Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.

This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright

Author's personal copy

Animal Reproduction Science 133 (2012) 123–128

Contents lists available at SciVerse ScienceDirect

Animal Reproduction Science

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

The effect of temperature and pH on the motility and viability of ostrich sperm

Maud Bonato[a,*], Charlie K. Cornwallis[b], Irek A. Malecki[c,d], Paulina K. Rybnik-Trzaskowska[e], Schalk W.P. Cloete[a,f]

[a] Department of Animal Sciences, University of Stellenbosch, Matieland 7600, South Africa
[b] Department of Biology, Lund University, SE-223 62 Lund, Sweden
[c] School of Animal Biology M085, Faculty of Natural and Agricultural Sciences, University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia
[d] UWA Institute of Agriculture, University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia
[e] Department of Genetics and Animal Breeding, Warsaw University of Life Sciences, Ciszewskiego 8, 02-786 Warsaw, Poland
[f] Institute for Animal Production: Elsenburg, Private Bag X1, Elsenburg 7607, South Africa

ARTICLE INFO

Article history:
Received 26 January 2012
Received in revised form 27 May 2012
Accepted 15 June 2012
Available online 21 June 2012

Keywords:
Semen diluent
Sperm storage
Sperm viability
Ostrich
Motility
CASA system

ABSTRACT

As the chemical environment of semen diluents can have a profound effect on sperm quality, we examined the effect of temperature and pH on the motility and viability of sperm in the ostrich. Semen was collected from four males, each male being replicated three times. Ejaculates were diluted and incubated for 10 min at 20 °C and 40 °C in four different buffers, temperature adjusted at pH 6, 7, 8 and 9 respectively. Average path velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL), linearity (LIN), beat cross frequency (BCF) and amplitude of lateral displacement (ALH) were then recorded for each sample using CASA. The viability of sperm was assessed using nigrosin–eosin staining. Sperm incubated at 40 °C had higher motility parameters, except for ALH. At 40 °C, VAP, VSL and LIN increased with pH while VCL, BCF and ALH were higher for lower pHs. The viability of sperm was not affected by temperature but decreased at pH values > 7. A pH in the neutral range appeared to yield higher quality sperm after in vitro storage at 20 °C. However, the effect of different pH levels and temperatures on sperm longevity needs to be investigated further to develop viable ostrich specific diluents.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Assisted reproductive technologies like artificial insemination (AI), semen holding and cryopreservation are needed to preserve and disseminate livestock germplasm. In domestic birds, these techniques were first established in chickens, and then extended to other poultry species (Blesbois and Brillard, 2007; Donoghue and Wishart, 2000; Lake, 1986). Commercial ostrich farming is constrained by low fertility, high levels of embryo and chick mortality and inadequate genetic improvement (Cloete et al., 1998; Cloete and Malecki, 2011; Malecki et al., 2008). The advent of animal and human friendly methods to collect semen from male ostriches and artificially inseminate females (Malecki et al., 2008; Rybnik et al., 2007) resulted in new interest in developing assisted reproductive technology to augment industry development. Recently, Bonato et al. (2011) established that semen can be collected frequently without depleting male sperm reserves or impairing their libido. This ample supply of semen allows AI technology to move to the next phase, which is to design a protocol for semen holding and preservation.

Sperm motility is a critical factor in determining semen quality and fertilizing ability. In female birds, spermatozoa

* Corresponding author at: Department of Animal Sciences, University of Stellenbosch, Private Bag X1, South Africa. Tel.: +27 44 272 6077; fax: +27 44 279 1910.
E-mail address: mbonato@sun.ac.za (M. Bonato).

0378-4320/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.anireprosci.2012.06.008

Author's personal copy

are stored for extended periods of time in specialized structures, the sperm storage tubules (SSTs) localized mainly in the utero-vaginal junction of the oviduct (Bakst, 2011). These structures have evolved to release sperm over time to insure sufficient sperm numbers at the site of fertilization, even in the absence of males. For instance, female ostriches were found to lay fertilized eggs for up to 4 weeks after the last copulation (Malecki et al., 2004). A single insemination led to maximum fertile periods of 12 days in Japanese quails (Reddish et al., 1996) and 70 days in turkeys (Lorenz, 1950).

Changes in the external medium are believed to regulate sperm motility by complex interactions among factors like temperature, pH and ion composition. For instance, an increase of temperature from 30 °C to 40 °C resulted in the inactivation of sperm motility in domestic chickens (*Gallus gallus*). An alkalinization of the external pH, following an addition of calcium, then restored sperm motility at 40 °C (Ashizawa and Wishart, 1987; Ashizawa et al., 1994). Similar results were obtained in the turkey and quail (Holm and Wishart, 1998), whereby the percentage of motile sperm and sperm velocity was stimulated by an alkaline pH particularly at 40 °C (the average avian body temperature) compared to 30 °C. Changes in pH of the vaginal mucosa after oviposition have also been observed in both broilers and turkeys (Bakst, 1980), indicating that such variation in pH may change the motility of sperm after insemination. Therefore, the effect of storage medium pH, storage temperature, and their interaction needs consideration during the development of a species specific semen diluent to maintain sperm viability and activity in vitro.

To date, only a few studies have been conducted on sperm motility and survival in the ostrich after short term storage. Undiluted ostrich semen coagulates rapidly at room temperature, leading to a deterioration of sperm quality (Ciereszko et al., 2010; Malecki et al., 2008). Furthermore, changes in motility and viability of ostrich sperm were investigated by storing diluted ostrich semen at 5 °C in Minimal Essential Medium diluent (Ya-jie et al., 2001), in EK, Lake's and NaCl diluents (Malecki et al., 2008) or Ovodyl and EK (Ciereszko et al., 2010). Sperm motility and viability could only be maintained for 4–12 h in these studies, suggesting the need for the development of a suitable diluent specific for ostrich sperm. The next crucial step in developing a workable AI program for ostriches is designing a storage medium that prolongs sperm viability. A key to developing such a medium involves determining the optimal pH for maximal preservation of sperm viability and motility in vitro.

Changes in sperm motility and viability over a biologically relevant pH range were thus assessed at temperatures of 20 °C and 40 °C, using computer-assisted semen analysis (CASA) for sperm velocity and nigrosin–eosin staining for sperm viability.

2. Material and methods

2.1. Study population, semen collection and evaluation

The study was carried out at the Oudtshoorn Research Farm, South Africa, in December 2008 on four South African black ostrich males (2–4 years of age). Males were trained prior to the experiment to mount a dummy female and ejaculate into an artificial cloaca (Rybnik et al., 2007). Three ejaculates were collected on consecutive days (24 h interval) from each male. Ejaculate volume was measured with an automatic pipette, and sperm concentration was determined with a haemocytometer in 20 μL of semen diluted 1:400 (v/v) with a phosphate buffered saline solution containing 1% formalin. The total number of sperm per ejaculate was then calculated by multiplying ejaculate volume and concentration. Finally, samples of neat semen were mounted onto a glass slide and the proportion of live normal (membrane intact with normal morphology, eosin non-permeable), live abnormal (membrane intact, eosin impermeable but with alteration to normal morphology) and dead sperm (membrane damaged – eosin permeable) was estimated after counting 500 sperm stained with nigrosin–eosin (Lake and Stewart, 1978) on duplicate slides. Sperm forming a complete unit of a slightly curved head, mid-piece and tail were considered as live normal (as described by Soley and Roberts, 1994), while any sperm showing a deviance from this structure was considered as live abnormal (i.e. giant sperm, double headed sperm, head bent on the tail, swollen head, broken head, straight sperm).

2.2. Sample preparation

Neat semen ostrich ejaculate has been previously estimated to have a pH of 7.0 (Ya-jie et al., 2001), hence for this experiment, each ejaculate was tested at four pH levels at the two temperatures as follows: 20 μL of sperm was diluted in 80 μL (1:4) of each of four buffers, temperature adjusted to 20 °C and 40 °C. All buffers contained 150 mM of NaCl and one of the following compounds: 20 mM of 2-(N-morpholino) ethane sulphonic acid (MES) for pH 6; 20 mM of *N*-tris-[hydroxymethyl]-methyl-2-aminoethanesulfonic acid (TES) for pH 7; 20 mM of Tris[hydroxymethyl] aminomethane hydrochloride (TRIZMA) for pH 8 and; 20 mM of 3[cyclohexylamino]-2-hydroxyl-1-propane-sulfonic acid for pH 9. Samples were then incubated for 10 min at either 20 °C or 40 °C. The order of the pH treatments was randomised but our experimental procedure did not allow for randomising temperature treatments.

2.3. Assessment of sperm motility

After incubation, 10 μL of the 1:4 sperm solutions were further diluted to 10 million sperm/mL with their original buffer held at the same temperature. Sperm velocity was measured with a ×10 objective under negative phase contrast using a BX 41 Olympus microscope (Olympus Corporation, Tokyo, Japan). For each sample, sperm velocity was recorded by loading 5 μL of solution onto a microscope slide placed on a heated stage at either 20 °C or 40 °C and taking three readings from three separate fields of view. This procedure was repeated twice for each sample. The digital video files were analyzed using a Sperm Class Analyzer (SCA v.3, Microptic S.L., Spain). Average path velocity (VAP, velocity over a calculated smoothed

Author's personal copy

path), curvilinear velocity (VCL, velocity over the actual sperm track, including all deviations of sperm head movement), straight line velocity (VSL, velocity over the straight line distance between the beginning and the end of the sperm track), linearity (LIN, departure of sperm track from a straight line [VSL/VCL] × 100), beat-cross frequency (BCF, frequency with which the sperm crosses the smoothed path), and amplitude of lateral displacement (ALH, time average of absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory) were calculated for each sample from the two recordings of at least 100 sperm tracks each. The medians for all parameters were strongly correlated with their means (Pearson's correlation coefficient $R > 0.95$), but we used the median to ensure that central tendency of all measures on ejaculates were not influenced by non-normal distribution of motility.

Finally, samples of each of the sperm suspensions were stained with nigrosin–eosin and mounted onto a glass slide to determine the proportion of live normal, live abnormal and dead sperm in the same way as for neat sperm.

2.4. Statistical analysis

To investigate the effects of temperature and pH on sperm velocity, General Linear Models (GLM) were performed on all CASA parameters (medians of VAP, VCL, VSL, LIN, BCF and ALH calculated from all tracked sperm over the 3 fields measured) entered as response variables, while temperature, pH and their interaction were entered as fixed factors. Male number was entered as a fixed factor to control for individual variation. Similar GLMs were performed on mean percentage of live normal, live abnormal and dead sperm to evaluate the effect of temperature and pH on viability of sperm. All statistical analyses were performed using Genstat version 13 (VSN International Ltd., UK).

3. Results

3.1. Descriptive statistics of ejaculate parameters

The mean ejaculate volume (±SEM) across the whole experiment was 1.11 ± 0.26 mL, with an average concentration of $3.20 \pm 0.29 \times 10^9$ sperm/mL, and contained $4.13 \pm 1.36 \times 10^9$ sperm. The mean proportion of live normal, live abnormal and dead sperm was 72.63 ± 4.93%, 18.15 ± 3.57% and 9.22 ± 2.29% respectively. While no age effect was detected for any of the variable measured ($P > 0.05$), a significant male effect was found for all ejaculate parameters ($P < 0.001$).

3.2. Effect of temperature and pH on the motility of sperm

The interaction between temperature and pH was significant for all CASA traits ($P < 0.05$; Table 1). Velocity parameters (VAP, VSL and VCL) as well as LIN and ALH were independent of pH at 20 °C ($P > 0.05$; Fig. 1), but the BCF of sperm showed an increase as pH increased from 6 to 7 ($F_{3,41} = 3.81$, $P = 0.02$; Fig. 2). Furthermore, we detected a male effect on LIN ($F_{3,41} = 5.98$, $P = 0.002$) and ALH ($F_{3,41} = 4.81$, $P = 0.006$), with one male showing a higher sperm LIN and a lower sperm ALH compared to the rest of the males.

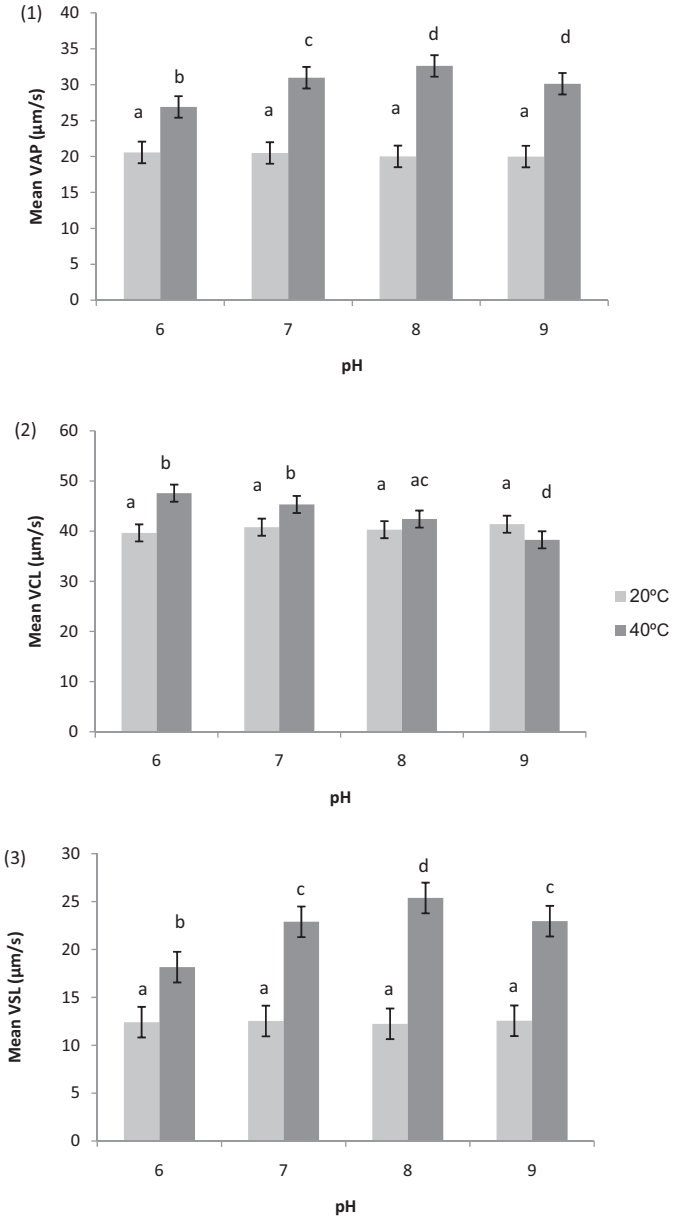

Fig. 1. Effect of pH and temperature on the velocity of sperm of four male ostriches: (1) mean average path velocity (VAP); (2) mean curvilinear velocity (VCL) and; (3) mean straight line velocity (VSL). Vertical lines about the means denote standard errors. For any temperature and pH, those results which are significantly different ($P < 0.05$) are assigned a different letter.

Sperm in all pH treatments incubated at 40 °C had a higher ($P < 0.05$) velocity than at 20 °C. In contrast to the tendency at 20 °C, all velocity parameters at 40 °C were affected by pH, but to different extents (Table 1). VAP and VSL increased with pH with a maximum at pH 8 (mean ± SE = 32.61 ± 1.71 µm/s, $F_{3,41} = 1.98$, $P = 0.035$ and mean ± SE = 25.38 ± 1.79 µm/s, $F_{3,41} = 2.84$, $P = 0.040$, respectively; Fig. 1), while VCL was highest at lower pH values, ($F_{3,41} = 8.20$, $P = 0.001$). In addition, LIN reached a maximum at pH 8 (mean ± SE = 67.34 ± 3.03%, $F_{3,41} = 18.16$, $P = 0.001$; Fig. 2), while BCF and ALH of sperm were higher at pH 6 (mean ± SE = 5.05 ± 0.2 Hz, $F_{3,41} = 10.67$, $P = 0.001$ and

Author's personal copy

Table 1
Degrees of freedom, *F*-values and significance levels denoting the effect of temperature and pH on the motility of the ostrich sperm.

Variable	Temperature			pH			Temperature × pH		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
VAP	1,85	134.14	**0.001**	3,85	1.68	0.17	3,85	2.34	0.080
VCL	1,85	11.15	**0.001**	3,85	4.01	**0.010**	3,85	7.31	**0.001**
VSL	1,85	122.28	**0.001**	3,85	2.78	**0.046**	3,85	2.93	**0.038**
LIN	1,85	295.24	**0.001**	3,85	15.52	**0.001**	3,85	19.20	**0.001**
BCF	1,85	11.80	**0.001**	3,85	1.11	0.348	3,85	14.47	**0.001**
ALH	1,85	144.22	**0.001**	3,85	40.59	**0.001**	3,85	28.15	**0.001**

Bold values are significant in the analysis.
Sperm motility was assessed using computer-assisted sperm analysis system (CASA system). VAP: average path velocity; VCL: curvilinear velocity; VSL: straight line velocity; LIN: linearity; BCF: beat-cross frequency; and ALH: amplitude of lateral displacement.

mean ± SE = 2.17 ± 0.06 μm, $F_{3,41}$ = 61.82, P = 0.001, respectively) and lower at pH 9 (mean ± SE = 4.01 ± 0.2 Hz, mean ± SE = 1.4 ± 0.06 μm respectively). No male effect was detected for any of the CASA parameters at 40 °C (P > 0.05).

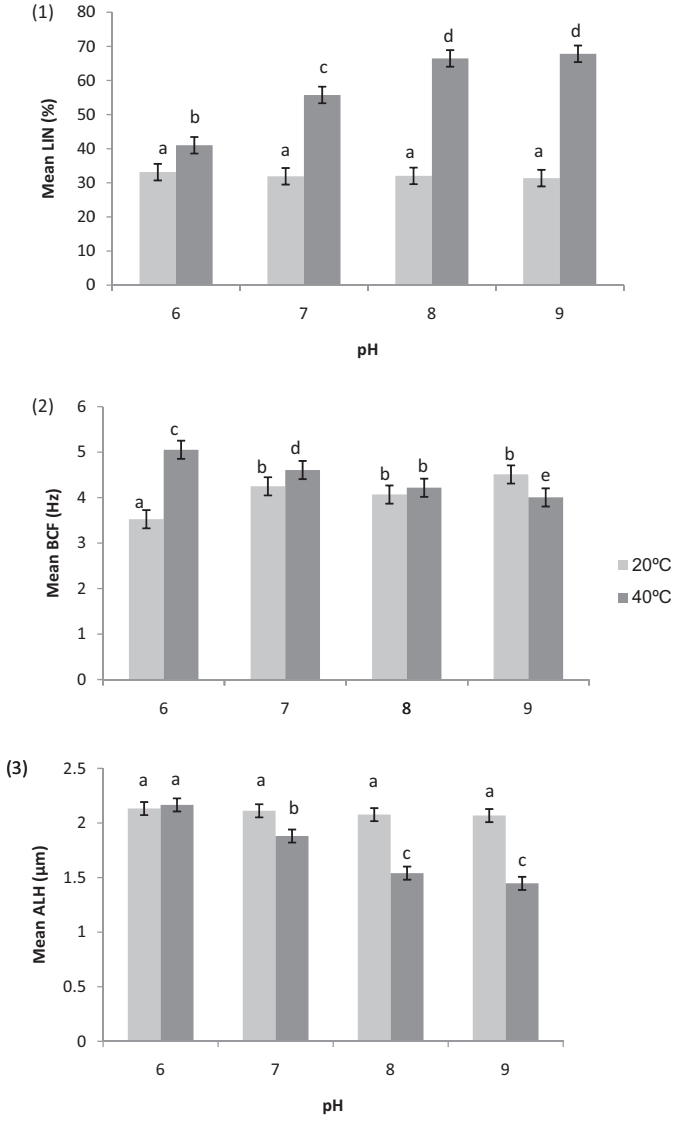

Fig. 2. Effect of pH and temperature on the motility of sperm of four male ostriches: (1) mean linearity (LIN); (2) mean beat cross frequency (BCF) and; (3) mean amplitude of lateral displacement (ALH). Vertical lines about the means denote standard errors. For any temperature and pH, those results which are significantly different (P < 0.05) are assigned a different letter.

3.3. Effect of temperature and pH on the viability of sperm

Sperm viability was not affected by temperature ($F_{1,85}$ = 0.01, P = 0.90). However, with an increase in pH, the percentage of live normal sperm decreased ($F_{3,85}$ = 8.19, P = 0.001; Fig. 3), while the percentage of dead sperm increased ($F_{3,85}$ = 6.85, P = 0.001) and the percentage of abnormal sperm remained unchanged throughout treatments ($F_{3,85}$ = 0.52, P = 0.672). Furthermore, we found variation between males, both in the percentage of live normal and live abnormal sperm ($F_{3,41}$ = 4.18, P = 0.01 and $F_{3,41}$ = 15.61, P = 0.001 respectively), but not in the percentage of dead sperm ($F_{3,41}$ = 0.98, P = 0.41).

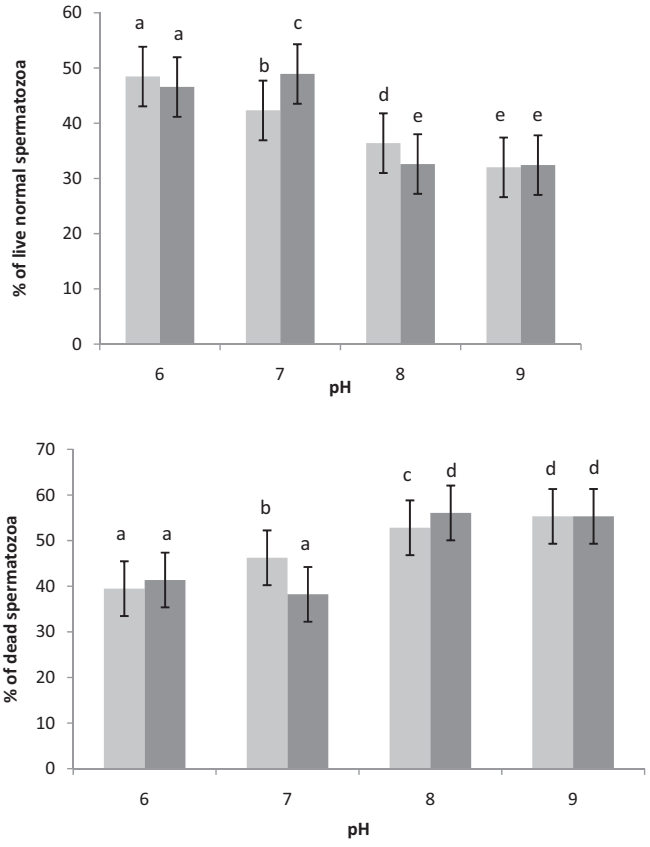

Fig. 3. Effect of pH and temperature on the percentages of live normal and dead sperm of four male ostriches. Vertical lines about the means denote standard errors. For any temperature and pH, those results which are significantly different (P < 0.05) are assigned a different letter.

Author's personal copy

4. Discussion

Our findings reveal that a combination of a high temperature (40 °C) and a pH 8 appear to maximize all velocity parameters, with the exception of curvilinear velocity. LIN showed a similar pattern but BCF and ALH had an opposite trend with lower values observed with higher pH. Temperature did not affect the viability of sperm, but higher proportions of live normal sperm were observed at lower pH values. Motility parameters have been extensively measured by CASA to investigate sperm motility in boars (Holt et al., 1997), bulls (Amann, 1989) and turkeys (Bakst and Cecil, 1992) and have been found to correlate well with fertility in avian species (chickens: Blesbois and Brillard, 2007; turkeys: King et al., 2000). For instance, fertilization success was found to be correlated to VAP in the chicken (Whishart and Palmer, 1986), and in the turkey, VSL, LIN and BCF described the overall sperm mobility phenotype, and VSL, VAP, VCL, LIN and BCF were higher in males described as having high mobility sperm, compared to males with low mobility sperm (King et al., 2000).

Ostrich sperm appear to be stimulated by near body temperature (in the ostrich: average: 38.3 °C with a range between 37.9 and 40.7 °C: Whittow, 1976) and slightly alkaline conditions. Previous research on chickens, turkeys and quails show a similar response whereby sperm motility became more vigorous for pH values higher than 7 (Holm and Wishart, 1998). However, no sperm movement was reported for any of these species in acidic conditions (pH 5–6) and sperm motility was only initiated at 40 °C from a pH of 7.8 for turkey sperm and 7.2 for quail sperm. Ostrich sperm seems to differ from this pattern, as sperm movement and velocity were detected for pH-values as low as 6 at both temperatures.

Originally, sperm were thought to remain motionless during their stay in the female's SSTs (chicken and turkey: Zavaleta and Ogasawara, 1987), until their motility was reinitiated upon release from the SSTs and their migration to the fertilization site. However, it has been argued (Froman, 2003) that sperm should stay motile during their stay in the SSTs as they have to maintain their position against a constant current generated by the epithelial cells of the SSTs. Our results are consistent with this being a possibility in ostriches, as sperm motility was observed, even for low pH values. In the ostrich, carbonic anhydrase, an enzyme that may regulate pH through bicarbonate and hydrogen secretion, was detected in the surface of the epithelium of the UVG and the SSTs (Holm et al., 2000) suggesting that females might be able to regulate sperm activity and storage through changing the pH. At pH 6 and 40 °C, high VCL and low VSL and LIN values that we recorded indicates sperm movement may be become near circular, suggesting a pH dependent regulatory mechanism that may enable sperm to turn around inside SST and move out.

In vitro studies have also demonstrated that the motility and metabolic rate of sperm, and consequently the lifespan of sperm, can be altered by the pH of diluents. For instance, in the chicken (Bogdonoff and Schaffner, 1954) low pH decreases motility, lactic acid production and oxygen uptake of sperm whereas a high pH may increase metabolic rate and subsequently affect sperm motility and viability. Further evidence arises from experiments showing that sperm from both chickens and turkeys can be kept immotile at an acidic pH and have their motility restored by diluting the medium with an alkaline buffer (Holm and Wishart, 1998). Our results appear to be in accordance with this, as the combination of a higher temperature and alkaline pH significantly increased the motility of sperm, while an acidic pH seems to impair sperm velocity.

Interestingly, while temperature did not affect sperm viability, we observed a greater proportion of live sperm for low to neutral pH values (6–7). pH is known to affect metabolic rate and it is generally accepted that a reduction in pH of diluents is required to store semen in vitro to maintain viability and fertilizing ability. For instance, in turkeys, sperm mixed with diluents at pH 6.5 in comparison to pH 7.5 had higher rates of fertility after 6 h in vitro storage at 15 °C (Giesen and Sexton, 1982). Although the exact mechanisms whereby low pH during in vitro storage promotes viability are unknown, it is thought that the reduction in sperm metabolism conserves energetic resources promoting lifespan. This is consistent with the results from our study, as higher pH values seemed to stimulate sperm motility, but sperm viability was compromised. This highlights the need to further investigate if the addition of compounds to diluents that may be metabolized by sperm can alleviate the negative effects of higher pH on sperm viability.

In conclusion, body temperature and slightly alkaline conditions seem to stimulate ostrich sperm motility, but it appears that an acidic to neutral pH range for the ostrich-specific diluents is required to ensure better sperm survival for artificial insemination during in vitro storage. These results are therefore the first milestone in our understanding of the sperm function in the in vitro environment for this species. However, the processes of in vivo sperm storage in the oviduct are a challenge to the development of appropriate methods to preserve sperm in vitro. Semen diluents must provide relatively constant physico-chemical stability to sperm during in vitro storage, including iso-osmotic and nearly neutral pH conditions, as well as appropriate energy substrates (Blesbois and Brillard, 2007; Donoghue and Wishart, 2000). However, as the fertilizing ability of sperm kept in vitro at avian body temperature cannot be maintained for long, sperm metabolism must be reduced by progressively decreasing temperature and potentially pH levels. Our results suggest that to maximize fertility in artificial insemination programs of ostriches, different temperature storage might be required: a lower temperature for long term storage that maximizes viability and longevity, and a body-like storage temperature for insemination purposes that stimulates sperm motility to maximize sperm storage.

Acknowledgments

We gratefully thank the Oudtshoorn Research Farm and especially Anel Engelbrecht, Stefan Engelbrecht, Zanell Brand and Basie Pfister for assistance in taking care of the birds, as well as Cecelia Mutlow at the Klein Karoo Laboratory for assistance with laboratory equipment. Funding was provided by the Western Cape Agricultural Research Trust

Author's personal copy

and the National Research Foundation (NRF) of South Africa through their THRIP program. M. Bonato was supported by a NRF Innovation Postdoctoral Fellowship.

References

Amann, R.P., 1989. Can the fertility potential of a seminal sample be predicted accurately? J. Androl. 10, 89–98.

Ashizawa, K., Wishart, G.J., 1987. Resolution of the sperm motility-stimulating principle of fowl seminal plasma into Ca^{2+} and an unidentified low molecular weight factor. J. Reprod. Fertil. 81, 495–499.

Ashizawa, K., Wishart, G.J., Nakao, H., Okino, Y., Tsuzuki, Y., 1994. Inhibition of temperature-dependent immobilization of fowl spermatozoa at body temperature by an increased intracellular pH. J. Reprod. Fertil. 101, 593–598.

Bakst, M.R., 2011. Role of the oviduct in maintaining sustained fertility in hens. J. Anim. Sci. 89, 1323–1329.

Bakst, M.R., 1980. Chicken and turkey oviductal pH at known times of oviposition. Poult. Sci. 59, 2793–2796.

Bakst, M.R., Cecil, H.C., 1992. Effect of bovine serum albumin on motility and fecundity of turkey spermatozoa before and after storage. J. Reprod. Fertil. 94, 287–293.

Blesbois, E., Brillard, J.P., 2007. Specific features of in vivo and in vitro sperm storage in birds. Animal 1, 1472–1481.

Bogdonoff, P.D., Schaffner, C.S., 1954. The effect of pH on in vitro survival, metabolic activity, and fertilizing ability of chicken semen. Poult. Sci. 33, 665–669.

Bonato, M., Rybnik, P.K., Malecki, I.A., Cornwallis, C.K., Cloete, S.W.P., 2011. Twice daily collection yields greater semen output and does not affect male libido in the ostrich. Anim. Reprod. Sci. 123, 258–264.

Ciereszko, A., Rybnik, P.K., Horbanczuk, J.O., Dietrich, G.J., Deas, A., Slowinska, M., Liszewska, E., Malecki, I.A., 2010. Biochemical characterization and sperm motility parameters of ostrich (*Struthio camelus*) semen. Anim. Reprod. Sci. 122, 222–228.

Cloete, S.W.P., Van Schalkwyk, S.J., Brand, Z., 1998. Ostrich breeding – progress towards scientifically based strategy. In: Huchzermeyer, F.W. (Ed.), Ratites in a Competitive World. Proceedings of the 2nd International Scientific Ratite Conference. Oudsthoorn, South Africa, 21–25 September, pp. 55–62.

Cloete, S.W.P., Malecki, I.A., 2011. Breeder welfare: the past, present and future. In: Glatz, P., Lunam, C., Malecki, I. (Eds.), The Welfare of Farmed Ratites. Springer, Berlin, pp. 13–43.

Donoghue, A.M., Wishart, G.J., 2000. Storage of poultry semen. Anim. Reprod. Sci. 62, 213–232.

Froman, F., 2003. Deduction of a model for sperm storage in the oviduct of the domestic fowl (*Gallus domesticus*). Biol. Reprod. 69, 248–253.

Giesen, A.F., Sexton, T.J., 1982. Beltsville poultry semen extender: 7. Comparison of commercial diluents holding turkey semen six hours at 15 °C. Poult. Sci. 62, 379–381.

Holm, L., Ruziwa, S.D., Dantzer, V., Ridderstrale, Y., 2000. Carbonic anhydrase in the utero-vaginal junction of immature and mature ostriches. Br. Poult. Sci. 41, 244–249.

Holm, L., Wishart, G.J., 1998. The effect of pH on the spermatozoa from chicken, turkey and quail. Anim. Reprod. Sci. 54, 45–54.

Holt, C., Holt, W.V., Moore, H.D., Reed, H.C., Curnock, R.M., 1997. Objectively measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: results of two fertility trials. J. Androl. 18, 312–323.

King, L.M., Holsberger, D.R., Donoghue, A.M., 2000. Correlation of CASA velocity and linearity parameters with sperm mobility phenotypes in turkeys. J. Androl. 21, 65–71.

Lake, P.E., Stewart, J.M., 1978. Artificial insemination in poultry. In: Ministry of Agriculture Fisheries and Food, Bulletin 213. Her Majesty's Stationery Office, London.

Lake, P.E., 1986. The history and future of the cryopreservation of avian germ plasma. Poult. Sci. 65, 1–15.

Lorenz, F.W., 1950. Onset and duration of fertility in turkeys. Poult. Sci. 29, 20–26.

Malecki, I.A., Rybnik, P.K., Martin, G.B., 2008. Artificial insemination technology for ratites: a review. Aust. J. Exp. Agric. 48, 1284–1292.

Malecki, I.A., Cloete, S.W.P., Gertenbach, W.D., Martin, G.B., 2004. Sperm storage and duration of fertility in female ostriches (Struthio camelus). S. Afr. J. Anim. Sci. 34, 158–165.

Reddish, J.M., Kirby, J.D., Anthony, N.B., 1996. Analysis of poultry fertility data. 3. Analysis of the duration of fertility in naturally mating Japanese quail. Poult. Sci. 75, 135–139.

Rybnik, P.K., Horbanczuk, J.O., Naranowicz, H., Lukaszewicz, E., Malecki, I.A., 2007. Semen collection in the ostriches using a dummy or a teaser female. Br. Poult. Sci. 48, 635–643.

Soley, J.T., Roberts, D.C., 1994. Ultrastucture of ostrich (Struthio camelus) spermatozoa II. Scanning electron microscopy. Ondersterpoort J. Vet. Res. 61, 239–246.

Whishart, G.J., Palmer, F.H., 1986. Correlation of the fertilising ability of semen from individual male fowls with sperm motility and ATP content. Br. Poult. Sci. 27, 97–102.

Whittow, G.C., 1976. Regulation of body temperature. In: Sturkie, P.D. (Ed.), Avian Physiology. Bailliere, Tindal & Cassel, London.

Ya-jie, J.I., Yan-bo, Y., Wu-zi, D., 2001. Studies on ostrich semen character and semen storage at low temperature. J. Econ. Anim. 5, 49–54.

Zavaleta, D., Ogasawara, F., 1987. A review of the mechanism of the release of spermatozoa from the storage tubules in the fowl and turkey oviduct. Worlds Poult. Sci. J. 43, 132–139.