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INVESTIGATIONS ON DIFFERENT SEMEN EXTENDERS FOR COCKATIEL SEMEN

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ABSTRACT

The aim of the present study was the modification and evaluation of three different semen extenders for cockatiel semen in order to achieve a long survival time for transport, examination purpose and for potential cryopreservation, respectively. Therefore, individual and pooled semen samples of 30 cockatiels (*Nymphicus hollandicus*) were investigated for pH and osmolality values and subsequently pH and osmolality values of the semen extenders were adjusted to those values in the semen. Pooled semen samples were then partitioned into four equal parts and diluted with the three different semen extenders in 1:4 and 1:8 dilution. 1% glucose-Ringer's solution was used as control, respectively. A total of 64 incremental diluted semen samples were obtained for investigation. Each dilution was investigated regarding sperm motility immediately after dilution and another four times every 30 minutes. Sperm viability was evaluated 0 and 120 minutes after dilution via eosin B-stain on the diluted semen samples and in pure semen samples. Additionally, the fluorescence stain SYBR® Green/propidium iodide was used to assess sperm viability. The results indicate that cockatiel spermatozoa are highly sensitive to variations in pH and osmolality, requiring adjustment of commercial diluents to pH = 7.42 and osmolality = 300 mOsm/kg. Modified Lake diluent maintained higher viability and motility than other diluents tested. Sperm morphology was indicated to be least adversely affected by modified Lake diluent in 1:4 concentration compared to other semen extenders and concentrations used.

Keywords: psittacine spermatozoa, parrots, semen analysis, sperm motility, assisted reproduction.

INTRODUCTION

More than one third of all psittacine species are listed as threatened by the International Union for Conservation of Nature and Natural Resources (IUCN 2014). The main risk and cause of this decline is, apart from illegal trade and slight clutch size in some species, the steadily progressing loss of habitat (Snyder *et al*. 2000, Wright *et al*. 2001). Captive breeding programs have been demonstrated as important tools for species conservation (Collar and Butchart 2014) using assisted reproduction (AR) to improve the breeding success (Blanco *et al*. 2009, Lierz *et al*. 2013, Fischer *et al*. 2014a). Assisted reproduction includes semen collection and artificial insemination (AI) which have been practiced effectively in domestic fowl for almost 80 years (Quinn and Burrows 1936) but experiences in AR in psittacine birds are scarce. Artificial insemination has been described in cockatiels (Neumann *et al*. 2013), budgerigars (Samour 2002) and some larger parrot species (Brock 1991, Lierz *et al*. 2013, Fischer *et al*. 2014a) but up to now AI in psittacines is mostly only possible with fresh semen (Brock 1991, Neumann *et al*.

2013). However, fresh semen cannot be stored for a prolonged period of time to overcome transport or seasonal dependences. To preserve physiological properties and functions of spermatozoa during storage the composition of the semen extender (SE) is fundamental (Salamon and Maxwell 2000). An appropriate diluent has to provide an energy source for spermatozoa and maintain pH and osmolality levels preferably identical to those of seminal plasma, the natural medium for sperm (Siudzinska and Lukaszewicz 2008, Blanco *et al*. 2012). Diluents should be able to compensate eventual pH-value deferrals caused by sperm metabolic products through their buffering capacity. Osmolality can also affect sperm function as low osmolality values cause the spermatozoa to swell when water is drawn into the cells, whereas in a hyperosmotic solution, spermatozoa lose water and shrink (Bakst 1980). Important parameters for the evaluation of a semen extender are the assessment of total motility (MOT) and progressive motility (PMOT) as well as the percentage of viable and morphological normal spermatozoa (MNS) (Fischer *et al*. 2014b). These parameters enable the estimation of long-term storage of spermatozoa and fertilization following AI (Froman 2000, Blesbois *et al*. 2008). Semen extenders have been studied in the domestic fowl and diluents such as Lake diluent (Lake and Stewart 1978) and Beltsville Poultry Semen Extender (BPSE) (Sexton 1977) are commercially available for poultry. Turkey semen diluted with Beltsville Poultry Semen Extender has been demonstrated to be less deteriorated during short time storage (3 h) at 5 °C compared to Lake diluent, although no significant differences in motility, viability and membrane integrity have been confirmed (Iaffaldano *et al*. 2010). Other findings demonstrated a positive influence on the survival and motility of crane and turkey spermatozoa through supplementation of 0.1 M betaine hydrochloride and 30 mM adenosine triphosphate (ATP) (Blanco *et al*. 2011). Extenders in domestic fowl are the basis for studies on semen extenders for non-domestic birds due to the lack of species specific studies, especially in psittacines. In budgerigars, Lake diluent and modified Biggers, Whitten and Whittinghams medium have been evaluated (Samour *et al*. 1988) whereas in cockatiels 1% glucose-Ringer's solution (1% G) demonstrated best results in PMOT in comparison with five other semen extenders after a storage time of 120 minutes (Stelzer 2004). Lake diluent has also been used as a semen extender in Hispaniolan parrots (*Amazona ventralis*) prior to AI (Brock 1991). However, only one study exists on the evaluation of semen extenders with psittacine semen over a defined period of time (Stelzer 2004).

The aim of the present study was to modify semen extenders for cockatiel semen in order to achieve an appropriate dilution for dependable short and long time semen storage. Cockatiels were chosen as model animals for psittacines due to their almost year-round seasonality (Arndt 1996) so that semen can be collected quite reliably through the established massage method. Moreover, basic spermatological data are available for this species (Fischer *et al*. 2014b) and AR techniques have been applied successfully before (Neumann *et al*. 2013). This study should be a first step towards an efficient protocol for cryopreservation of psittacine semen which may be easily modified for large parrots.

MATERIALS AND METHODS

Cockatiels and semen collection: In this study, 30 healthy, male, two to four year old cockatiels were randomly selected out of a breeding flock and clinically examined. All birds were experienced breeding birds, which were paired with female birds and had produced fertile clutches in previous breeding seasons. Good body condition score (BCS 3 or 4 on a scale of 1 to 5) and absence of infectious agents (*Chlamydia* sp., *Candida* sp., parrot bornavirus, avian polyomavirus, psittacine circovirus, paramyxovirus 1 & 3 and endoparasites) were attested to all birds. The cockatiels were marked with colored legbands and housed in three separated indoor (3 x 2.4 x 2.4 m) - outdoor (3 x 2.5 x 2.5 m) combined aviaries placed in their original flock. Ambient temperature in the indoor aviaries was approximately 21 °C and a photoschedule of 13:11 LD assisted by artificial light (Arcadia 20 W Fluorescent Bird Lamp, 2,4% UVB / 12% UVA; Arcadia Products plc, Redhill, United Kingdom) was applied. Nest boxes were offered in all aviaries to stimulate nesting activity. Water was offered ad libitum. Birds were fed a commercial, mineral supplemented cockatiel diet plus fresh fruits and vegetables in the morning.

Semen was collected once a week during the months of February to November using a massage method as described before (Neumann *et al*. 2013). Briefly, the birds were manually restrained in dorsal recumbency by an assistant and the cloaca was cleaned with tissues if faecal contamination was visible. Thumb and index

finger of the examiner were positioned on both sides of the cloaca and the middle finger dorsally near the uropygial gland. Gentle opposed rhythmic movements of the thumb and index finger for approximately 30 seconds induced the ejaculation. If no active ejaculation could be evoked, semen could yet be massaged out of the deferent duct if present. Semen was then collected in a graduated microcapillary tube (Wiretrol® II 1 to 5 µl, Drummond Scientific Co, Broomall, PA, USA).

This study was conducted in accordance with national laws and the methods were approved by the competent regional authority (Regierungspraesidium) Giessen, Germany, with the permission number GI 18/9 No. 07/2012.

Semen analysis and adjustment of semen extenders

Initial semen analysis and osmolality measurements: Collecting the semen in a microcapillary tube permitted evaluation of volume, semen color, sperm density and estimated sperm motility. Until investigation, individual samples were stored in a cool box filled with cold water (+ 4 °C). Constancy of temperature was verified by a digital thermometer. Only semen samples without macroscopically visible contamination of feces and urates were used for analysis. Sperm density and motility were evaluated immediately after semen collection by placing the microcapillary tube onto a slide using a Leica DM2500 microscope (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) and X 100 magnification. A pre-estimation of the amount of spermatozoa was performed by grading sperm density inside the capillary from 0 to 5 (0: no spermatozoon per visual field, 1: 1 to 20 spermatozoa per visual field, 2: 21 to 100 spermatozoa per visual field, 3: 101 to 200 spermatozoa per visual field, 4: > 201 spermatozoa per visual field, 5: not countable). Sperm motility in the microcapillary tube was subjectively estimated and classified from 0 to 4 by judging the velocity of spermatozoa heading towards the liquid/air barrier (0: no movement, 1: slow, 2: sedate, 3: at pace, 4: extremely fast).

The pH-value was then measured from a small drop of semen (≤ 0.5 µl) out of individual samples by using indicator test-strips (Spezialindikatorpapier, pH 5.5 – 9.0; 5.4 – 7.0 & 6.4 – 8.0; Macherey-Nagel GmbH & co. KG, Dueren, Germany). Sperm concentration was assessed in 100fold diluted (distilled water) semen in a Neubauer improved counting chamber and total sperm count was calculated by multiplication of the sperm concentration with the semen volume (Behncke 2002).

For osmolality measurement, semen of cockatiels was pooled in an Eppendorf® Safe-lock microcentrifuge tube (Eppendorf AG, Hamburg, Germany) by adding non contaminated individual samples until a volume of at least 100 µl was reached. Samples were cooled to + 4 °C until measurements were performed approximately 60 minutes after collection with a semi-micro osmometer (Loeser Osmometer Typ 6, Loeser Messtechnik, Berlin, Germany). The measurements were performed according to the manufacturer's specifications in nine replicates in order to achieve statistically sound values.

Adjustment of semen extenders: Three semen extenders, Lake diluent (SE 1) (Lake and Stewart 1978), a semen extender described by Blanco *et al*. (SE 2) (Blanco *et al*. 2008) and Beltsville Poultry Semen Extender (SE 3) (Sexton 1977), were prepared with modifications to the original formula by dissolving the chemicals in water for injection (Wasser fuer Injektionszwecke, Serumwerk Bernburg AG, Bernburg, Germany). All used chemicals were obtained from Sigma® Life Science as BioXtra or BioUltra products and were dissolved without heating. PH and osmolality values of the solutions were measured (S20 SevenEasy™ pH, Mettler Toledo GmbH, Giessen, Germany; Loeser Osmometer Typ 6, Loeser Messtechnik, Berlin, Germany) and adjusted accordingly to pH = 7.42 by addition of 1 M NaOH or 1 M HCl solution and to osmolality = 300 mOsm/kg by varying the amount of soluble substances (for final composition see Table 1). Glucose solution 1% (Ringer Loesung B. Braun Ecoflac® plus & Glucose 5% B. Braun Ecoflac® plus, B. Braun, Melsungen AG, Melsungen, Germany) was used as control according to previous studies and not adjusted to the mentioned values to assure better comparability. All solutions were sterile-filtered (Millex® Syringe Filter Units, 0.22 µm, 33mm, Merck Millipore, Merck KGaA, Darmstadt, Germany) and bottled in small portions of each 10 ml into sterile tubes (CELLSTAR® TUBES, Greiner Bio-One GmbH, Frickenhausen, Germany). Until use, the modified semen extenders were stored at – 22 °C. To SE 2, ATP was added immediately before dilution of the pooled semen sample. The semen extenders were refrozen immediately after use.

Table 1. Components of different semen extenders after adjustment to pH and osmolality (except for 1% G).

1% G[a]		SE 1[b]		SE 2[c]			SE 3[d]	
constituent	(ml)	constituent	weight (g)	constituent	weight (g)	molarity	constituent	weight (g)
Ringer's solution	4	D-fructose	***0.70***	D-fructose	***0.575***		D-fructose	***0.333***
Glucose solution 5%	1	sodium glutamate	1.92	sodium glutamate	***2.00***		sodium glutamate	***0.578***
		magnesium acetate (tetrahydrate)	0.08	***potassium acetate (anhydrous)***	***0.25***		sodium acetate trihydrate	***0.287***
		potassium acetate (anhydrous)	0.50	***betaine hydrochloride***		0.1 M	potassium citrate	***0.0427***
		polyvinyl-pyrrolidone (MW 10,000)	***0.30***	polyvinyl-pyrrolidone (MW 10,000)	***0.15***		magnesium chloride hexahydrate	***0.0227***
				ATP		30 mM	dipotassium hydrogen phosphate	***0.8467***
							potassium dihydrogen phosphate	***0.0433***
						N-tris (Hydroxy-methyl) methyl-2-aminoethane sulfonic acid		***0.195***

Bold italic font indicates modification from original formula. Dilute in sterile water and dissolve to 100 g.
Abbreviations: G, glucose-Ringer's solution; SE, semen extender. [a] based on (Stelzer 2004) [b]based on (Lake and Stewart 1978) [c] based on (Blanco *et al*. 2008) [d] based on (Sexton 1977).

Evaluation of semen extenders: All evaluations were performed by a single investigator with proven intra-observer reliability and experiences in semen analysis for more than two years. Investigated and pooled semen samples were partitioned into four aliquots and diluted 1:5 with 1% G as a control and three different modified semen extenders Lake diluent, Blanco and BPSE (SE 1 – 3) in 1:4 and 1:8 dilution, respectively. 30 mM ATP was added to SE 2 shortly before mixing with the semen sample to reveal possible positive effects on cockatiel spermatozoa motility and survival. Each semen extender in each dilution was evaluated at least eight times regarding sperm motility, viability and morphology which results in a total of 64 investigations including the samples diluted with 1% G.

Motility assessment: Sperm motility was checked manually after 0, 30, 60, 90 and 120 minutes by pipetting 8 µl of the semen/diluent mixture onto a slide, covering it with a cover slip and counting the spermatozoa in a defined area of each 5 visual fields as previously described (Fischer *et al*. 2014b). They there classified into non-motile and motile spermatozoa (MOT), whereby forward progression (PMOT), local motility and circular motility were differentiated. Motility was not evaluated in pure samples as they were too dense to differentiate between the individual spermatozoa and motility may have been altered by high interspermatozoal interferences. Between the evaluation times samples were stored in a cool box filled with cold water at + 4 °C.

Viability assessment: Viability stains were made of pure semen samples and semen compounded with the different diluents after 0 and 120 minutes (0 min = immediately after mixing). For staining eosin B 2% and the fluorescence stain SYBR® Green/propidium iodide (LIVE/DEAD® Sperm Viability Kit; Invitrogen™, Molecular Probes™, Eugene, OR, USA) were used according to previous studies (Fischer *et al*. 2014b). Briefly, 2 µl of semen were mixed with eosin B 2% on a glass slide, incubated for 15 seconds and smeared according to standard procedures (WHO 2010). In the dried smear 200 spermatozoa per slide were evaluated. Thereby dead spermatozoa (colored) were distinguished from live spermatozoa (unstained or white) and subsequently a live/dead ratio was calculated.

Furthermore 5 µl of semen were added to 2.5 µl of the SYBR® Green/propidium iodide solution prepared as specified by the manufacturer and incubated at room

temperature for 10 minutes in a brown microfuge tube to protect the reagents from light. 7 µl of the mixture were then pipetted onto a slide, covered with a cover slip and evaluated at X 400 magnification with a fluorescence microscope with a fluorescent light source (Leica DM2500; Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany). Green spermatozoa were interpreted as live sperm cells (excitation: 488 nm, emission: 516 nm), red spermatozoa were counted as dead sperm cells (excitation: 530 nm, emission: 617 nm).

Morphologic evaluation of spermatozoa: A morphologic evaluation of 200 spermatozoa in the eosin B smear was performed at X 1000 magnification using oil immersion with a Leica DM2500 microscope (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany). Spermatozoa were classified into morphologic normal and abnormal spermatozoa. The different abnormalities were further classified according to a detailed description previously published on gander spermatozoa (Marvan *et al*. 1981), which had been used in psittacine semen samples before (Behncke 2002, Fischer *et al*. 2014a). According to standardized evaluation of sperm morphology, the classification of abnormalities was performed using specific categories (1. loose heads, 2. other head abnormalities, 3. acrosome abnormalities, 4. midpiece abnormalities, 5. tail abnormalities) in order of priority (WHO 2010).

Statistical analysis: In accordance to the study design each pooled semen sample formed a statistical unit in the analysis. Statistically significant differences in mean for sperm motility parameters (MOT and PMOT), sperm viability values and sperm morphology using the different semen additives were tested based on two- or three-factorial analysis of variance (ANOVA) with repeated measures using the program BMDP2V from the statistical software package BMDP (Dixon 1993). To obtain nearly normal distributions of the analyzed variables, in morphology analysis an arcsine transformation of the data was performed prior to the analysis. Correlation was assessed using the Pearson correlation coefficient (r). In general, a significance level of $\alpha = 0.05$ was used, so p-values less or equal to 0.05 indicated statistical significant effects.

RESULTS

Initial semen analysis and osmolality measurements: The time interval between the commencement of semen collection and the beginning of the initial semen analysis did not exceed 41 minutes. Volume reached from 0.1 to 19.4 µl (n = 262; $\tilde{x}$ = 2.20 µl; Q1 = 1.00, Q3 = 4.125) while in 38.2% of the samples semen color was between grayish and whitish and in 31.3% of the samples transparent. Median sperm density in the capillary tube was 3.5 (n = 262; Q1 = 2.5, Q3 = 4.0; range: 0 to 5). Mean estimated sperm motility in the microcapillary tube was 2.39 ± 1.19 (n = 262; $\bar{x}$ ± SD; range: 0 to 4). Sperm concentration ranged between 30,000 and 5,950,000 spermatozoa/µl (n = 51; $\tilde{x}$ = 350,000; Q1 = 121,500, Q3 = 740,000), while measurements of pH revealed a range of pH from 6.4 to 8.5 (n = 70; $\bar{x}$ ± SD = 7.42 ± 0.42) in individual and a range of osmolality from 290 to 320 mOsm/kg (n = 9; $\bar{x}$ ± SD = 298.62 ± 9.56) in pooled semen samples.

Adjustment of semen extenders: Semen extenders 1 to 3 were adjusted according to pH and osmolality values of the semen samples. The final composition after adjustment of the three semen extenders is shown in Table 1. All modifications of semen extenders from the original description are indicated by bold italic font. To guarantee comparability to previous studies pH (range: 4.9 to 6.13; $\bar{x}$ ± SD = 5.55 ± 0.41) and osmolality (range: 304 to 337 mOsm/kg; $\bar{x}$ ± SD = 319.22 ± 10.43) of 1% G were not adjusted.

Evaluation of semen extenders: By partitioning of the 16 pooled semen samples 64 samples were investigated for sperm motility, sperm viability and sperm morphology. The results of the global comparison of semen extenders, concentrations and observation time by three-way ANOVA with repeated measures in respect to time is provided in Table 2.

Motility analysis: The global mean comparison revealed significant differences between extenders and between time (both $p < 0.0001$) while the differences between concentration were barely not significant ($p = 0.08$) and all interactions were not statistically significant (Table 2). Over the investigation period of 120 minutes a decline in sperm motility (MOT and PMOT) was detected in the control samples as well as in all samples using semen extenders in each dilution (Tables 3 & 4). Modified SE 1 in 1:4 dilution initially started with a higher ($p < 0.05$) MOT ($\bar{x}$ ± SD = 77.31% ± 7.01%) value compared to the control and all other semen extenders in both dilutions (Table 3). Similar results were observed in initial PMOT ($\bar{x}$ ± SD = 70.74% ± 7.82%) (Table 4). Pairwise comparison of the

different semen extenders with 1% G revealed SE 1 in 1:4 dilution to maintain MOT and PMOT better than 1% G ($p < 0.01$). Additionally, PMOT, but not MOT was maintained better by SE 1 in 1:8 dilution and SE 3 in 1:4 dilution compared to 1% G ($p \leq 0.01$) (Table 5). The comparison of both dilutions (1:4 and 1:8) within SE 1 showed differences in PMOT ($p < 0.05$), but not in MOT ($p > 0.05$). Again, 1:4 dilution achieved better results.

Table 2. Resulting p-values from the three-way ANOVA with repeated measures in respect to time; here: global comparison.

Variable		main effects (p-value)			interaction effects (p-value)			
		SE	c	t	SE x c	SE x t	c x t	SE x c x t
	MOT	< 0.0001	0.0803	< 0.0001	0.6429	0.5114	0.1303	0.5630
	PMOT	< 0.0001	0.0072	< 0.0001	0.3166	0.4152	0.1558	0.8506
	Viability	< 0.0001	0.8077	< 0.0001	0.8961	0.5662	0.0884	0.4643
Morphology	normal	< 0.0001	0.0651	0.0003	0.0610	0.2421	0.4525	0.1540
	Acrosome abnormalities	0.2514	0.1114	0.8845	0.8892	0.6415	0.9614	0.4499
	Head abnormalities	0.0017	0.3772	0.0216	0.5464	0.9238	0.9816	0.9004
	Midpiece abnormalities	0.2062	0.3276	0.4114	0.0312	0.7616	0.4649	0.6417
	Tail abnormalities	0.1044	0.2270	0.0021	0.4406	0.1195	0.1709	0.1570
	Multiple abnormalities	0.0648	0.7190	0.8837	0.7190	0.4524	0.9833	0.7510

Abbreviations: SE, semen extender; c, concentration; t, time; x, interaction; MOT, total motility; PMOT, progressive motility.

Table 3. Sperm motility (MOT) in percent by comparison of different semen extenders and dilutions (n=64; ± SD).

Evaluation time	1% G (1:5)	SE 1 (1:4)	SE 1 (1:8)	SE 2 (1:4)	SE 2 (1:8)	SE 3 (1:4)	SE 3 (1:8)
0 min	56.63 ± 13.57	77.31 ± 7.01	65.00 ± 10.68	52.61 ± 10.48	49.87 ± 14.75	64.17 ± 9.92	60.46 ± 5.68
30 min	56.36 ± 17.34	74.11 ± 9.35	62.89 ± 13.39	52.14 ± 6.01	49.52 ± 12.03	64.25 ± 9.11	61.66 ± 8.13
60 min	48.88 ± 14.49	68.28 ± 9.50	62.31 ± 13.04	47.84 ± 5.68	41.64 ± 14.10	59.34 ± 6.19	56.24 ± 9.76
90 min	45.27 ± 13.72	63.55 ± 11.07	56.14 ± 12.09	44.53 ± 2.98	38.43 ± 15.82	54.05 ± 6.89	49.80 ± 12.04
120 min	37.03 ± 16.65	59.14 ± 9.69	54.31 ± 12.06	38.15 ± 4.79	36.45 ± 13.56	44.72 ± 7.95	46.76 ± 12.26

Abbreviations: G, glucose-Ringer's solution; SE, semen extender.

Table 4. Progressive sperm motility (PMOT) in percent by comparison of different semen extenders and dilutions (n=64; ± SD).

Evaluation time	1% G (1:5)	SE 1 (1:4)	SE 1 (1:8)	SE 2 (1:4)	SE 2 (1:8)	SE 3 (1:4)	SE 3 (1:8)
0 min	41.89 ± 15.00	70.74 ± 7.82	55.03 ± 10.06	40.69 ± 9.17	37.90 ± 14.57	54.04 ± 11.34	48.13 ± 11.32
30 min	41.13 ± 16.27	65.93 ± 9.32	50.66 ± 13.67	38.93 ± 4.14	36.23 ± 13.40	53.66 ± 7.23	48.82 ± 8.83
60 min	33.75 ± 12.09	59.42 ± 9.45	48.70 ± 12.65	34.69 ± 3.62	30.28 ± 12.65	47.43 ± 6.34	41.81 ± 9.02
90 min	30.87 ± 10.21	54.07 ± 10.25	41.09 ± 10.49	31.05 ± 6.78	23.69 ± 13.53	41.48 ± 6.79	35.62 ± 7.17
120 min	21.82 ± 13.21	47.35 ± 11.00	41.10 ± 12.02	22.77 ± 8.86	20.76 ± 11.44	30.42 ± 7.38	29.42 ± 8.33

Abbreviations: G = glucose-Ringer's solution; SE = semen extender.

Table 5. Resulting p-values from the two-way ANOVA with repeated measures in respect to time; here: pairwise comparison of SE's with 1% glucose-Ringer's solution.

p-value	SE 1 (1:4)	SE 1 (1:8)	SE 2 (1:4)	SE 2 (1:8)	SE 3 (1:4)	SE 3 (1:8)
Total motility	0.0014	0.0529	0.7287	0.3487	0.1206	0.2651
Progressive motility	< 0.0001	0.0073	0.9476	0.4185	0.0129	0.1252
Viability	0.0028	0.0045	0.8983	0.9707	0.1725	0.3086

Abbreviation: SE, semen extender.

Viability analysis: The comparison of the percentage of viable spermatozoa counted in 46 eosin B-smears and 46 fluorescence stains SYBR® Green/propidium iodide demonstrated a strong correlation of both evaluation methods (n = 46; r = 0.893; p < 0.001). However, due to volume limitation, the remaining samples had to be stained using eosin B only (Figure 1 and Table 6). Pairwise comparison of the different semen extenders with 1% G showed that only SE 1 obtained higher viability values (p < 0.05). The comparison between SE 1 (1:4) and SE 1 (1:8) did not point out any differences (p > 0.05).

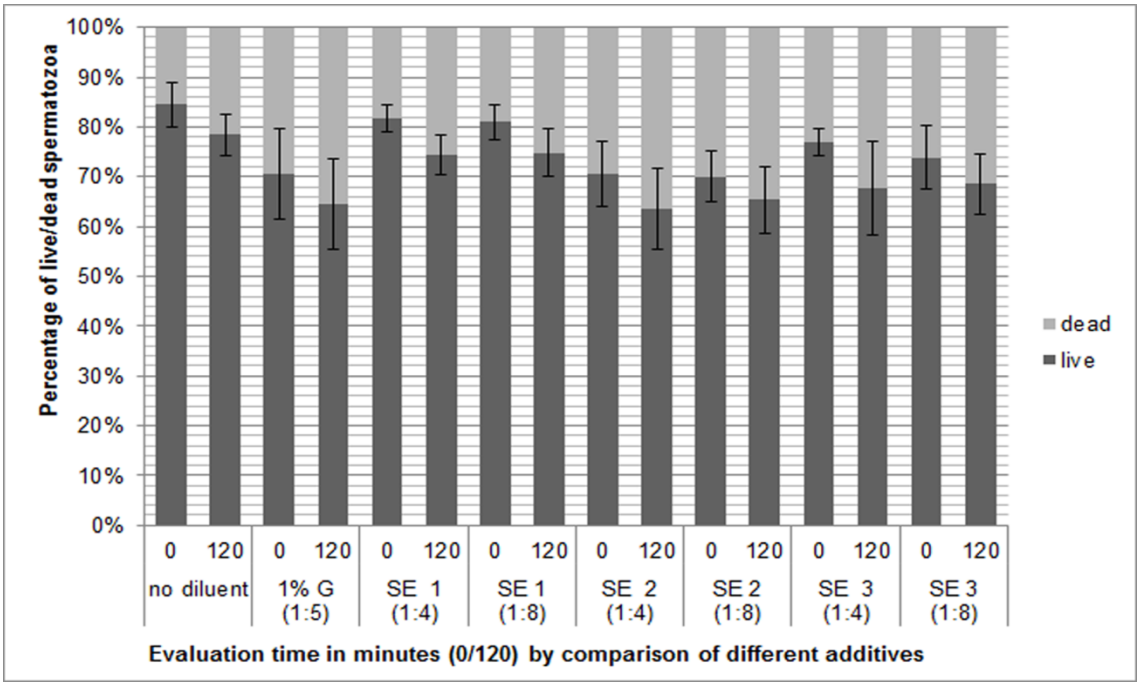

Figure 1. Mean viability (including standard deviation) in eosin B smears by comparison of pure semen samples and different semen extenders in different dilutions (n=140; ± SD).
Abbreviations: G, glucose-Ringer's solution; SE, semen extender.

Table 6. Mean sperm viability (eosin B) in percent by comparison of the different semen extenders (n=64; ± SD).

evaluation time	no diluent	1% G (1:5)	SE 1 (1:4)	SE 1 (1:8)	SE 2 (1:4)	SE 2 (1:8)	SE 3 (1:4)	SE 3 (1:8)
0 min	84.45 ± 4.42	70.53 ± 8.98	81.69 ± 2.78	81.00 ± 3.45	70.50 ± 6.63	70.06 ± 5.16	77.00 ± 2.79	73.88 ± 6.31
120 min	78.45 ± 4.00	64.56 ± 9.15	75.25 ± 3.81	74.81 ± 4.69	63.69 ± 8.04	65.31 ± 6.76	67.75 ± 9.32	68.50 ± 5.95

Abbreviations: G, glucose-Ringer's solution; SE, semen extender.

Morphology analysis: Morphological evaluation of 200 spermatozoa in each of 140 stains revealed multiple changes in cockatiel spermatozoa (Figure 2). Taken all together, mainly the tail region (34.8%) and the head region (21.3%) were morphologically altered, followed by abnormalities in the acrosome (1.99%) and midpiece section (1.6%). In 9.83% of the morphological abnormal spermatozoa multiple abnormalities occurred within one spermatozoon. A wide range (10% to 61%; $\bar{x}$ ± SD = 30.40% ± 13.13%) of morphologically normal spermatozoa (MNS) was found. Morphology was significantly influenced by the different semen extenders and evaluation times (Table 7). The percentage of MNS depended on the utilisation of the different semen extenders and the investigation times (p < 0.0001) (Table 7). Again, experimentally modified SE 1 in 1:4 dilution achieved a higher amount of MNS. Statistical analysis revealed that abnormalities concerning head abnormalities were influenced by the use of the different SE (p < 0.05) but not by storage time, whereas SE 1 showed the least rate of those abnormalities. However, tail abnormalities were not influenced by the different semen extenders (p > 0.05) but by time (p < 0.001). Acrosome, midpiece and multiple abnormalities were neither influenced by semen extender, nor by storage time (p > 0.05). In general, none of the morphological characteristics was significantly influenced by treatment by time interaction.

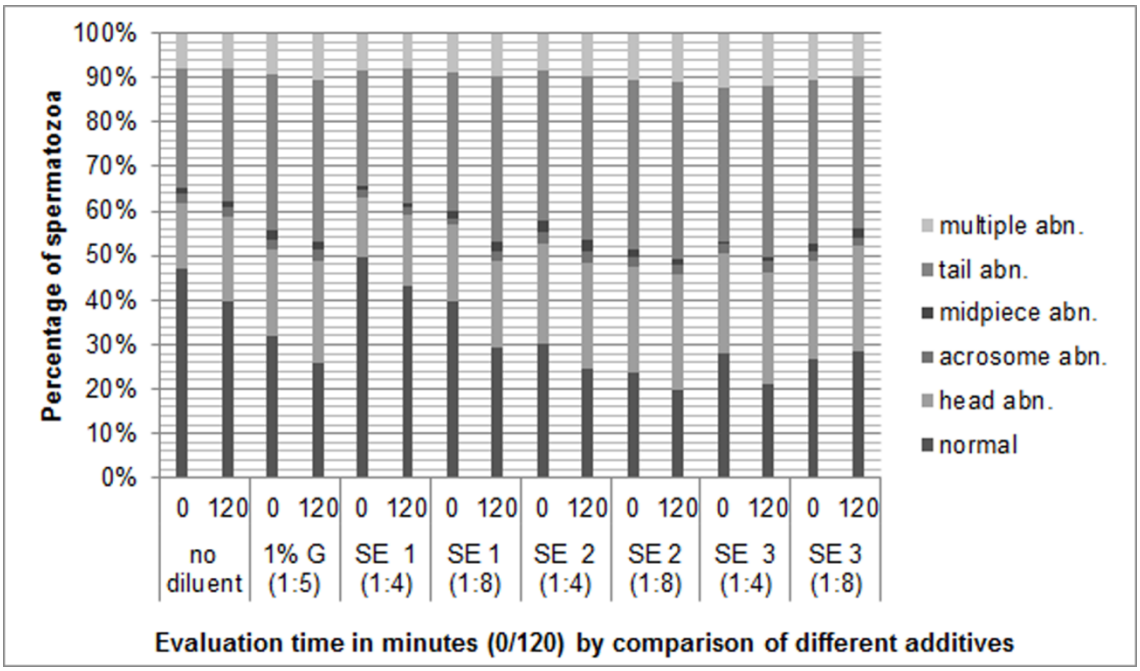

Figure 2. Graphical presentation of mean sperm morphology (time 0 and 120) in semen stains by comparison of different additives (n = 140).
Abbreviations: G, glucose-Ringer's solution; SE, semen extender; abn., abnormality.

Table 7. Resulting p-values from a two-way ANOVA with repeated measures in respect to time; here: sperm morphology.

p-value	Normal	Head abn.	Acrosome abn.	Midpiece abn.	Tail abn.	Multiple abn.
Treatment	<0.0001	0.0214	0.4102	0.0956	0.2247	0.1430
Time	<0.0001	0.0036	0.9274	0.6613	0.0008	0.5960
Interaction treatment by time	0.3173	0.9701	0.8853	0.5366	0.0980	0.6990

Abbreviation: abn., abnormality.

DISCUSSION

In our study, SE 1 allowed higher MOT and PMOT than previously used ones in psittacine AR. Before, 1% G solution was reported to maintain sperm motility the best (Stelzer 2004), which could not be confirmed in this study. Firstly, our modified SE 1 did start with a higher initial PMOT ($\bar{x}$ ± SD = 70.74% ± 7.82%) than 1% G diluted cockatiel semen in the previous study (approx. 61%). Secondly, it also retained PMOT over a 120 minutes investigation period remarkably higher compared to the best dilution of previous investigations (decrease approx. 56%) (Stelzer 2004). Additionally, semen osmolality values have been investigated for the first time in psittacines and impacts of the different semen extenders on sperm viability and morphology were evaluated. Usually, semen samples are evaluated individually to exclude interferences between spermatozoa of different males. Due to the little volume obtained from the cockatiels, detailed studies into the course of semen quality were only possible with pooled semen samples. Therefore, a potential impairment of semen quality in the consequence of sperm competition interactions cannot be excluded in the present study.

Motility analysis of cockatiel semen diluted with 1% G revealed an average MOT and PMOT in the range of previous studies (Fischer *et al*. 2014b), although it did neither boast best results concerning sperm motility (MOT and PMOT), nor viability compared to the used SE 1 in our study. In budgerigars the addition of three different semen extenders was subjectively evaluated judging half-life period and quality (+ - +++) of budgerigar sperm movement (Behncke 2002). 1% G maintained MOT with constant quality (++) over a period of several minutes better than Ringer's solution and 5% glucose solution. Similar findings were reported through comparing the effect of six different semen extenders on cockatiel sperm motility (Stelzer 2004), where 1% G retained PMOT over a 30 minute period between 40% and 60%, followed by a linear decrease to below 10% within the next 90 minutes. Despite the

distinct degradation, 1% G was reported to achieve the best results in comparison with Ringer's solution and 5% glucose solution, as well as diluents described for poultry (Bechstedt *et al*. 1974, Schramm and Löhle 1984, Bakst and Dymo 2013). Lake diluent was reported to start with an estimated PMOT of 15% within the first ten minutes of investigation, followed by a decline to 0% during investigation. In a study on Hispaniolan parrots initial MOT values between 41% and 82% were subjectively estimated using Lake diluent as a semen extender (Brock 1991). It has to be mentioned that the comparability of these findings is limited, as they all affect different psittacine species. Distinctive values could consequently arise from various reasons like individual differences of the used animals, different methods of the semen extraction, different methods of semen treatment or varying time intervals between semen collection and investigation and cannot only be ascribed to influences of the semen extender. All motility values in previous studies have been estimated in broadly defined groups (Behncke 2002) or in decadic percent ranges (Stelzer 2004) while in our study motility values have been calculated, allowing elaboration. Nevertheless, both methods are highly observer-dependent and need expert experience (Verstegen *et al*. 2002). Unfortunately, the little sample volumes were insufficient for a computer assisted semen analysis (CASA) and therefore had to be performed manually. Comparison of the percentage decline of PMOT within SE 1 during 120 minutes revealed a lower decrease in 1:8 dilution (Δ = 13.93%) than in 1:4 dilution (Δ = 23.39%), while initial PMOT was significantly higher in 1:4 dilution. This may be attributed to the relatively higher amount of nutrients and energy providers in 1:8 dilution guaranteeing a lower decrease in MOT and PMOT, which indicates that a higher dilution might be beneficial for the purpose of long-term storage. The contradictory outcome between our study and previous studies on psittacine semen extenders might most possibly be due to the distinct osmolality values and pH numbers of the used semen extender. In our study the three semen extenders were adjusted especially to cockatiel semen while 1% G remained unchanged to ensure better comparability to previous studies (Behncke 2002, Stelzer 2004, Fischer *et al*. 2014b). It cannot be excluded that the difference between mean pH and osmolality of cockatiel semen and 1% G impaired sperm motility in this way. A previous study reported hypertonic semen extenders to unveil positive effects on turkey spermatozoa survival (Giesen and Sexton 1983). On the contrary, a reduction of osmolality below 200 mOsm/kg irreversibly harmed turkey and fowl spermatozoa (Bakst 1980). The worse results of 1% G in comparison with the other semen extenders might be due to the osmolality differences between cockatiel seminal plasma values and 1% G and the low pH-value of 1% G. Strikingly, pH-values measured in our study (n = 70; $\tilde{x}$ = 7.5; range: 6.4 to 8.0) differed from previous studies in cockatiels (n = 128; $\tilde{x}$ = 9.0; range 8.0 to 9.5) (Stelzer 2004). In those studies more alkaline pH-values have been measured, similar to values in budgerigars (n = 126; $\bar{x}$ ± SD = 8.37 ± 0.24; range: 8.1 to 9.0) (Behncke 2002) and monk parakeets (*Myiopsitta monachus*) (n = 8; range: 8.05 to 8.5) (Anderson *et al*. 2002). However, our semen pH-values are not out of range of other psittacine species. Semen of Spix's macaws (*Cyanopsitta spixii*) (n = 31; $\tilde{x}$ = 7.0; range: 6.4 to 8.0) (Fischer *et al*. 2014a), of cockatoos ($\tilde{x}$ = 7.0; range: 5.4 to 8.0), of Amazon parrots ($\tilde{x}$ = 7.3; range: 5.7 to 8.0), of other macaw species ($\tilde{x}$ = 6.8; range: 5.4 to 7.8) and of Eclectus parrots ($\tilde{x}$ = 7.0; range: 5.4 to 7.8) (Bublat et al. 2017) was also pH-neutral or slightly alkaline, respectively. Although macroscopically contaminated samples (feces and urates) were excluded from further investigation, it cannot be ruled out that a contamination with transparent fluid (Nishiyama 1951), a lymp-like fluid which is part of the seminal plasma, occurred. Contamination with blood, feces and transparent fluid would increase the pH-value, whereas a contamination with urates would decrease it. However, the same applies for other studies too, in which the exclusion of contaminated samples has not been mentioned by the authors in all studies. Moreover, differences due to the usage of different pH indicator strips should be considered.

Particularly minding usual cell death during future freezing and thawing procedures a viability of ≥ 70% at both investigation times (0; 120 min) was aimed at in the present study (Holt 2000). This was only fulfilled by SE 1 in both dilutions. Unfortunately, sperm viability after dilution has not been evaluated in psittacines before, rendering comparison with previous studies on psittacine semen impossible. Contrary to our expectations, the addition of ATP to SE 2 did not seem to have a similar positive influence on motility or viability

of cockatiel spermatozoa as demonstrated on turkey and crane sperm (Blanco *et al*. 2011). However, in the mentioned study semen values were determined after thawing of semen and not for the purpose of short-time liquid storage as in the present study. Surprisingly, pure samples resulted in higher viability values after 120 minutes of storage than diluted ones, although we hypothesized it to be the other way around. As it is known that the quality of staining may be influenced by many factors, a possible reaction of the semen extenders with the staining solution might be an explanation for the misinterpretation as dead spermatozoa, thus leading to false low values.

In this study a relation between semen extenders, storage time and effects on sperm morphology have been investigated for the first time in psittacine species. During the time of storage, a decrease in live, morphologically normal spermatozoa and an increase in dead spermatozoa and spermatozoa with bent heads were observed in all semen extenders, which was in agreement with previous studies on fowl spermatozoa (Blesbois *et al*. 1999, Siudzinska and Lukaszewicz 2008). Our findings demonstrate that every semen extender seems to have a significant influence on abnormalities of the sperm head, a region which is known to be highly sensitive to osmotic variances (Bakst 1980). As this effect is suspected to be independent from storage time an optimization of semen extenders was regarded essential – even for very short storage time. In contrast, tail abnormalities did not show any dependence on the used semen extender in the present study but occurred significantly more frequent after prolonged storage time. This might be related to energy losses during storage. As acrosome and midpiece sections were not influenced by treatment or time these regions seem to be less sensitive. Morphological alterations are important because the percentage of viable, MNS has been strongly correlated to fertilization success ($r = 0.66$, $p < 0.01$) in the domestic chicken (Blesbois *et al*. 2008). In this regard, various morphologic abnormalities are reported to have a negative impact on sperm velocity (Lüpold *et al*. 2009), while sperm motility (MOT and PMOT) are positively correlated with fertilization success (Birkhead *et al*. 1999, Froman *et al*. 1999, Blesbois *et al*. 2008). This underlines the importance to investigate sperm morphology in course of a proper evaluation of semen diluents. Moreover, this demonstrates that semen diluted with SE1 is most promising to achieve fertilization following AI if other influencing factors are not altering this.

CONCLUSION

The evaluation of semen quality is of great importance regarding the prediction of survival of spermatozoa during long-term storage and cryopreservation as well as for the prediction of fertilization success following AI (Blesbois *et al*. 2008). Frequently utilized in vitro quality tests for semen are the evaluation of sperm viability as well as the percentages of MNS and – most important – motile spermatozoa (Birkhead *et al*. 1999, Froman 2000). Based on the current results, we conclude that our modified SE 1 had the least harmful effect on cockatiel spermatozoa and is therefore considered as the most appropriate semen extender for short time storage in cockatiels. Hopefully, this may be taken as a solid basis for cryopreservation of psittacine spermatozoa. However, species-specific differences in sperm metabolism must be considered and further investigations concerning basic semen parameters and effects of semen extenders on semen of different psittacine species are required.

DECLARATION OF INTEREST

The authors confirm that there are no conflicts of interest.

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