swimming velocities and no hyperactivation. In comparison to heterozygous mice the total number of motile sperms was decreased.
(Khatun et al. 2018)	Inhibition of aminopeptidase N with 100 μM leuhistin in adult male mice led to an increased total sperm motility, but did not change velocity parameters (VAP, VSL, VCL) or the ability for capacitation. The finding was protein kinase A dependent and on the other hand increased the level of reactive oxygen species, which negatively affected early embryonic development.
(Miki et al. 2004)	Male Glyceraldehyde 3-phosphate dehydrogenase-S (GAPDS) knockout mice hardly showed progressive motility. Only weak movements increased their incidence during 4h of incubation. All CASA parameters had clearly lower values in comparison with wildtype mice.

Reference	Fundamental result of the study
(Nayernia et al. 2002)	Sperm from Smcp$^{-/-}$ mice showed significant lower velocity values in the CASA analysis, except of LIN. Notably, Smcp-deficient male mice on a mixed genetic background (C57BL/6J x 129/Sv) were able to reproduce naturally, whereas Smcp-deficient male mice on a pure 129/Sv genetic background did not show productive mating at all. When zona-free oocytes were inseminated artificially, sperm from both genetic backgrounds was able to fertilise the eggs.
(Nayernia et al. 2003)	Two triple knockout lines (Tnp-2$^{-/-}$/Acr$^{-/-}$/H1.1$^{-/-}$ and Tnp-2$^{-/-}$ /Acr$^{-/-}$/H1t$^{-/-}$) did not show any significant differences in sperm quantity, motility or fertilising ability when compared with wildtype mice. However, it is conjectured that it makes a difference when it comes to direct competition between spermatozoa with or without the null mutation.
(Odet et al. 2008)	Lactate Dehydrogenase C knockout (Ldhc$^{-/-}$) male mice showed normal sperm morphology and initial motility, but after 1.5 h of incubation there was a significant difference when compared to wildtype mice and no signs for hyperactivity could be detected. Additional this knockout led to male infertility.
(Pitetti et al. 2013)	Mice lacking the insulin and IGF1 receptors showed drastically reduced testes weight (72.4 %), resulting in a decreased number of Sertoli cells and produced sperms when compared to wildtype mice. No effects on sperm viability, motility or on fertility of the mutant mice have been detected.
(Raspa et al. 2018)	Two-week oral administration of D-aspartate decreased the number of morphological abnormal sperm cells.
(Shang et al. 2016)	Epididymal sperm from Atg7$^{-/-}$ knockout mice showed morphological abnormalities, such as bent heads or coiled tails and had a decreased motility rate of only 15.67 %,

Reference	Fundamental result of the study
	whereas the control wild type group showed motility values of about 83.5 %.
(Shao et al. 2008)	The absence of the Junctional Adhesion Molecule A (Jam-A) affected total, progressive and hyperactivated motility to the detriment of the knockout animals. This led to reduced litter size and fertility for the affected mice.
(Stenz et al. 2017)	Prenatal exposure of bis(2-ethylhexyl)phthalate (DEHP) during testicular differentiation (embryonic day 9–19) led to decreased sperm concentration and VCL and ALH values in C57BL/6J adult male mice and might therefore be an appropriate model for the human testicular dysgenesis syndrome.
(Sztein et al. 2000)	This study investigated the effects of cryopreservation on the fertility rate of different inbred and hybrid mouse strains. There was no significant difference in the mean sperm concentration from all strains. After freezing and thawing a decrease in motility was detected, more serious in the inbred strains and also the post-thawed fertilisation rate dropped significantly, while the hybrid strain sperm showed similar results.
(Takeo et al. 2014)	Sperm motility of C57BL/6J male mice decreased indirectly proportional to the duration of the cold storage of the epididymides, significant differences were only noticed after a period of 72h.
(Vosoughi et al. 2013)	Short-term analysis after exposure to formaldehyde vapor caused a decrease in progressive motility and sperm viability only if a concentration of 20 ppm was reached (exposure 8h/day for ten consecutive days). In long-term analysis (mice were killed 35 days after exposure) there was a significant decrease in sperm number, velocity parameters and an increase in morphological abnormalities in the low and the high concentration group (10 resp. 20 ppm).

Reference	Fundamental result of the study
(Wang et al. 2007)	In contrast to wildtype mice, Aldh2 $^{-/-}$ knockout mice did not show a dose-dependent decrease in total and progressive motility of sperm after administration of ethylene glycol monoethyl ether (EGEE) for seven days. This demonstrates the effect of Aldh2 activity on the toxic effect of EGEE in sperm cells and its importance as a major metabolising enzyme of the ethoxyacetic aldehydes.

7. Discussion

Spermatozoa swim in a multivariate pattern, making a detailed analysis difficult and complex. Consequently, a precisely performed qualitative and quantitative measurement is obligatory to study these motile cells. Computer assisted sperm analysis led to the discovery and understanding of so far unidentified variables and influencing factors. More detailed information about male germ cells could be achieved, which is important for the improvement of the automated analysis of spermatozoa. By now it is common knowledge that the mere determination of a mean value or median is not enough for a valid and significant scientific evaluation. For the subdivision of the total sperm population of a sample into subpopulations, defined cut-off values need to be determined. Ideally this is performed separately for each laborator as there is a list of varying and influencing factors. Ranking among the important ones are: optic system, degree of dilution and used medium, chamber type, incubation period or time interval between sampling and measuring, image acquisition rate, magnification, number of cells and field examined and so on. This should clarify and make aware of the impact of the whole sample processing, as most of the steps are carried out prior to the actual analysis itself. In order to enable comparison, a number of authors postulate it is important to give every detail of the setting (Amann and Waberski 2014, Verstegen et al. 2001).

Even the most precise instrument always leads to a certain degree of measurement inaccuracy, as each spermatozoon's flagellum shows slight differences in movement when compared to others. Yet, only one smoothing algorithm is applied for the whole sample. This undeterrable limitation needs to be minimised, facilitated by the use of adaptive smoothing (Davis and Siemers 1995). Moreover, each software is programmed differently and therefore reconstructing the sperm trajectory individually, which can lead to diverging kinematic values if the same sample is analysed with two CASA devices. The differentiation between fresh and thawed sperm samples is an important factor, as the cryopreservation process influences the motility performance of sperm cells among other factors by the medium used.

Advanced and higher sophisticated classification by using fractal dimensions for hyperactive sperm characterisation leads to more precise results, but is not routinely used yet. The same issue appears with cut-off values: Only a thoughtful selection lead to the desired success of clear assignment for every single sperm cell. Here a comparative study would help to determine the extent of the differences more precisely.

Considering sperm collection in mice, there is clearly one main approach, namely the sacrification of the animal. The other methods mentioned (e.g. percutaneous sperm aspiration or electroejaculation) bring the advantages of being repeatable and not fatal for the mouse. This would go hand in hand with the implementation of the 3Rs, which must be aspired to by every scientist. On the contrary, a sacrification is not considered as a burden and therefore does not count as an animal experiment. Thus it falls to the scientist's responsibility to balance this fact against the repeated use and the accompanying higher burden of the same animal.

Advancing maturation and enhancing sperm motility with pharmacologically active substances holds the potential to increase IVF success and decrease the number of animals further. This is from even more significance when performed in younger mice (7–8 weeks old) as Raspa et al. (2018) showed, because fewer animals are needed and only for a shorter time period, bringing advantages in terms of animal reduction and economy. The investigation of drug therapies on germ cells is an essential tool of CASA to demonstrate toxic effects and resulting reduced fertility. The gained knowledge is benefiting humans and genetically modified mouse lines, as they often suffer from poor sperm motility.

In addition, it is noticeable that motility or morphology are usually only a part of the investigations in a study, combined with e.g. immunohistochemistry, western blot or histology. Depending on the respective value of the individual methods, this can be a possible reason that data are not always given in detail. Especially morphology is rarely considered solely when CASA is used, whereas Tablado et al. (1998) focuses exclusively on this characteristic. Some authors, such as Mortimer et al. (2015) claim that for detailed analysis an improvement in morphometric measurements needs to be implemented in order to obtain highly reliable results. Modern methods and techniques enable to create multiple knock out mouse lines and thus allow to evaluate synergistic effects of mutations, which is likely to lead to more credible and better comparable models for complex syndromes.

The field of application for CASA devices ranges from human fertility and IVF clinics, reproduction centres for livestock to laboratory settings, which shows that the capability is versatile and broad. All these areas require an individual setup and software as the intended outcome is different. Therefore, it is understandable and correct that different devices are available. The costs and the range of features for the equipment vary greatly, and therefore each institute should consider carefully before a decision is made.

In order to ensure correct application, one must also be aware of the limitations and difficulties: One disadvantage is the fact that researchers tend to not validate their data within their own laboratory and setting and seem to have “blind faith” in the computer. But even in automated systems, quality control is indispensable to guarantee precision and accuracy. Conflict of interest can be an obstacle to progress, and therefore every researcher should reflect upon further utility or use for improving existing protocols to ensure that his findings contribute to the continuous advancement and knowledge gain of science. Still, declarations for the choice of a particular medium or the reason why default settings are adjusted are rarely given. Computer-assisted sperm analysis is reducing bias in analysing cells, yet it is not a universal solution. The expertise and skills of the user have a significant effect on the outcome, what makes it utmost important to bring in the necessary competence and high standard of knowledge to ensure precise and accurate analyses and to achieve repeatable and reliable results at the same time.

For high quality and justified research, an obligation to provide detailed information on hardware and software settings as well as the validation method would help to increase comparability and standardisation. When authors write several papers on one topic, the procedure descriptions are often kept short and only refer to previous papers. It is also striking that many times one scientific work is cited as reference in different publications repeatedly. In addition, general information such as housing conditions (light program, type of food, individual or group housing, cage type) is poorly mentioned. There are also great differences in the aspect of how detailed the results are presented: Some authors write them down meticulously, whereas others only show a graph or just present significant findings. Of course, it is not always possible to provide particularised data in the paper, but they can be added as supplementary data to allow interested readers to get to know these details, as Miki et al. (2004) implements it. However, it also depends on how data are interpreted, so the reader should always be aware of the various influencing factors in the process of a computer-assisted sperm analysis as many authors point out (e.g. Verstegen et al. 2001; Schleh and Leoni 2013).

In future, CASA will continue to play an important role in the area of reproduction and related research, because clinical studies are only performed after preclinical trials have preceded and dead ends are sorted out. The often-used argument that merely research within one species provides useful data is not tenable, because only preclinical *in vivo* studies allow to test the possible toxicity of certain substances or advantageous effects of a new fertility treatment.

Concluding, computer-assisted sperm analysis is a valuable and indispensable method for generating objective and accurate data. It still should not be disregarded that validation and quality control must be performed to minimise procedural artefacts and to ensure precision and reproducibility that is essential in contemporary and high-level research.

8. Summary

a. English

The intention to obtain increasingly detailed information about a sperm sample led to the development of various computer-assisted sperm analysis (CASA) methods, being differently suitable for a variety of species. In mice, the hook-shaped acrosome at the sperm head represents a characteristic and a challenge primarily for the morphologic analysis, which has not yet been completely overcome. So far, a lot of knowledge has been gained by technical progress and a vast number of experiments which were conducted in mice and led to a more profound knowledge in a sperm cell's physiology and lifespan. If influencing methodological factors are considered and minimised, it is possible to make predictions about fertility, to study the influence of mutations and of toxic substances on sperm quality. In order to achieve the greatest possible gain of knowledge from an experiment, it is important to provide precise data and metadata (e.g. analysis conditions and setup parameters). The choice of CASA device as well as method of sperm collection depends on the intended purpose (e.g. cryopreservation, genetic analysis or IVF), the expertise and facilities of the research team. Although much more detailed results can be obtained by an evaluation based on subclasses within the sperm population, it is still important to carry out quality controls even with this objective measuring method in order to guarantee precision and accuracy and to contribute to the continuous development and gain of knowledge in science. To this end, it is important to state reasons for decisions and to provide itemised information about the data obtained, since the best possible comparison can only be achieved by taking all factors into account.

b. German

Die Absicht, immer detailliertere Informationen über eine Spermienprobe zu erhalten, führte zur Entwicklung verschiedener Methoden der computergestützten Spermienanalyse, welche für unterschiedliche Spezies verschieden gut geeignet sind. Bei Mäusen stellt das hakenförmige Akrosom am Kopf des Spermiums ein Charakteristikum und eine Herausforderung v.a. bei der morphologischen Analyse dar, die noch nicht vollständig bewältigt ist. Durch den technischen Fortschritt und eine Vielzahl von Experimenten an Mäusen konnte bereits umfassendes Wissen über die Physiologie und Lebensdauer von Spermien gewonnen werden. Das Berücksichtigen und Minimieren aller methodischer Einflussfaktoren ermöglicht es, Vorhersagen über die Fruchtbarkeit einer Spermienprobe zu

treffen und z.B. den Einfluss von Mutationen und toxischen Substanzen auf die Spermaqualität zu untersuchen. Um den größtmöglichen Erkenntnisgewinn aus einem Experiment zu erzielen, ist es wichtig, detaillierte Daten und Metadaten (z.B. Analysebedingungen und Set-up-Parameter) zu liefern. Die Wahl des CASA-Gerätes sowie der Methode der Samengewinnung hängen vom Verwendungszweck (z.B. Kryokonservierung, genetische Analyse oder IVF), dem Fachwissen und der Ausstattung des Forschungsteams ab. Obwohl durch eine Auswertung auf Basis von Unterklassen innerhalb der Spermienpopulation wesentlich genauere Ergebnisse erzielt werden können, ist es auch bei dieser objektiven Messmethode wichtig, Qualitätskontrollen durchzuführen, um Präzision und Genauigkeit zu gewährleisten und zur kontinuierlichen Weiterentwicklung und zum zusätzlichen Erkenntnisgewinn der Wissenschaft beizutragen. Dazu ist es notwendig, Entscheidungen zu begründen und umfassende Informationen über die gewonnenen Daten zu liefern, da nur unter Berücksichtigung aller Faktoren ein bestmöglicher Vergleich erzielt werden kann.

9. List of abbreviations

ALH:	amplitude of lateral head displacement
ANOVA:	analysis of variance
ASMA:	automated sperm morphology analysis
ARC:	total curve shaped head length
ATP:	adenosine triphosphate
BCF:	beat cross frequency
BSA:	bovine serum albumin
CASA:	computer-assisted sperm analysis/computer-aided sperm analysis
CPA:	cryoprotectant agent
ENU:	Ethyl Nitroso Urea (or N-ethyl-N-nitrosourea)
HTF:	human tubal fluid
Hz:	hertz (frames per second)
ICSI:	intracytoplasmic sperm injection
IVF:	*in vitro* fertilisation
IVOS:	Integrated Visual Optical System
LIN:	linearity
MAD:	mean angular displacement
MESA:	microsurgical epididymal sperm aspiration
PBS:	phosphate-buffered saline
PCA:	principal component analysis
PESA:	percutaneous epididymal sperm aspiration
ROC:	receiver operating characteristics
SCA:	Sperm Class Analyzer
SMQ:	Sperm Motility Quantifier
STR:	straightness
SVM:	support vector machine
VAP:	average path velocity
VCL:	curvilinear velocity
VSL:	straight line velocity
WOB:	wobble
WT:	wild type

10. List of tables

11. List of figures

12. References

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