

Semen cryopreservation in black-footed (*Spheniscus demersus*) and gentoo (*Pygoscelis papua*) penguins: Effects of thawing temperature on semen characteristics



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ABSTRACT

In this study, there was an examination of the effect on the characteristics of cryopreserved black-footed (*Spheniscus demersus*) and gentoo (*Pygoscelis papua*) penguin semen, of thawing at 37 and 5 °C. For two consecutive years, semen was collected and frozen during the April-June period from six gentoo penguins, and during the October-November period from 13 black-footed penguins. After thawing, sperm motility variables were examined by computer-assisted sperm analysis. Propidium iodide and SYBR-14 were used as fluorochromes for the examination of membrane integrity. For the gentoo penguins, no differences were detected in the values of frozen-thawed semen characteristics after thawing at 37 or 5 °C. For the black-footed penguins, however, thawing at 5 °C resulted in greater values ($P < 0.05$) for straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), and wobble (WOB) as compared with thawing at 37 °C. After thawing at 37 °C, there were greater values with gentoo penguin sperm for percentage motile sperm, progressive motility, curvilinear velocity (VCL), VSL VAP, LIN, STR, WOB and beat-cross frequency (BCF; $P < 0.05$) than that for black-footed penguin sperm. After thawing at 5 °C, there were no differences in values for any variables between the two species. In conclusion, thawing temperature affects semen characteristics in a species-specific manner. The present data strongly suggest that cryopreservation procedures should be adapted for use with each penguin species. Cryopreserved black-footed penguin semen should be thawed after cryopreservation at 5 °C, while that of gentoo penguins can be thawed at either 5 or 37 °C.

1. Introduction

Of the 18 species of penguins, 11 are threatened with extinction. The black-footed penguin (*Spheniscus demersus*) is an endangered species (IUCN, 2018) endemic to southern Africa. There is currently occurring a rapid population decrease of numbers of this species as a result of commercial fishing, changes in prey populations, and environmental fluctuations (Crawford et al., 2011). During the early 20th century, human activity and egg-collecting were also important factors in the decrease in animal numbers for this species (Shannon and Crawford, 1999). Guano collection, for example, resulted in major disturbances at many colonies, and

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deprived the birds of nest-burrows (Frost et al., 1976). Currently there are about 25,000 pairs of black footed penguins remaining - representing a reduction in numbers of over 50% in just three generations (IUCN, 2018). There is no indication that these decreases will be abating, immediate conservation action is needed, including *ex situ* conservation.

The gentoo penguin (*Pygoscelis papua*) has a circumpolar distribution with breeding occurring at a latitude that exists from the Fish Islands of the Antarctic Peninsula (66 °S) to the Crozet Islands (46 °S) (Lynch, 2013). This species is currently listed as 'of least concern', but there are major threats such as tourism, marine traffic and environmental change that are affecting breeding in this species (Lynch et al., 2009; Crawford et al., 2014).

Spermatozoa are the cells usually preserved in genetic resource banks with the aim of maximising genetic diversity and sustainability of captive penguin populations. There have been few reports on the use of assisted reproductive technologies in penguins, but there have been acceptable results for semen collection (in the rockhopper penguin *Eudyptes chrysocome chrysocome* [Waldoch et al., 2007]), semen chilling, semen freezing (in the Magellanic penguin *Spheniscus magellanicus* [O'Brien et al., 1999]) and artificial insemination [in the rockhopper penguin, *Eudyptes chrysocome chrysocome* (Waldoch et al., 2012)]. The likely challenges in penguin semen management and cryopreservation include breeding seasonality (Groscolas et al., 1986; Mauget et al., 1994), individual responses to the semen collection technique, urate contamination, and the presence of immature sperm cells (O'Brien et al., 1999). The cooling rate (Santiago-Moreno et al., 2011), semen packaging type (e.g., straws or pellets) (Tselutin et al., 1999), cryoprotectant used, and freezing temperature (Abouelezz et al., 2015), however, all need to be taken into account when optimising freezing-thawing protocols for any bird species. The rate at which frozen semen are warmed after cryopreservation before use may be more important than the cooling rate. Furthermore, the optimum thawing temperature for bird semen varies with the freezing procedure utilised (Abouelezz et al., 2015). Interspecific variation in the biochemical composition of the sperm cell membrane, and its fluidity, also influence whether an extender can be successfully used in one species and will be useful in another species (Blesbois, 2012). A varied response to cryopreservation should, therefore, be expected among penguin species. In the present study, therefore, there was comparison in the response of cryopreserved black-footed and gentoo penguin semen to thawing at different temperatures.

2. Materials and methods

All diluents and media were prepared in the laboratory using reagent-grade chemicals purchased from Panreac Química S.A. (Barcelona, Spain) and Sigma Chemical Co. (St. Louis, Missouri, USA).

2.1. Animals

The gentoo penguins used in this study were housed in an indoor exhibition enclosure at *Faunia* nature park (Madrid, Spain) with the photoperiod and temperature conditions being controlled, with annual changes programmed to mimic those of the Antarctic habitat for this species. The black-footed penguins were housed in an outdoor exhibition enclosure at the Madrid Zoo-Aquarium (Madrid, Spain) and there were natural photoperiod and temperature conditions that prevailed for this species during the study. All birds were fed frozen-thawed sprat, capelin and herring, and received a vitamin supplement (Aquaminivits IZVG LLP, Keighley, UK; one tablet per day). All birds were managed in ways consistent with the Spanish Policy for Animal Protection (RD53/2013), which conforms to European Union Directive 86/609 regarding the protection of animals used in scientific experiments. All procedures of this study were evaluated and approved by the internal animal welfare committee of each institution based on the EAZA (European Association of Zoos and Aquariums) Code of Ethics.

2.2. Semen collection and assessment of fresh semen variables

During two consecutive seasons, semen samples were collected in the respective breeding season of each species. Semen was collected once-twice weekly from six gentoo penguins between April and June, and from 13 black-footed penguins between October and November. The first year, the semen samples from both species were frozen, and the effects of thawing at 37 °C on semen variables was assessed. In the second year, the same animals were used to evaluate the effect of thawing at 5 °C. All birds were subjected to the massage technique of Burrows and Quinn (1937; adapted to these species). Briefly, two research personnel were required for sperm collection. One individual with skills for working with penguins caught a male and held the bird firmly over a U-shaped wooden cradle – one hand holding the legs and the other immobilizing the body and wings. The other individual who was skilled in collecting semen from penguins stimulated the bird, simultaneously stroking the back with the right hand and the abdomen with the left hand following by a mild stimulation of the vent area. Semen was collected by capillarity using a microhaematocrit tube (Brand® GMBH + Co KG, Wertheim, Germany). Semen volume was determined by measuring the length of the semen column in the microcapillary tube using a plastic ruler (accuracy \pm 1 mm) and calculating the equivalent in volume units (μ L). The seminal content of the microcapillary was then emptied into 1.5 mL Eppendorf microcentrifuge tubes (Eppendorf Ibérica SLU, Madrid, Spain), and diluted 1:1 (v:v) at an ambient temperature using a glutamate-polyvinylpyrrolidone-based medium composed of sodium-L-glutamate (1.92 g), glucose (0.8 g), magnesium acetate 4H₂O (0.08 g), potassium acetate (0.5 g), polyvinylpyrrolidone (PVP, relative molecular mass = 10,000; 0.3 g), and H₂O (100 ml) (final pH 7.08, final osmolality 343 mOsm/kg). The diluted semen samples were pooled and immediately refrigerated at 5 °C (cooling rate: 0.2 °C/min).

All semen was examined within 45 min of dilution in freshly pooled samples. Sperm concentrations and motility were assayed using previously described procedures (Santiago-Moreno et al., 2012) for a computer-aided sperm analysis (CASA) system coupled to a Nikon Eclipse model 50i phase contrast microscope (Nikon Instruments Europe B.V., Izasa S.A., Barcelona, Spain; negative contrast

mode) and a Sperm Class Analyzer (SCA®, Barcelona, Spain) v.4.0. software (Microptic S.L., Barcelona, Spain). For motility analysis, semen samples were diluted to a concentration of approximately 40 million sperm/mL and loaded onto warmed (38 °C) 20 µm Leja® 8-chamber slides (Leja Products B.V., Nieuw-Vennep, The Netherlands). The CASA evaluations were made within 1 min after filling the Leja chamber. The percentage of motile spermatozoa and the percentage showing progressive motility (spermatozoa swimming forward quickly in a straight line) were recorded. Sperm movement characteristics – curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH), and beat-cross frequency (BCF) – were also recorded. Three progression ratios, expressed as percentages, were calculated from the velocity measurements described above: linearity (LIN = VSL/VCL × 100), straightness (STR = VSL/VAP × 100), and wobble (WOB = VAP/VCL × 100). CASA settings for motility were: progressive motility > 75% STR, circular movement < 50% LIN. A minimum of three fields and 500 sperm tracks were evaluated at a magnification of 100x for each sample (image acquisition rate 25 frames/s).

Propidium iodide (PI) and SYBR-14 were used as fluorochromes in the examination of membrane integrity with 200 cells being examined (Chalah and Brillard, 1998). When conducting this procedure, 4 µl of SYBR-14 and 10 µl of the semen sample were added to an Eppendorf tube containing 200 µl of HEPES medium (20 mM Hepes, 197 mM NaCl, 2.5 mM KOH, and 10 mM glucose), and there was incubation at 5 °C for 10 min. Immediately afterwards, 2 µl of PI were added to the solution, followed by incubation for 2 min at 5 °C. The samples were then examined using an epifluorescence microscope at 1000× (wavelength: 450–490 nm). Spermatozoa stained green (no PI staining) were considered to be alive, while red coloured spermatozoa (PI-positive) and spermatozoa with red and green colours were considered to be dead (any red colour means that the membrane is impaired and has lost function).

2.3. Semen freezing

All semen samples were diluted using the previously described glutamate-polyvinylpyrrolidone-based medium to a concentration of 700×10^6 sperm/mL for freezing. Glycerol was then added to a final concentration of 8%, and the samples allowed to equilibrate for 10 min at 5 °C (Santiago-Moreno et al., 2011) before loading into 0.25 mL straws (Minitüb®, Landshut, Germany). These straws were then frozen by placing them in nitrogen vapour 5 cm above the surface of a liquid nitrogen bath (a 1.1 L expanded polythene box with a top surface area of 560 cm²) for 10 min. This provides for a slow freezing rate: from 5 to –85 °C at 10 °C/min. These samples were then plunged into the liquid nitrogen. Because the freezing rate with nitrogen vapour may vary depending on the size of the bath, and time that elapses between when the bath is first filled and the straws being suspended in the nitrogen vapour, the method was standardised using a freezing-resistant Ventix® K/J/T thermometer (Ventix, China). The time elapsed between the filling of the bath and the straws being suspended in the nitrogen vapour was 30 min.

2.4. Thawing at different temperatures, and assessment of frozen-thawed semen characteristics

Frozen semen samples from each collection year and penguin species were assigned to be thawed in a water bath at either 37 °C for 30 s, or 5 °C for 3 min. The contents of the straws were then decanted into polystyrene tubes and immediately assessed for semen characteristics as previously described in this manuscript.

2.5. Statistical analysis

Values for semen characteristics are reported as means ± SE. For several variables, there was not a normal distribution when there was assessment using the Shapiro-Wilk's test. The non-parametric Mann Whitney *U* test for unmatched samples, therefore, was used to assess differences between species and between thawing temperatures. The Wilcoxon test for matched pairs was used to compare fresh and frozen-thawed semen variables within the same species. To assess the response to freezing-thawing in both species, a cryoresistance ratio (Esteso et al., 2006) was determined for each of the semen variables assessed, as follows:

$$\text{Cryoresistance ratio (CR)} = (\text{value after thawing (post)} / \text{value before thawing (pre)}) \times 100$$

Differences in the CRs for each thawing temperature were compared using the Mann Whitney *U* test. All calculations were performed using Statistica software v.13 (Dell Statistica, StatSoft Inc., Tulsa, OK, USA).

3. Results

No differences were detected between years for semen volume and sperm concentration in either species. Semen volume in gentoo penguins ($36.8 \pm 3.4 \mu\text{L}$) was greater ($P < 0.001$) than for black-footed penguins ($21.7 \pm 2.2 \mu\text{L}$), but there were no differences between the gentoo and black-footed birds in sperm concentration ($1809.2 \pm 355.6 \times 10^6$ sperm/mL and $1649.3 \pm 541.2 \times 10^6$ sperm/mL, respectively) and in the remaining freshly diluted semen characteristics (Table 1).

Fig. 1 depicts the effects of the freezing-thawing process on the semen characteristics of each species. In both species, semen values were less whether the thawing temperature was at 37 or 5 °C, except for the percentage of sperm with progressive motility in gentoo penguins. In black-footed penguins ALH was less after thawing at 5 °C, however, there was only a tendency for this difference ($P = 0.09$).

After thawing at 37 °C, there were differences between the gentoo and black-footed penguins for the CR of motile sperm,

Table 1

Semen variables in freshly pooled samples diluted without cryoprotectant (mean \pm standard error for samples collected over two consecutive years), for gentoo and black-footed penguins.

Semen variables	Gentoo fresh semen (n = 32)	Black-footed fresh semen (n = 29)
Viability (%)	68.91 \pm 3.6	70.79 \pm 5.2
Motile sperm (%)	47.99 \pm 5.1	57.74 \pm 7.2
Progressive motility (%)	7.58 \pm 2.0	9.24 \pm 4.4
VCL ($\mu\text{m/s}$)	36.82 \pm 2.9	36.42 \pm 4.6
VSL ($\mu\text{m/s}$)	14.80 \pm 1.8	17.40 \pm 4.0
VAP ($\mu\text{m/s}$)	24.21 \pm 2.3	26.08 \pm 4.4
LIN (%)	37.84 \pm 2.2	44.60 \pm 3.7
STR (%)	58.02 \pm 2.3	63.11 \pm 2.8
WOB (%)	63.97 \pm 1.8	69.68 \pm 2.7
ALH (μm)	2.49 \pm 0.2	2.55 \pm 0.4
BCF (Hz)	6.65 \pm 0.6	5.39 \pm 0.8

Curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head (ALH), beat-cross frequency (BCF).

progressive motility, VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF. After thawing at 5 °C, the only difference between the species was for the CR of STR (Table 2). The CR values for sperm viability didn't vary between species for both the 37 and 5 °C thawing temperatures.

In the gentoo penguins, there were no differences in the values of the recorded variables between semen thawed at 37 and 5 °C. In the black-footed penguin samples, however, the values for the relative variable were greater ($P < 0.05$) after thawing at 5 than at 37 °C for VSL, VAP, LIN, STR, and WOB. There were no differences in the values for sperm viability, motile sperm, progressive motility, VCL, ALH, and BCF between semen thawed at 37 and 5 °C (Tables 2 and 3).

4. Discussion

Although there were no differences between the two penguin species in terms of fresh semen characteristics, the black-footed penguin semen appeared to be more sensitive to the freezing-thawing procedure. Certainly, the thawing temperature affected the sperm cells in a species-specific manner. There was no apparent effect on frozen-thawed gentoo semen, however, in the black-footed birds the 5 °C thawing temperature was associated with a more desirable response to the thawing process.

The values for the fresh semen quantitative and qualitative characteristics for both the present species were similar to those reported for Magellanic penguins (O'Brien et al., 1999), while mean sperm motility and sperm concentration were greater in both species than in the Rockhopper *Eudyptes chrysocome chrysocome* penguin (Waldoch et al., 2007, 2012). Surprisingly, the values for the kinetic variables (VAP, VSL, VCL, ALH and BCF) in the fresh samples, and consequently after thawing, were much less in the species evaluated in the present study than in those reported for either Magellanic (O'Brien et al., 2016) or king penguins (*Aptenodytes patagonicus*) (O'Brien and Robeck, 2014). This might be explained by the size of the sperm head. King penguins have long (13.8 μ), large (19.7 μ^2) sperm heads (Santiago-Moreno et al., 2016), and it has been reported that sperm with longer heads may be more motile (Gomendio and Roldan, 1991; Malo et al., 2006; Santiago-Moreno et al., 2015). The results of O'Brien and Robeck (2014) also suggest that king penguin sperm are more cryoresistant than that of gentoo or black-footed penguins. Although cryoresistance may be a species-specific response, the effects of the freezing-thawing procedure, and extenders as well as other factors, have to be taken into account. Furthermore, the use of additives such as BSA may stimulate sperm motility (Uto and Yamahama, 1996). In previous studies with the Magellanic and king penguins, there was use of Beltsville Poultry Semen Extender at a cooling rate of 0.35 °C/min, and 8% DMSO was used as the cryoprotectant (O'Brien et al., 2016; O'Brien and Robeck, 2014).

The findings in the present study suggest that for black-footed penguins the 37 °C temperature was more harmful during sperm thawing than the 5 °C temperature. The gentoo sperm did not appear to be affected by thawing temperature. The method of freezing may, however, influence the effect of thawing temperature. In chickens, for example, 5 °C is usually used when sperm are frozen in straws, however, 60–70 °C is recommended when ultra-rapid freezing procedures are used to store sperm as pellets (Abouelezz et al., 2015). The most desirable outcomes when thawing of mammalian sperm, however, is 37 °C for about 25 s, or 70 °C for 2–3 s (Holt, 2001; Penfold and Watson, 2001).

As described in chickens (Blesbois and de Reviers, 1992), the presence and composition of seminal plasma may actually prevent or enhance damage during thawing, depending on the species. In penguins, differences in the seminal plasma protein content, or of other plasma components might, therefore, affect sperm freezability (see Labas et al., 2015; Marzoni et al., 2013). Further research should be conducted to investigate effects of seminal plasma protein on cryopreservation of penguin sperm.

The thawing temperature may also affect the rate at which any osmotic changes occur, and thus the amount of osmotic damage during thawing. The cell damage is related to the decrease in the solute concentration of the extracellular milieu, the flux of water in the intracellular milieu, and subsequent increase of cell volume (Holt and North, 1994). At 5 °C the time over which these changes occur would be longer, which may perhaps be advantageous in preserving the filiform characteristics of bird sperm.

The size of sperm subpopulations as categorised by cell dimensions (Villaverde-Morcillo et al., 2017) can influence the osmotic

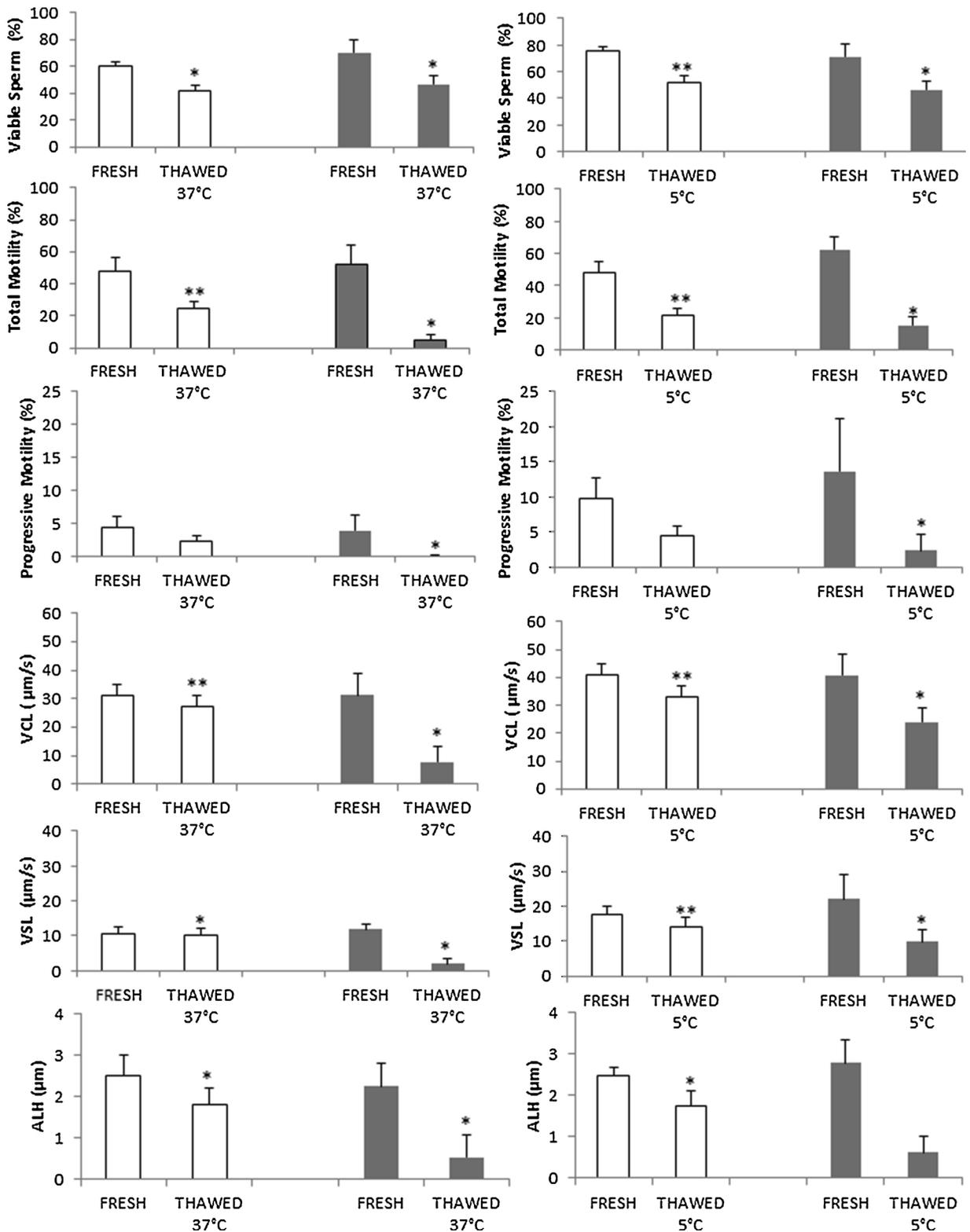


Fig. 1. Freshly diluted semen characteristics and that of semen thawed at 37 and 5 °C in gentoo (open bars, $n = 15$ at 37 °C and $n = 17$ at 5 °C) and black-footed penguins (solid bars, $n = 12$ at 37 °C and $n = 17$ at 5 °C); Asterisks indicate differences between fresh and thawed sperm variables (* $P < 0.05$; ** $P < 0.01$); Curvilinear velocity (VCL), straight line velocity (VSL), amplitude of lateral head (ALH).

Table 2Cryoresistance ratio (CR, mean \pm standard error) for cryopreserved gentoo and black-footed penguin pooled semen samples thawed at 37 and 5 °C.

CR	Gentoo semen thawed at 37 °C (n = 15)	Gentoo semen thawed at 5 °C (n = 17)	Black-footed semen thawed at 37 °C (n = 12)	Black-footed semen thawed at 5 °C (n = 17)
Viability (%)	71.4 \pm 12.6	69.3 \pm 6.1	66.0 \pm 13.6	80.6 \pm 19.6
Motile sperm (%)	44.8 A \pm 8.9	58.5 \pm 16.3	9.7 B \pm 9.4	31.2 \pm 7.7
Progressive motility (%)	141.1 A \pm 77.2	170.1 \pm 114.3	0.0 B \pm 0.0	7.5 \pm 6.0
VCL (μ m/s)	80.1 A \pm 14.9	85.3 \pm 11.7	17.1 B \pm 15.1	72.7 \pm 8.5
VSL (μ m/s)	81.3 A \pm 20.5	93.9 \pm 20.9	6.3 bB \pm 6.1	51.6 a \pm 6.4
VAP (μ m/s)	72.7 A \pm 16.1	81.9 \pm 14.0	10.9 bB \pm 10.3	61.8 a \pm 6.0
LIN (%)	89.5 A \pm 10.0	94.7 \pm 10.9	9.9 bB \pm 7.8	75.2 a \pm 3.7
STR (%)	140.0 A \pm 23.1	105.2 A \pm 5.5	18.0 bB \pm 11.9	87.3 aB \pm 3.3
WOB (%)	77.5 A \pm 11.2	87.3 \pm 7.1	19.6 bB \pm 13.4	86.1 a \pm 2.6
ALH (μ m)	68.2 A \pm 8.3	69.9 \pm 14.5	0.0 B \pm 0.0	34.5 \pm 21.9
BCF (Hz)	109.8 A \pm 23.3	81.8 \pm 17.4	0.0 B \pm 0.0	38.2 \pm 24.9

Different lower case letters (a–b) indicate differences ($P < 0.05$) within species for different thawing temperatures; Different upper case letters (A–B) indicate significant differences ($P < 0.05$) between species for the same thawing temperature. Curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head (ALH), beat-cross frequency (BCF).

Table 3Semen variables (mean \pm standard error) for cryopreserved gentoo and black-footed penguin pooled samples thawed at 37 and 5 °C.

Semen variables	Gentoo semen thawed at 37 °C (n = 15)	Gentoo semen thawed at 5 °C (n = 17)	Black-footed semen thawed at 37 °C (n = 12)	Black-footed semen thawed at 5 °C (n = 17)
Viability (%)	41.64 \pm 6.1	51.69 \pm 4.7	47.00 \pm 10.1	46.25 \pm 6.5
Motile sperm (%)	22.47 A \pm 4.5	21.52 \pm 4.7	4.87 B \pm 3.5	15.31 \pm 5.2
Progressive motility (%)	2.22 A \pm 0.7	4.53 \pm 1.4	0.08 B \pm 0.1	2.40 \pm 2.3
VCL (μ m/s)	24.63 A \pm 4.6	32.92 \pm 4.0	7.87 B \pm 4.4	24.05 \pm 5.2
VSL (μ m/s)	9.12 A \pm 2.0	14.30 \pm 2.4	1.95 bB \pm 1.3	9.91 a \pm 3.4
VAP (μ m/s)	14.56 A \pm 2.9	21.15 \pm 3.1	3.97 bB \pm 2.4	15.45 a \pm 4.2
LIN (%)	28.41 A \pm 4.7	38.20 \pm 4.3	9.29 bB \pm 5.7	39.00 a \pm 5.6
STR (%)	61.63 A \pm 7.5	64.25 \pm 2.9	19.09 bB \pm 10.1	60.61 a \pm 5.3
WOB (%)	45.29 A \pm 7.8	58.43 \pm 4.9	20.94 bB \pm 10.6	62.29 a \pm 3.8
ALH (μ m)	1.66 \pm 0.4	1.76 \pm 0.3	0.54 \pm 0.5	0.62 A \pm 0.4
BCF (Hz)	5.51 A \pm 1.3	5.84 \pm 1.2	0.84 B \pm 0.8	2.20 \pm 1.4

Different lower case letters (a–b) indicate differences ($P < 0.05$) within species for different thawing temperatures; Different upper case letters (A–B) indicate differences ($P < 0.05$) between species for the same thawing temperature. Curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head (ALH), beat-cross frequency (BCF).

changes that occur during thawing. Sperm head size influences the volume of water contained in cells, as well as the flux of water and cryoprotectant. Thus, cryodamage to spermatozoa might be directly related to sperm head dimensions (Esteso et al., 2006), with cells possessing smaller heads having less damage. The capacity of spermatozoa to survive a freeze/thaw cycle, therefore, could be species-specific. The sperm heads of king penguins have a surface area of around 19.7 μ m² while those of gentoo penguins are about 18.2 μ m² (Santiago-Moreno et al., 2016), but values for this variable have not been determined in black-footed penguins. The structure, elasticity and permeability of the sperm cell membrane, which are determined genetically (Songsasen and Leibo, 1997), will also affect water and glycerol fluxes, and thus the cryoresistance of sperm cells.

Differences in sensitivity to thawing might be related to the amount of consanguinity of captive populations. The capacity of sperm to withstand freezing and thawing is reported to be inversely related to the amount of inbreeding of a population (Garde et al., 2003). Consanguinity increases inbreeding and this affects reproductive functions, favours apoptotic processes (Hingst and Blottner, 1995), and thus reduces sperm cryoresistance. Single ejaculates of individuals with greater genetic diversity should be cryopreserved.

In conclusion, the thawing temperature affects frozen-thawed semen characteristics in a species-specific manner. Thawing at 5 °C is recommended for black-footed penguin semen, but either 5 or 37 °C can be used for gentoo penguin semen with similar outcomes. The present results strongly suggest that the freezing and thawing procedures used with different penguin species will need to be optimised for each species.

Conflict of interest

None of the authors have any conflict of interest to declare.

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