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Current perspectives of CASA applications in diverse mammalian spermatozoa

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Abstract. Since the advent of computer-aided sperm analysis (CASA) some four decades ago, advances in computer technology and software algorithms have helped establish it as a research and diagnostic instrument for the analysis of spermatozoa. Despite mammalian spermatozoa being the most diverse cell type known, CASA is a great tool that has the capacity to provide rapid, reliable and objective quantitative assessment of sperm quality. This paper provides contemporary research findings illustrating the scientific and commercial applications of CASA and its ability to evaluate diverse mammalian spermatozoa (human, primates, rodents, domestic mammals, wildlife species) at both structural and functional levels. The potential of CASA to quantitatively measure essential aspects related to sperm subpopulations, hyperactivation, morphology and morphometry is also demonstrated. Furthermore, applications of CASA are provided for improved mammalian sperm quality assessment, evaluation of sperm functionality and the effect of different chemical substances or pathologies on sperm fertilising ability. It is clear that CASA has evolved significantly and is currently superior to many manual techniques in the research and clinical setting.

Additional keywords: domestic mammals, mammals, morphology, motility, primates, rodents, wildlife.

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Introduction

The development of computer-aided sperm analysis (CASA) during the 1980s sparked great excitement, although the systems were predominantly little more than black boxes able to track sperm motion, with hardly any standardisation and verification of data generated (Mortimer *et al.* 2015). In the 1990s and early 2000s, advances in computer technology resulted in considerable improvements in CASA systems. During this time, the value of CASA, particularly in research applications, came to the forefront. However, its application in the clinical setting was largely distrusted, particularly for assessment of human sperm concentration and morphology. Nevertheless, CASA has advanced significantly during the past two decades due to major innovations in science and bioengineering, as well as development in software algorithms and increased computational power. This pioneering effort between industry and science helped establish CASA as a reliable and objective research and diagnostic instrument in the medical, veterinary, laboratory animal and wildlife fields (Holt *et al.* 2007). Over the past 30 years, the number of publications related to the use of CASA in human and animal sperm analysis (indexed in PubMed using the combined search terms 'CASA' and 'Sperm') increased dramatically, as shown in Fig. 1.

CASA systems have become very user friendly and are able to produce vast amounts of data in a short period of time. However, these seeming benefits are also a cause for concern, because the correct methodology, quality control and data interpretation have been largely neglected and accordingly distorted (Amann and Waberski 2014). Furthermore, CASA is no longer just used for standard sperm concentration, motility (percentages and kinematics) and morphology analysis, but is also rapidly evolving to become an indispensable tool to objectively assess numerous facets related to sperm functionality.

The latest generation of CASA systems incorporates fully quantitative modules to routinely measure sperm DNA fragmentation, vitality, acrosome intactness and reaction and hypo-osmotic swelling (semiquantitatively). More functional motility aspects, such as sperm mucous penetration and hyperactivation, can now also be routinely evaluated. New approaches to CASA vitality measurement include the use of a ×20 objective to rapidly analyse a large number of spermatozoa using either fluorescence or brightfield microscopy in combination with

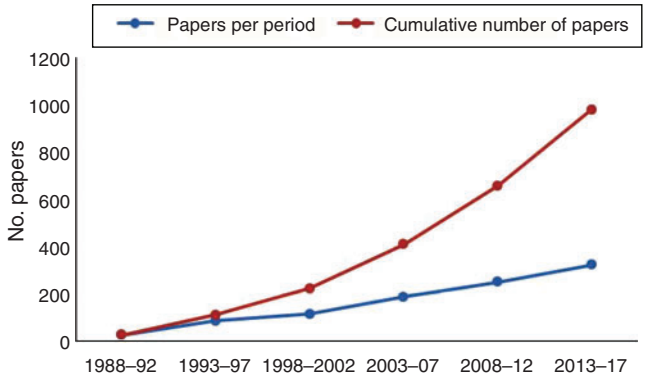

Fig. 1. Increased computer-aided sperm analysis (CASA)-related publications per 5-year period and cumulative number of publications between 1988 and 2017 as extracted from PubMed (October 2017) using the search terms 'CASA' and 'Sperm' in combination.

staining kits such as FluoVit and BrightVit (www.micropticsl.com, accessed 30 October 2017) respectively.

The aim of the present paper is to illustrate how CASA encompasses the diversity in mammalian spermatozoa at both structural and functional levels, as well as the importance of selecting species-specific analysis properties. Concomitantly, the potential of CASA to quantitatively measure essential aspects related to sperm subpopulations, hyperactivation, morphology and morphometry, which are not possible with manual analysis, is also demonstrated. Finally, CASA applications are showcased that cover improved mammalian sperm quality assessment, evaluation of sperm functionality and the effects of different chemical substances or pathologies on sperm fertilising ability in both research and clinical settings.

Materials and methods

Semen collection

In all instances, semen samples were collected according to ethical guidelines after institutional approval was received and informed consent was provided (humans).

Human and non-human primates

Human semen samples were obtained from either volunteers as part of a donor program in the Division of Medical Physiology, Faculty of Medicine and Health Sciences, Stellenbosch University, or from patients attending the andrology clinic at Tygerberg Hospital (South Africa). All samples were freshly collected according to World Health Organization (WHO 2010) guidelines and reached the research laboratories within 30 min of collection for further analysis. Semen samples from baboon (*Papio ursinus*), rhesus monkey (*Macaca mulatta*) and vervet monkey (*Chlorocebus aethiops*) were obtained from sedated animals via electroejaculation, as described by Maree (2011) and de Villiers (2014).

Laboratory rodents

Spermatozoa were retrieved from murine (Wistar and Sprague-Dawley rats (*Rattus norvegicus*) and C57 strain mice (*Mus musculus*)) and non-murine (naked mole-rat (*Heterocephalus glaber*)) animals postmortem after the animals had been humanely killed with a lethal dose of sodium pentobarbital, halothane or cervical dislocation. The testis and epididymis were subsequently removed and spermatozoa were harvested from the proximal cauda epididymis using a sperm isolation procedure as described by Maree (2011) for mouse and naked mole-rat and by Oyeyipo *et al.* (2018) for rat.

Domestic mammals

Semen samples from merino rams (ovine; *Ovis aries*), Kolbroek boars (porcine; *Sus scrofa*) and Arabian stallions (equine; *Equus caballus*) were collected using artificial vagina procedures. Electroejaculation by trained veterinary staff was used to obtain semen samples from Tankwa goat rams (caprine; *Capra hircus*) and Nguni bulls (bovine; *Bos taurus*).

Wildlife mammalian species

Semen samples were obtained from a wide range of wildlife species, including African elephant (*Loxodonta africana*; Luther 2016; Lueders *et al.* 2017), white rhinoceros (*Ceratotherium simum*; Luther 2016), mhorr gazelle (*Nanger dama*), rock hyrax (*Procavia capensis*), bottlenose dolphin (*Tursiops truncates*) and common warthog (*Phacochoerus africanus*) through electroejaculation, manual stimulation or epididymal retrieval. Semen from carnivores, including lion (*Panthera leo*) and wild dog (*Lycaon pictus*), were obtained by urethral catheterisation as described by Lueders *et al.* (2012).

Media and chemicals used

Semen samples were diluted to ideally contain a sperm concentration of 15–30 $\times$ 10^6 mL^{-1} in order to study sperm motility and morphology using CASA-Mot and CASA-Morph, respectively. Human tubal fluid (HTF) (Mortimer 1994) and Ham's F-10 (Merck) were routinely used to wash, incubate and obtain baseline human sperm motility information. For other mammals, Ham's F10 was also the medium of choice for most species, whereas BO medium (Brackett and Oliphant 1975) was used for bull spermatozoa. All media and chemicals used in treatment experiments were of analytical grade and purchased from Sigma-Aldrich.

Motility and hyperactivation

In all instances, CASA was performed with Sperm Class Analyzer (SCA) software versions 4.1, 5.1, 5.4, 6.1 and 6.2 (Microptic). Particle size and other important parameters were set for each species according to Table 1. Hardware configurations (cameras and microscopes) varied slightly between laboratory and fieldwork setups. Four different Basler cameras (A602fc, A312fc, SCA1024 and acA1300-200um) were used at frame rates between 50 and 100 frames per s (f.p.s.). Cameras were mounted on either a Nikon E50i, Nikon E200 or Olympus CH2 microscope ($\times$10 positive phase for human sperm and $\times$10 negative phase for all other species) equipped with a heating stage (37°C). Leja slides (Leja Products) with a chamber depth of 20 μm were used to standardise motility analyses. All media and consumables, including pipette tips and slides, were preheated to 37°C before analysis.

Table 1. Various parameter settings for the Sperm Class Analyzer (SCA®) computer-aided sperm analysis (CASA) system used in this investigation to study a wide range of mammals

VCL, curvilinear velocity; VAP, average path velocity; STR, straightness; f.p.s., frames per second

	Particle area (μm^2)	VCL ($\mu m\ s^{-1}$)			Progressiveness (% of STR)	Connectivity	VAP points	
		Slow	Medium	Rapid			50 f.p.s.	100 f.p.s.
Primates								
Human	>2, <80	>23, <60	>60, <89	>89	>80	12	7	9
Baboon	>20, <80	>64, <185	>185, <296	>296	>80	20	7	11
Rhesus monkey	>20, <80	>81, <170	>170, <240	>240	>80	20	7	9
Vervet monkey	>10, <90	>64, <164	>164, <280	>280	>80	12	7	11
Rodents								
Rat	>500, <1600	>50, <80	>80, <150	>150	>70	60–80	9	11
Mouse	>20, <70	>50, <146	>146, <242	>242	>70	18	9	11
Domestic mammals								
Ram	>1, <100	>54, <105	>105, <175	>175	>80	14	9	11
Goat	>3, <70	>25, <80	>80, <180	>180	>80	14	7	9
Bull	>10, <200	>25, <80	>80, <150	>150	>70	12	9	11
Boar	>10, <200	>40, <80	>80, <120	>120	>75	16	9	11
Horse	>10, <150	>25, <50	>50, <120	>120	>70	14	7	9
African wildlife								
Lion	>5, <80	>25, <65	>65, <100	>100	>75	14	7	9
Wild dog	>5, <80	>25, <65	>65, <100	>100	>75	14	7	9
African elephant	>15, <80	>10, <65	>65, <120	>120	>70	14	7	9
White rhinoceros	>4, <75	>10, <65	>65, <120	>120	>75	12	5	7
Mhorr gazelle	>15, <120	>25, <50	>50, <100	>100	>75	12	7	9
Rock hyrax	>5, <80	>25, <65	>65, <100	>100	>75	14	7	9
Bottlenose dolphin	>1, <60	>60, <80	>80, <120	>120	>80	14	7	9
Warthog	>1, <100	>25, <50	>50, <100	>100	>75	12	7	9

Hyperactivation was induced and quantified in several of the mentioned species using caffeine (5–10 mM), procaine hydrochloride (2–5 mM) or progesterone ($19\ \mu g\ L^{-1}$). For human, previously published CASA-Mot cut-off values for hyperactive motility were used (Mortimer 1997; Mortimer and Mortimer 2013). In several other mammalian species studied (vervet monkey, ram, bull, elephant and rhinoceros), kinematic parameter cut-off points for hyperactivation were determined using receiver operating characteristic (ROC) curve analysis. Those parameters with the best cut-off points in terms of specificity and sensitivity were combined in a Boolean argument and incorporated as a sort function in SCA. In most of these hyperactivation studies, a new flush technique was implemented as described by Ntanjana (2015). This technique involves inserting a small volume (1–2 µL) of semen in a Leja chamber and then displacing it towards the distal end of the slide with the hyperactivation medium. Spermatozoa would subsequently swim into the hyperactivation medium. This technique showed a high positive correlation with routine swim-up preparations and appeared to accelerate the process of hyperactivation. In most instances, hyperactivation could be quantified within 30–45 min.

Morphology and morphometry

Smears were prepared from either neat or diluted semen from the various species and stained with SpermBlue (Microptic) according to the manufacturer's instructions and a technique described by van der Horst and Maree (2010). This stain has been shown to be highly compatible with CASA-Morph across all species. The same SCA software, cameras and microscopes (×60 or ×100 brightfield) were used, as described previously, to perform detailed automated sperm morphology and morphometry analyses.

Statistical analysis

The appropriate statistical analysis was performed using unpaired Student *t*-tests or Mann–Whitney tests depending on whether variables exhibited Gaussian distribution, determined using the D'Agostino–Pearson normality (omnibus K2) test. All tests were two-tailed and analysis was performed using GraphPad Prism version 5 or MedCalc version 12.3.0 (Mariakerke). Results are expressed as either the mean ± s.d. or mean ± s.e.m., with results considered to be significant at $P < 0.05$. Qualitative multivariate analyses were performed using Statgraphics version XVII (Centurion), where grouped quantitative variables were visually represented using star glyphs (Fienberg 1979) to identify differences and similarities among animal species.

Results and discussion

Sperm motility

Motility is one of the most important characteristics associated with the fertilising ability of spermatozoa, thus the examination

of this parameter constitutes an integral part of semen analysis. Variations of between 30% and 60% have been reported in the subjective optical microscopic estimation of motility parameters in the same ejaculates of both humans and animals (Verstegen *et al.* 2002). CASA-Mot allows for the accurate and objective assessment and classification of sperm motility from various species and given populations with great repeatability (Fréour *et al.* 2010).

Apart from simply measuring sperm motility, CASA-Mot can further offer additional insights into sperm movement through various kinematic and velocity parameters, some of which have been shown to be closely related to fertility prediction and pregnancy outcomes in humans (Shibahara *et al.* 2004) and animals (Nagy *et al.* 2015; Ahmed *et al.* 2017). It is important to note that numerous factors can affect sperm motility and kinematic results, and should thus be standardised, including sample type (e.g. neat vs washed specimens), sample processing (frozen vs thawed), media added (capacitation vs hyperactivation), analysis temperature and CASA-Mot settings.

This section on sperm motility will not focus purely on the diversity observed in sperm motility among species, but also allude to sperm functionality in terms of sperm subpopulations, hyperactivation and applications in the clinical setting with the assistance of CASA-Mot.

Diversity in mammalian sperm motility

Sperm concentration, percentage motility and kinematics for selected primates, rodents, domestic and wildlife mammalian species are given in Table 2. Data represent washed and diluted semen samples from a normospermic human population; for most of the other mammals listed in Table 2, data represent males with known fertility. This selection of species provides baseline information of CASA kinematics that is not easily available in this comprehensive format. Furthermore, Table 2 includes values for several rapid-, medium- and slow-swimming sperm subpopulations.

In species that are laboratory bred or bred for high fertility (domestic mammals), as well as in most wildlife species where there is a risk of sperm competition, sperm concentration is high and sperm exhibit much higher velocities (curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL)) compared with spermatozoa from humans and naked mole-rats (two species with a low risk of sperm competition). This has important implications in assessing both the quality and fertilising ability, particularly in mammals with lower levels of sperm competition (van der Horst and Maree 2014). It may be that essentially so-called ‘poor human sperm quality’ may still have good fertilising ability as an adaptation to the lack of sperm competition.

Multivariate visualisation, such as star symbol plot analysis (Fig. 2), can assist in the identification of distinct similarities or differences among the mammals included in Table 2. In Fig. 2 it is evident that rhesus monkey, ram, African elephant and bottlenose dolphin show similarities in motility patterns, whereas bull and lion can also be grouped together. Mouse seems to be dissimilar to all other species, with human and naked mole-rat showing the smallest features for most parameters.

Motility subpopulations

Mammalian ejaculates are known to contain several sperm subpopulations with differences in terms of their functional, structural and biochemical characteristics (Buffone *et al.* 2004). It is recommended that such subpopulations be evaluated rather than relying on mean values for the entire ejaculate, which oversimplifies the analysis and could mask the effect of drug treatment on spermatozoa (Abaigar *et al.* 1999; Martinez-Pastor *et al.* 2005; Núñez-Martínez *et al.* 2006). In a clinical setting, evaluation of rapid progressive motility (Grade ‘a’; Sifer *et al.* 2005; WHO 2010) and cervical mucous penetration capabilities of spermatozoa (Mortimer and Mortimer 2013) can assist the andrologist to determine whether an ejaculate contains enough functional spermatozoa to potentially reach and fertilise the oocyte (Mortimer *et al.* 2015).

Maree and van der Horst (2013) described a method of using species-specific swimming speed (VCL) cut-off values for six species (see Table 1) to identify three motility subpopulations (rapid-, medium- and slow-swimming spermatozoa) within the total motile sperm population (see Table 2). This CASA-based technique is not only used to evaluate a male’s semen quality, but it can also be used to assess the effect of any treatment, which will manifest as changes in the distribution of spermatozoa among the three swimming speed classes. For example, after exposing three fractions of human spermatozoa (obtained through density gradient centrifugation) to different caffeine concentrations, it was found that the most motile (bottom) fraction not only had the highest percentage rapid-swimming spermatozoa, but also that this subpopulation did not change with an increase in caffeine concentration (Fig. 3). Conversely, the less motile sperm fractions could be stimulated by caffeine to increase swimming speed, indicating a possible therapeutic intervention for IVF (Ntanjana 2015). When vervet monkey spermatozoa were exposed to gonadotropin-releasing hormone (GnRH) for 30 min *in vitro*, percentage total motility was significantly decreased at a GnRH-II concentration of 10^{-8} M. However, this inhibition of motility was also evident with two higher GnRH-II concentrations (10^{-6} and 10^{-7} M) when the percentage of rapid-swimming spermatozoa instead of percentage total motility was evaluated (de Villiers 2014). Thus, these results highlight the gradient effect of this peptide on sperm motility, which is probably closer to the exposure range in the female reproductive tract, rather than just a sudden decrease at a specific GnRH concentration as was found for total motility.

Numerous studies have applied more detailed multivariate pattern analysis, principal component analysis and clustering methods using CASA-derived parameters to demonstrate the existence of motile sperm subpopulations in several domestic and wildlife mammalian species including rabbit, pig, goat, horse, gazelle, red deer, dog and fox (Abaigar *et al.* 1999, 2001; Quintero-Moreno *et al.* 2003, 2004; 2007; Martinez-Pastor *et al.* 2005; Núñez-Martínez *et al.* 2006; Dorado *et al.* 2010; Soler *et al.* 2014). However, a well-grounded statistical model to determine the number of clusters (subpopulations) to be considered is not yet available for CASA data (Martínez-Pastor *et al.* 2011). Most of these studies considered variations in the frequencies of determined motile subpopulations to assess

Table 2. Sperm concentration, percentage motility and kinematic values of semen or sperm subpopulations for nine mammalian species

Data are given as the mean ± s.d. VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; ND, not determined

	Primates		Rodents		Domestic mammals		African wildlife		
	Human ($n = 10$)	Rhesus monkey ($n = 10$)	Mouse ($n = 10$)	Naked mole-rat ($n = 10$)	Ram ($n = 10$)	Bull ($n = 7$)	Lion ($n = 10$)	African elephant ($n = 21$)	Bottlenose dolphin ($n = 3$)
Concentration ($\times 10^6$ mL^{-1})	63.9 ± 18.6	193.6 ± 122.9	ND	43.0 ± 45.2	3846.1 ± 2773.0	2001.0 ± 1200.0	1940.0 ± 1610.0	868.0 ± 201.0	330.0 ± 101.3
Motility (%)	72.8 ± 15.9	69.2 ± 10.2	89.7 ± 8.5	7.3 ± 6.7	74.1 ± 10.7	81.0 ± 13.5	84.0 ± 20.53	80.0 ± 21.5	75.5 ± 8.4
VCL (μm s^{-1})									
Mean	118.8 ± 17.3	292.3 ± 19.2	293.3 ± 30.3	35.6 ± 6.7	246.6 ± 38.4	141.9 ± 39.3	143.0 ± 40.2	241.0 ± 58.5	276.0 ± 90.1
Rapid	131.4 ± 18.2	315.3 ± 20.4	320.7 ± 32.4	ND	231.3 ± 48.4	218.0 ± 35.6	172.0 ± 38.5	299.7 ± 41.2	295.0 ± 80
Medium	76.4 ± 18.6	215.6 ± 26.3	193.3 ± 41.1	ND	134.0 ± 40.5	121.0 ± 18.6	98.3 ± 20.6	124.5 ± 37.2	113.9 ± 31.2
Slow	33.4 ± 9.9	92.4 ± 32.3	80.1 ± 12.4	ND	66.9 ± 19.3	59.7 ± 8.3	56.0 ± 7.5	66.2 ± 15.4	79.3 ± 14.5
VSL (μm s^{-1})									
Mean	85.8 ± 13.7	217.6 ± 34.3	119.7 ± 12.0	16.4 ± 5.8	182.3 ± 38.6	76.0 ± 12.8	120.0 ± 27.4	172.8 ± 49.5	155.0 ± 60.3
Rapid	96.6 ± 15.4	233.5 ± 37.6	128.8 ± 14.0	ND	188.9 ± 28.4	113.0 ± 12.1	91.2 ± 14.8	261.0 ± 53.2	206.2 ± 65.1
VAP (%)									
Mean	92.9 ± 13.7	251.6 ± 27.9	147.4 ± 13.4	25.9 ± 5.8	208.3 ± 39.1	87.0 ± 6.3	114.0 ± 11.8	201.0 ± 54.5	217.0 ± 93.0
Rapid	103.6 ± 15.1	270.2 ± 30.1	158.1 ± 15.5	ND	211.9 ± 34.8	129.4 ± 13.1	120.1 ± 21.1	273.0 ± 44.3	217.6 ± 86.0
LIN (%)									
Mean	72.3 ± 5.6	74.2 ± 9.3	41.3 ± 6.2	44.8 ± 8.4	74.4 ± 12.0	55.0 ± 7.1	63.2 ± 8.2	66.9 ± 16.4	57.2 ± 22.3
Rapid	73.6 ± 6.2	73.9 ± 10.0	40.5 ± 6.0	ND	82.5 ± 5.1	52.4 ± 4.5	79.4 ± 5.9	87.5 ± 15.5	83.8 ± 6.5
STR (%)									
Mean	92.2 ± 2.6	86.1 ± 5.4	81.3 ± 4.1	61.7 ± 9.0	87.2 ± 5.1	87.0 ± 10.6	80.2 ± 11.0	85.7 ± 5.7	40.6 ± 27.0
Rapid	93.1 ± 2.7	86.0 ± 5.7	81.5 ± 4.1	ND	89.3 ± 1.3	87.0 ± 7.3	91.3 ± 15.2	95.36 ± 14.1	95.3 ± 20.6
WOB (%)									
Mean	78.3 ± 4.3	85.9 ± 5.8	50.6 ± 5.6	72.3 ± 4.1	84.8 ± 10.3	62.7 ± 4.8	76.0 ± 5.3	82.5 ± 5.7	78.3 ± 16.4
Rapid	78.9 ± 4.7	85.6 ± 6.3	49.6 ± 5.4	ND	92.4 ± 5.1	60 ± 4.3	86.5 ± 4.3	84.6 ± 7.2	90.3 ± 20.3
ALH (μm)									
Mean	2.4 ± 0.2	4.0 ± 0.4	9.1 ± 1.2	0.6 ± 0.4	3.6 ± 0.8	2.4 ± 0.6	1.8 ± 0.5	3.7 ± 1.0	4.1 ± 2.2
Rapid	2.5 ± 0.2	4.2 ± 0.6	9.8 ± 1.3	ND	3.8 ± 0.9	2.8 ± 0.6	1.2 ± 0.4	4.1 ± 2.3	2.9 ± 1.9
BCF (Hz)									
Mean	18.7 ± 1.7	17.3 ± 1.3	10.5 ± 1.4	6.6 ± 5.8	17.4 ± 2.9	41.6 ± 10.1	50.5 ± 9.4	20.7 ± 3.1	15.9 ± 7.5
Rapid	19.5 ± 1.8	17.7 ± 1.5	10.3 ± 1.4	ND	18.1 ± 3.2	48.2 ± 11.9	35.1 ± 12.8	18.3 ± 6.3	17.5 ± 8.9

differences among individual or repeated ejaculates (Vázquez *et al.* 2015; Kanno *et al.* 2017), possible fertility of ejaculates (Quintero-Moreno *et al.* 2007; García-Álvarez *et al.* 2014) and the effect of short-term storage and cryopreservation on sperm quality (Martinez-Pastor *et al.* 2005; Núñez-Martínez *et al.* 2006; Dorado *et al.* 2011).

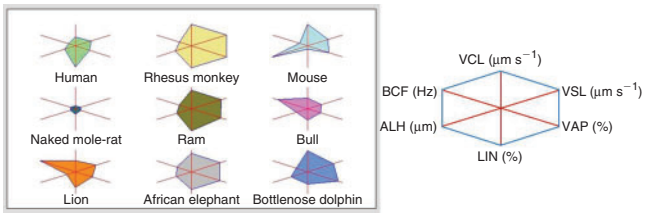

Fig. 2. Multivariate visualisations allow for recognition of similar kinematic patterns among species. The diagram on the left shows star glyphs including a selection of kinematic parameters for nine mammalian species. The diagram on the right is the key to kinematic parameters used to construct the star glyphs. VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

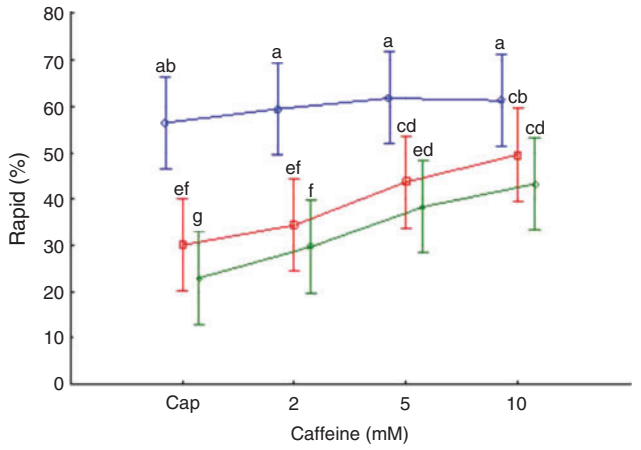

Fig. 3. Effect of capacitating medium (Cap) and increasing caffeine concentrations (2–10 mM) on the percentage of rapid-swimming spermatozoa in three human sperm fractions. Fractions were separated by 40%, 60% and 80% discontinuous PureSperm gradient: blue line, bottom fraction; red line, middle fraction; green line, top fraction. Data are the mean ± s.e.m. ($n = 10$). Different letters indicate significant differences among treatments as well as among different fractions ($P < 0.05$).

Hyperactivation

Hyperactivation can be induced *in vitro* by incubating spermatozoa under capacitating conditions (Suarez 2008), with or without the use of specific chemicals as agonists to open the cation channels of spermatozoa (CatSper channels), release internally stored Ca^{2+} or raise intracellular pH (Strünker *et al.* 2011; Alasmari *et al.* 2013). Ranges of hyperactivation for normospermic populations of selected mammalian species are presented in Table 3 after semen or spermatozoa were challenged with caffeine, procaine hydrochloride or progesterone. Although different hyperactivation patterns were evident for each species, Fig. 4 shows mostly typical starspin tracks in the vervet monkey, merino ram, white rhinoceros and African elephant. In all these species, hyperactivated sperm tracks were characterised by elevated VCL (>200 μm s^{-1}), decreased linearity (LIN; <30%) and elevated amplitude of lateral head displacement (ALH; >5.7 μm; 11.4 μm for other CASA-Mot systems, because SCA measures true ALH).

Burkman (1984) and Mortimer and Mortimer (1990) have shown that semen samples with 20% hyperactivation refer to human spermatozoa with fertilising ability. This figure seems to be a commonality among mammalian species, because hyperactivating stimulants also induced, on average, more than 20% hyperactivation in so-called normospermic samples (Table 3). In African elephant and white rhinoceros, lower hyperactivation levels (<20%) appear to be associated with non-breeding males or seasonal effects. Using four different agonists to induce hyperactivation, Alasmari *et al.* (2013) also reported that percentage hyperactivation was significantly lower in IVF patients (10–20%) compared with donor samples (15–30%).

The stimulants indicated in Table 3 induced hyperactivation in a species-specific manner, emphasising evident differences in their Ca^{2+} mobilisation pathways. For example, caffeine (5–10 mM) was a potent stimulator of hyperactivation in human, bull, rhinoceros and elephant spermatozoa, but not in ram and goat spermatozoa. However, procaine hydrochloride (2–5 mM) induced hyperactivation in human, ram and goat spermatozoa. Previous studies on ram (Colás *et al.* 2010), bull (Marquez and Suarez 2004) and stallion (McPartlin *et al.* 2009) spermatozoa, using caffeine and procaine respectively, reported that hyperactivation is probably induced by increasing the permeability of

Table 3. Hyperactivation in response to various stimulants in six mammalian species
Unless indicated otherwise, data are given as the mean ± s.d.

Parameters	Primates		Domestic mammals		African wildlife	
	Human ($n = 20$)	Vervet monkey[A] ($n = 6$)	Ram[A] ($n = 25$)	Bull[A] ($n = 7$)	African elephant[B] ($n = 21$)	White rhinoceros[B] ($n = 14$)
Stimulant	Caffeine	Progesterone	Procaine	Caffeine	Caffeine	Caffeine
Motility (%)	72.8 ± 15.9	69.2 ± 10.2	74.1 ± 10.7	81.0 ± 13.5	80.0 ± 21.5	81.7 ± 7.5
Hyperactivation range (%)	16–62	31–76	26–72	21–75	8–53	17–86

[A]Preliminary data.
[B]Data from African elephant and white rhinoceros in conjunction with Luther (2016).

the plasma membrane for calcium or the release of calcium from internal stores.

Thus, care should be taken as to which agonists are selected for inducing hyperactivation, as well as the incubation time before assessing hyperactivation. For example, Ntanjana (2015) investigated human sperm hyperactivation in the most motile PureSperm-separated fraction. After 15 min incubation, there was no significant difference in percentage hyperactivation between caffeine and procaine stimulation but, after 60 min, caffeine induced more sperm to hyperactivate than procaine ($23.0 \pm 3.8\%$ vs $10.9 \pm 3.0\%$, mean $\pm$ s.e.m.). Apart from evaluating only the most motile sperm fraction, it is also important to compare other sperm subpopulations for their ability to hyperactivate. Sperm subpopulations with lower sperm motility typically showed a deficient capacity to hyperactivate (Buffone *et al.* 2004; Ntanjana 2015).

Clinical outcomes

As part of the clinical work-up, total and progressive motility estimations are used as indirect measures of male fertility potential (WHO 2010). This section focuses specifically on the application of CASA-Mot as part of the diagnosis for sexually transmissible infections (STIs) and identifying semen samples of improved sperm quality.

From a total of 120 ejaculates from patients attending the andrology clinic at Tygerberg Hospital, 15.8% ($n = 19$) tested positive for *Neisseria gonorrhoea*, 20.8% ($n = 25$) tested positive for *Trichomonas vaginalis* and 9.2% ($n = 11$) tested positive for *Chlamydia trachomatis*. All samples that were identified as polymicrobial (WHO 2005) were omitted from the study and CASA-Mot analysis was performed to identify any differences in motility parameters between the various infections. As can be observed from Table 4, all three STIs negatively affected various sperm motility parameters compared with controls (uninfected).

In order to increase pregnancy success rates, one of the main aims in the clinical setting is to assist subfertile men to improve the quality of their sperm samples. Recently, a few inconclusive studies appeared on the contributing effect of abstinence time on sperm function (Ayad *et al.* 2018). As part of our ongoing investigations, we compared the effect of long (4 days) and short (4 h) abstinence periods on the motility parameters of 100 donors. From the data (Table 5), it is noticeable that shorter abstinence periods significantly enhanced total motility, progressive motility and the population of rapid cells. The kinematic parameters VCL, VSL, VAP, LIN, straightness (STR) and beat cross frequency (BCF) were also significantly increased. This clearly shows that shorter abstinence periods could be beneficial to the functional quality of sperm movement

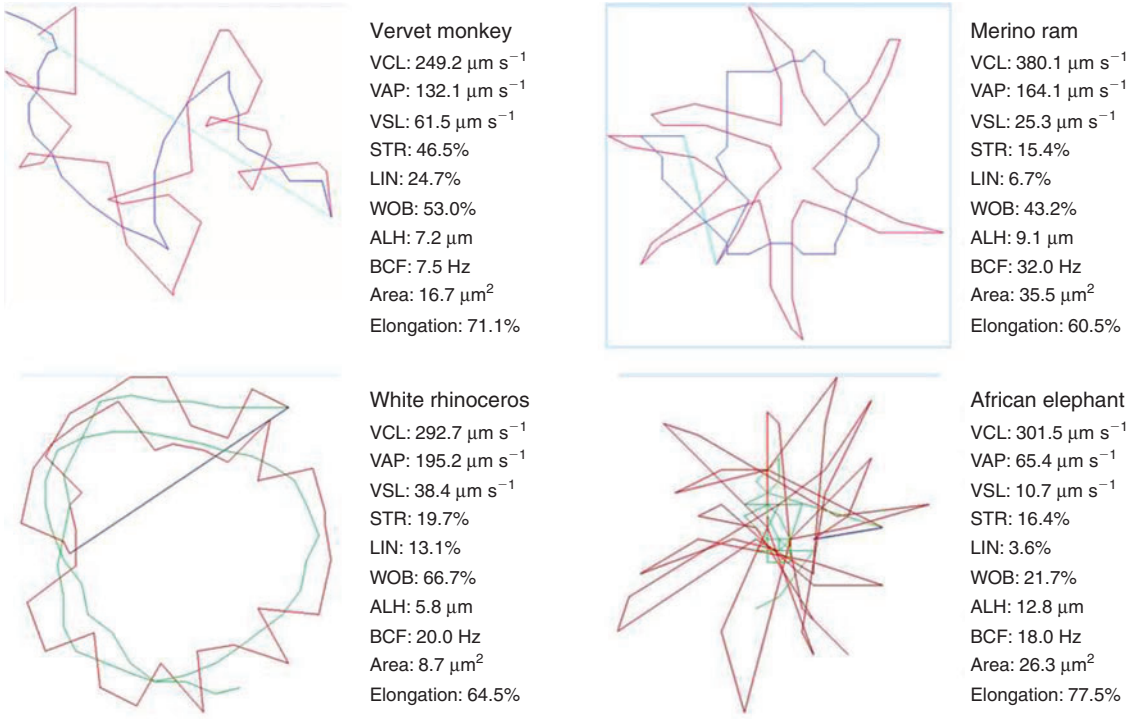

Fig. 4. Examples of individual hyperactivated sperm tracks of four mammalian species with detailed kinematics to the right. Tracks were captured at 50 frames per second (f.p.s.), except for Merino ram, with a frame rate of 100 f.p.s. VCL, curvilinear velocity (red line); VAP, average path velocity (blue (monkey, ram) or green (rhinoceros, elephant) lines) VSL, straight-line velocity (turquoise (monkey, ram) or blue (rhinoceros, elephant) lines); STR, straightness; LIN, linearity; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

Table 4. Effects of three sexually transmissible infections on various computer-aided sperm analysis (CASA) parameters as measured by SCA® (Microptic) (adapted from Flint 2016)

Data are the mean ± s.e.m. Within rows, values with different superscript letters differ significantly ($P < 0.05$). VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble

	Control ($n = 65$)	*Neisseria gonorrhoea* ($n = 19$)	*Chlamydia trachomatis* ($n = 11$)	*Trichomonas vaginalis* ($n = 25$)
Total motility (%)	82.1 ± 1.1[a]	37.1 ± 3.2[b]	37.8 ± 4.2[b]	47.6 ± 2.2[b]
Progressive motility (%)	51.8 ± 2.5[a]	13.4 ± 1.9[b]	13.6 ± 2.3[b]	15.8 ± 2.0[b]
Non-progressive motility (%)	30.4 ± 1.5	23.7 ± 1.3	24.1 ± 1.9	32.3 ± 0.2
Rapid (%)	42.4 ± 6.1[a]	6.4 ± 3.2[b]	14.0 ± 7.1[b]	15.9 ± 3.0[b]
Medium (%)	29.5 ± 4.2[a]	12.3 ± 2.2[b]	11.1 ± 7.4[b]	20.3 ± 1.9[b]
Slow (%)	13.4 ± 2.5	9.2 ± 2.9	15.8 ± 6.1	18.6 ± 3.3
VCL (μm s^{-1})	42.4 ± 27.8[a]	20.6 ± 5.1[b]	22.9 ± 14.9[b]	25.4 ± 18.5[b]
VSL (μm s^{-1})	19.1 ± 2.0[a]	9.2 ± 3.8[b]	10.4 ± 2.5[b]	11.4 ± 1.8[b]
VAP (μm s^{-1})	28.6 ± 3.7[a]	10.5 ± 2.6[b]	12.2 ± 1.9[b]	18.7 ± 1.8[b]
LIN (%)	48.5 ± 6.3	25.7 ± 4.6	31.0 ± 4.1	39.5 ± 1.5
STR (%)	69.1 ± 4.9	47.3 ± 5.9	58.1 ± 5.7	63.8 ± 1.9
WOB (%)	69.0 ± 3.7	52.5 ± 6.0	62.7 ± 4.0	59.7 ± 1.3

Table 5. Effects of long (4 days) and short (4 h) abstinence in the same donors ($n = 100$) on various computer-aided sperm analysis (CASA) parameters as measured by SCA® (Microptic) (adapted from Ayad *et al.* 2017)

Data are the mean ± s.e.m. Types of progressive motility (A–D) are according to WHO (WHO 2010). VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency

	Long abstinence	Short abstinence	*P*-value
Total motility (%)	58.9 ± 1.5	62.3 ± 1.5	0.0013
Progressive motility (%)	45.0 ± 1.4	49.6 ± 1.5	<0.0001
Fast progressive (Type A) (%)	11.2 ± 0.7	14.0 ± 0.7	<0.0001
Slow progressive (Type B) (%)	33.8 ± 1.2	35.6 ± 1.5	0.056
Non-progressive (Type C) (%)	13.9 ± 0.4	12.7 ± 0.4	0.0015
Immotile (Type D) (%)	41.2 ± 15.1	37.7 ± 15.3	0.0012
Rapid (%)	44.0 ± 1.4	48.6 ± 1.5	<0.0001
Medium (%)	6.4 ± 0.2	6.1 ± 0.2	0.0851
Slow (%)	8.5 ± 0.3	7.7 ± 0.3	0.0016
VCL (μm s^{-1})	76.2 ± 1.1	82.0 ± 1.7	<0.0001
VSL (μm s^{-1})	29.8 ± 0.5	32.9 ± 0.6	<0.0001
VAP (μm s^{-1})	49.5 ± 0.6	53.4 ± 0.8	<0.0001
LIN (%)	39.4 ± 0.6	40.9 ± 0.8	0.0055
STR (%)	60.2 ± 0.7	61.8 ± 0.8	0.0132
WOB (%)	65.2 ± 0.4	65.8 ± 0.6	0.0601
ALH (μm)	1.95 ± 0.03	1.97 ± 0.04	0.2202
BCF (Hz)	13.6 ± 0.2	14.9 ± 0.2	<0.0001

(Ayad *et al.* 2017). Similar findings were also observed in bottlenose dolphins (data not shown). A second and third ejaculate collected after 30 and 60 min also produced semen with better quality than at initial collection. Interestingly, not only did sperm motility improve, but both sperm concentration and semen volume were also increased in subsequent samples from these dolphins.

Sperm morphology and morphometry

The spermatozoon is the most diverse cell type known and the importance of the diversity in sperm morphology as a marker of fertility has already been recognised since the early 1950s (MacLeod and Gold 1951). However, as highlighted by van der Horst and du Plessis (2017), manual assessment of morphology remains problematic due to human subjectivity as well as intra- and intertechnician inconsistency. These diagnostic errors make interpretation of results difficult and can affect treatment options.

The development of automated sperm morphology analysis (ASMA) software to process sperm morphology samples has advantages over manual microscopic analysis because it provides faster, quantifiable and accurate morphometric measurements. Assessing morphology and morphometry through CASA, also commonly referred to in the past as computer-aided sperm morphology analysis (CASMA), but now referred to as CASA-Morph is a complex process, but technological development in image processing and analytical tools currently allows for the derivation of morphometric parameters and accurate, as well as objective, determination of sperm shape.

In a study by Maree *et al.* (2010), two SCA® systems (Microptic), operated by two independent technicians in different laboratories, were used to analyse the same stained slides. No major significant interlaboratory differences in the automatic analyses of various sperm head parameters (length, perimeter and acrosome coverage) were observed between the two laboratories, thereby highlighting the ability of CASA-Morph to accurately reproduce sperm morphology assessments independent of staining technique.

Determining percentage normal sperm morphology using morphometry cut-off points

van der Horst *et al.* (2018) introduced and verified a method to establish CASA morphometric cut-off points for determining the percentage of normal spermatozoa in a semen sample. This

Table 6. Minimum and maximum morphometric values to determine normal sperm morphology in four mammalian species
The curious hook-shaped spermatozoa of rodents, such as the rat, allows measurement of additional features such as chord and linearity related to the sperm head (see Fig. 5). H, head; ARC, total length of the sperm head; Chord, straight line from anterior tip of acrosome to base of head; MP, midpiece

	Human ($n = 10$)		Rat ($n = 49$)		Boar ($n = 5$)		Stallion ($n = 8$)	
	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum
H length = ARC (μm)	4.05	5.05	19	23	8.5	10.5	5	6.7
H width (μm)	2.55	3.55	0.9	1.9	4	5.5	1.8	3
H perimeter (μm)	11.7	15.7	40	50	18.5	22.6	10.5	14.5
H area (μm^2)	9.3	15.9	15	23	33	44.5	7.7	17
Chord (μm)	–	–	8	14	–	–	–	–
H angle (degrees)	–	–	50	87	–	–	–	–
H linearity (%)	–	–	40	65	–	–	–	–
H roughness	0.8	1.32	0.08	0.3	0.5	1.5	0.5	1.5
H regularity	0.8	1.15	–	–	0.8	1	0.75	1
H ellipticity	1.3	1.8	–	–	1.5	2.5	1.9	2.8
Acrosome cover (%)	40	70	–	–	53	61	45	75
MP width (μm)	0.05	1.45	0.4	1	0.7	1.2	0.6	1.5
MP angle (degrees)	0	60	0	60	−1	4.75	−1	5.5

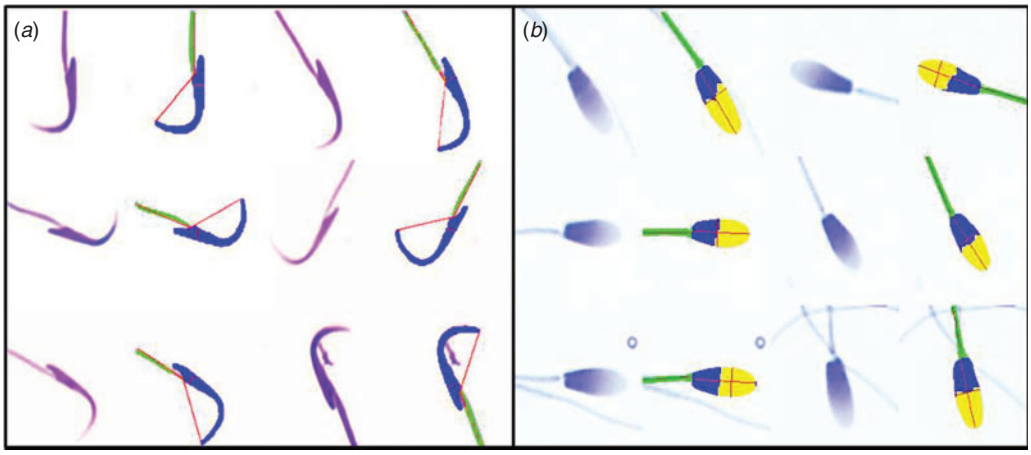

Fig. 5. Computer-aided sperm morphology analysis (CASMA) analysis of (*a*) rat and (*b*) stallion spermatozoa. In each instance, the actual spermatozoon is on the left, with the image immediately to the right after thresholding, indicating the sperm head (blue), acrosome (yellow) and midpiece (green). Spermatozoa were stained with SpermBlue (Microptic) and analysed with the Morphology module of SCA® (Microptic) (rat: ×60 objective with primary magnification of ×600; stallion: ×100 objective with primary magnification of ×1000). The accuracy in thresholding of different sperm components allows for calculation of 13 morphometric parameters (see Table 6).

technique is particularly valuable in cases where no criteria exist for a species to distinguish normal from abnormal spermatozoa. Essentially the method involves determining the percentile minimum and maximum cut-off points for as many as 15 morphometric parameters (e.g. length, width, perimeter, surface area, elongation and roughness of the head, as well as some midpiece and tail features). These cut-off values are then incorporated in the Sort function of SCA for automatic decision making of normal versus abnormal.

We have recently been most successful in objective assessment of the percentage of normal spermatozoa in Tankwa goats and in two strains of laboratory rats (van der Horst *et al.* 2018; A. Ngcauzele, G. van der Horst and L. Maree, unpubl. obs.). van der Horst *et al.* (2018) has shown that the percentage normal sperm morphology was vastly overestimated in the laboratory rat when comparing manual analysis with CASA-Morph (95% vs 68–75% respectively). Table 6 shows minimum and maximum values for 13 morphometric parameters in order to define normal sperm morphology in diverse mammalian species. For example, it can be seen that spermatozoa of most domestic mammals have large elongated heads, whereas the sperm head is considerably smaller in the African elephant and African white rhinoceros, and even smaller in most carnivores such as lions. Furthermore, Fig. 5 shows how accurately the SCA system detects various sperm components for morphology and morphometry assessment.

Morphometry subpopulations

Because automatic analysis of sperm morphometry allows for detailed measurements of individual sperm dimensions, species-specific sperm head morphometry subpopulations can be determined using similar statistical procedures as for motility data (Vicente-Fiel *et al.* 2013; Yániz *et al.* 2015). However, Soler and Cooper (2016) warn that regardless of the specific CASA-Morph system used, only a few research groups are using the proper statistical analyses to assess the large volumes of data generated with sperm morphometry analysis.

There has been a wide application for such subpopulation evaluations in domestic animals, including prediction of ejaculate fertility (for a review, see Yániz *et al.* 2015), post-thaw semen quality (Thurston *et al.* 2001; Peña *et al.* 2005) and correlation with sperm chromatin integrity (Núñez-Martinez *et al.* 2007). Even though it is well known that human spermatozoa are heteromorphous in nature, surprisingly little information is available on morphometry subpopulations in men (Santolaria *et al.* 2016; Vásquez *et al.* 2016). This is probably due to the fact that andrologists primarily focus on percentage normal sperm morphology and the most prevalent sperm abnormalities when evaluating human semen. With recent advances in CASA-Morph software, it is now possible to also evaluate the dimensions of other sperm components (e.g. midpiece and tail), which should be included in future studies to determine more intricate morphometry subpopulations. Furthermore, by combining kinematic and morphometric datasets to determine sperm subpopulations, a more holistic view of the relationship between sperm structure and function is provided (Soler *et al.* 2017).

Apart from the reported interspecies differences in head morphometry subpopulations, it is interesting to note that many studies also found interindividual differences in these subpopulations (Thurston *et al.* 2001; Vicente-Fiel *et al.* 2013), which makes it an important tool for individual male fertility assessment. A wider and evolutionary implication of individual differences, such as found in adult marmosets (Valle *et al.* 2012), would be that there is a low risk for sperm competition in certain (often monogamous) species and could be the reason for the high degree of pleomorphisms observed (van der Horst and Maree 2014).

Teratozoospermic index

In general, morphologically abnormal spermatozoa show multiple defects that can be calculated and expressed as indices such as the teratozoospermic index (TZI), multiple anomalies index (MAI) and sperm deformity index (SDI) (Auger 2010). These indices have been shown to correlate with fertilisation *in vivo* and *in vitro* (Jouannet *et al.* 1988; Aziz *et al.* 1996; Menkveld *et al.* 2001; Slama *et al.* 2002).

The TZI is useful because, in addition to percentage abnormal spermatozoa, it indicates the coexistence of more than one sperm compartmental defect per morphologically classified abnormal spermatozoon and is expressed as an index. Most domestic and wildlife species usually have a high percentage of normal spermatozoa and a low TZI, particularly where the risk of sperm competition is high. However, in human spermatozoa, it is not uncommon to find, for example, 6% normal spermatozoa, but a TZI of 1.7. Menkveld *et al.* (2001) compared the semen parameters of a fertile and subfertile population. Using ROC curve analysis and taking into consideration that the male contributes to the infertility problem in approximately 50% of cases, the cut-off points for normal sperm morphology and TZI to identify subfertile males were calculated to be $\leq$3% and $\geq$2.09 respectively, with the lower 10% of the fertile population at 2% and 1.33 respectively (Menkveld *et al.* 2001). It may well be that TZI and the other sperm indices also automatically measured by SCA provide more useful information regarding sperm quality and fertility potential than sperm morphology on its own.

In a recent study conducted in separate and independent laboratories, we used CASA-Morph to calculate the TZI of two populations of Wistar rats and found this index to be significantly similar, with mean ($\pm$ s.d.) values of 1.02 ± 0.04 ($n = 20$) and 1.03 ± 0.04 ($n = 15$) respectively, whereas the results from another laboratory showed the TZI index of Sprague-Dawley rats to be 1.00 ± 0.00 ($n = 14$). The mean ($\pm$ s.d.) percentage of morphologically normal spermatozoa for these three groups was $74.00 \pm 8.29\%$, $69.14 \pm 9.23\%$ and $67.92 \pm 8.59\%$ respectively (van der Horst *et al.* 2018).

In a human study (Kochman *et al.* 2016), we set off comparing manual sperm morphology analysis with CASA-Morph as well as evaluating the various morphology indices as sperm quality predicting factors. Bland and Altman analyses, as well as Passing and Bablok regressions, confirmed that there were no significant differences between manual sperm morphology analysis and CASA-Morph ($P = 0.48$). Highly significant correlation coefficients (Spearman rank) between calculated indices and percentage normal morphology were established in both manual comparisons (TZI: $P < 0.008$, $r = -0.37$; SDI: $P < 0.0001$, $r = -0.52$; MAI: $P < 0.0002$, $r = -0.50$) and for CASMA (TZI: $P < 0.0005$, $r = -0.47$; SDI: $P < 0.0001$, $r = -0.76$; MAI: $P < 0.0001$, $r = -0.53$). Similarly, significant correlations were established when these indices were compared with motility parameters (data not shown here but included in Kochman *et al.* 2016). These results clearly show that the choice of diagnostic method (manual or CASA-Morph) does not affect the outcome of sperm morphology evaluation. Furthermore, inversely proportional correlations were found between sperm morphology indices, normal morphology and sperm motility (Kochman *et al.* 2016). It is therefore recommended that these indices are calculated with the aid of CASA-Morph during routine semen analysis.

Possible future additions to CASA technology

It is pre-empted more sperm functional tests will be incorporated into CASA systems in the foreseeable future, which, together with basic semen parameters, will provide a more comprehensive and quantitative approach to better understand the quality and fertilising potential of a semen sample.

Assessment of sperm mitochondria

In addition to evaluation of midpiece length, width, area and angle of implantation, sperm mitochondrial structure and function are currently not assessed in much detail using CASA-Morph (Yániz *et al.* 2015). Several reviews published on the role of sperm mitochondria in sperm quality highlighted the importance

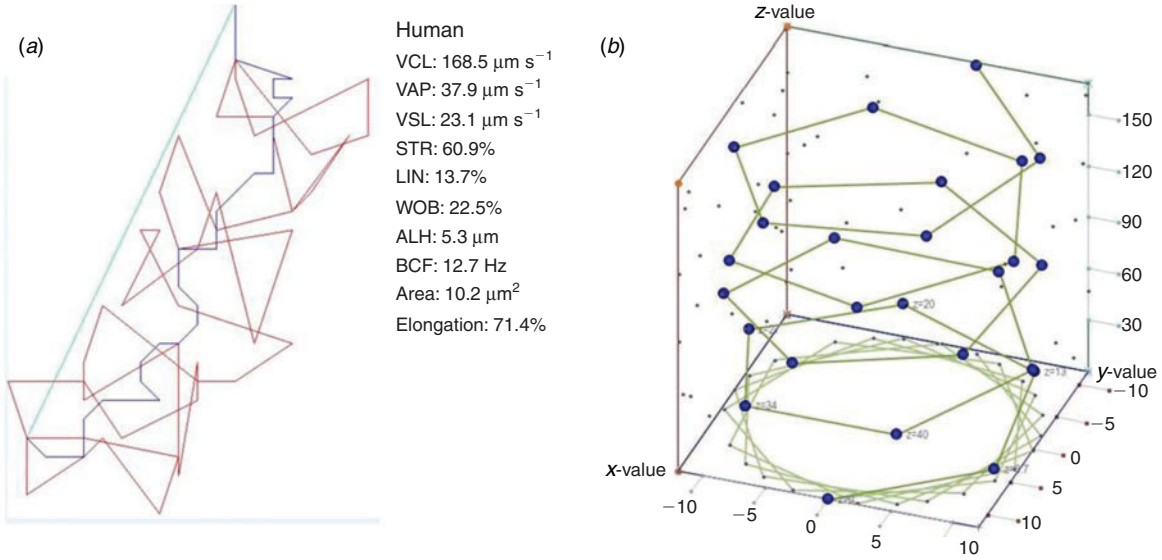

Fig. 6. (*a*) Human sperm computer-aided sperm analysis (CASA) track reconstructed from *x* and *y* coordinates every 50th of a second. VCL, curvilinear velocity (red line); VAP, average path velocity (blue line); VSL, straight-line velocity (turquoise line). (*b*) A four-dimensional construction of the same sperm track (see text for explanation). STR, straightness; LIN, linearity; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

of these organelles in energy production, production of reactive oxygen species, apoptosis and possibly even in Ca^{2+} signalling (Ramalho-Santos *et al.* 2009; Piomboni *et al.* 2012; Amaral *et al.* 2013). Moreover, sperm subpopulations with active mitochondria had a better fertilisation potential (Barroso *et al.* 2006; Sousa *et al.* 2011). Comparing normozoospermic with asthenozoospermic semen samples, Amaral *et al.* (2014) indicated that 29% of the differences in proteins between these samples were related to sperm mitochondria. Because the presence, membrane intactness and protein activity of mitochondria can be assessed using fluorescent probes (biomarkers), these characteristics of sperm mitochondria should be easy to assess with CASA-Morph.

Four-dimensional motility

Initially, tracking of spermatozoa in three dimensions was performed by high-speed image stack acquisitions in sea urchin spermatozoa (Corkidi *et al.* 2008) and a lens-free on-chip imaging technique in human spermatozoa (Su *et al.* 2012) and boar (Soler *et al.* 2018) spermatozoa. Newly developed CASA software enables the modelling and simulation of four-dimensional sperm motility tracks from the *x* and *y* coordinates obtained from normal two-dimensional tracks (Fig. 6; van der Horst and Sanchez 2016). This model is based on the assumption that spermatozoa swim in a helix that is more or less spherical. We foresee that this will become part of the routine CASA motility evaluations that will contribute significantly to assessment of sperm velocity and functioning of the sperm flagellum.

Conclusion

CASA systems have evolved substantially over the past three decades to become powerful tools for the rapid and objective assessment of sperm concentration, motility and kinematics, as well as morphology, in almost any mammalian species, including humans. Within a few seconds, hundreds to thousands of spermatozoa can be analysed with great accuracy. In this regard, CASA is far superior to subjective manual assessments; it quantitatively measures different aspects of sperm velocity, hyperactivation and morphometry, which cannot be done manually. New-generation CASA modules have recently been developed that automatically quantify diverse aspects of sperm functionality, such as sperm DNA fragmentation, sperm vitality and acrosome integrity and reaction.

In this investigation we focused primarily on studies pertaining to sperm motility and morphology of primates and a wide range of domestic animals, as well as diverse wildlife species. Not only do we provide parameter settings, but we also provide novel baseline data for primarily normospermic samples. We showed that even the most challenging samples, such as rat and mouse spermatozoa, can now be routinely analysed for motility and morphology, thereby providing a powerful tool for toxicology studies. As evident from our own results as well as the current literature, there are numerous applications for the routine use of CASA in the research laboratory, wildlife management and clinical fertility assessment setting (human and veterinary).

Conflicts of interest

Gerhard van der Horst is a senior consultant for Microptic SL (Barcelona, Spain), manufacturers of the SCA CASA software used in this investigation. However, all analyses were performed independently and Microptic SL had no bearing on the outcome.

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