

1 **Validation of the sperm chromatin dispersion (SCD) test in the Amphibian *Xenopus***
2 ***laevis* using *in situ* nick hybridisation and comet assay**

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1 **ABSTRACT**

2

3 The integrity of sperm DNA is becoming increasingly recognised as an important
4 parameter of semen quality, but there are no published reports of this phenomenon or
5 procedure for any amphibian. The primary aim of this study was to apply a modified
6 sperm chromatin dispersion (SCD) test (Halomax[®]) to an amphibian sperm model (African
7 clawed frog; *Xenopus laevis*) and to validate the assay against *in situ* nick translation
8 (ISNT) and the double comet assay procedures. Inactivated spermatozoa were collected
9 from fresh testes (n = 3). Sperm DNA fragmentation (SDF) for each sperm sample was
10 conducted immediately following activation (T0) and again following one hour (T1) and
11 24 hours (T24) of incubation at room temperature in order to produce a range of
12 spermatozoa with differing levels of DNA damage. The SCD procedure resulted in the
13 production of three nuclear morphotypes; amphibian sperm morphotype 1 (ASM-1) and
14 ASM-2 showed no evidence of DNA damage, whereas ASM-3 sperm were highly
15 fragmented with large halos of dispersed DNA fragments and a reduced nuclear core.
16 ISNT confirmed that ASM-3 nuclei had damaged DNA. There was a significant
17 correlation ($R = 0.972$; $P = 0.00001$) between the levels of ASM-3 exposed by the SCD
18 test and SDF revealed by the double comet assay.

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1 INTRODUCTION

2

3 The integrity of sperm DNA is becoming increasingly recognised as an important
4 parameter of semen quality and essential for embryonic development and ultimately
5 reproductive success (D'Occhio *et al.* 2007). During spermiogenesis, the DNA molecule
6 undergoes a series of structural changes that allow the chromatin to be remodelled into a
7 highly compacted form. This tight configuration is susceptible to disruption by sperm
8 DNA fragmentation (SDF). SDF is regarded as damage to the topology and function of the
9 chromatin complex via single and/or double-stranded DNA breaks that ultimately lead to
10 impaired fertility (López-Fernández *et al.* 2009). SDF may include both inherent
11 constitutive DNA modifications that are observed immediately following sperm emission,
12 mostly associated with the normal chromatin remodelling process and inducible or
13 iatrogenic modifications to the DNA that arise as a function of incubation time *ex vivo* and
14 which are a result of external factors such as the conditions of sperm storage during
15 processing for assisted reproductive technologies (ART; Gosálvez *et al.* 2011a; Alvarez
16 and Gosálvez 2012).

17

18 As DNA integrity is poorly correlated with standard parameters of semen quality, a
19 means to identify existing or potential damage to the inheritable genetic material is a
20 necessary component of ART (López-Fernández *et al.* 2008). The sperm chromatin
21 dispersion (SCD) test measures the susceptibility of sperm DNA to fragmentation and is
22 based on the principle that controlled denaturation and removal of nuclear proteins using a
23 lysing solution, results in nucleoids with a central core and a peripheral halo from the
24 dispersion of damaged chromatin (Fernández *et al.* 2003). Given the vast differences in the
25 protein composition and structure of sperm DNA across the different vertebrate taxa, it is

1 not surprising that species-specific protocols are required to ensure a targeted and
2 validated approach to DNA fragmentation assessment (Gosálvez *et al.* 2011a).

3

4 Originally developed for assessing DNA fragmentation in human spermatozoa
5 (Halosperm[®]; Fernández *et al.* 2003), the SCD test (as the Halomax[®]) has been now been
6 successfully adapted to the unique chromatin structure of a range of domestic and wildlife
7 species including the koala (*Phascolarctos cinereus*; Johnston *et al.* 2007), short-beaked
8 echidna (*Tachyglossus aculeatus*; Johnston *et al.*, 2009), stallion (López-Fernández *et al.*
9 2007 and *Equus asinus*; Cortés-Gutiérrez *et al.* 2008), boar (López-Fernández *et al.* 2008),
10 rhinoceros (*Diceros bicornis*, *Rhinoceros unicornis* and *Ceratotherium simum*; Portas *et*
11 *al.* 2009) and bony fish (*Tinca tinca*; López-Fernández *et al.* 2009); the sperm chromatin
12 dispersion test was validated in each of these species separately and employs a species-
13 specific modified lysing solution for protein depletion.

14

15 There are extremely limited studies that have investigated DNA fragmentation in
16 amphibians, with the majority of this work focused on the effects of environmental toxicity
17 on somatic cells (Marselli *et al.* 2010; Wang and Jia 2009; Valencia *et al.* 2011); there
18 have been none that have specifically examined DNA fragmentation in amphibian
19 spermatozoa. A DNA fragmentation assay applied and validated for amphibian sperm
20 would be a useful tool for not only comprehending the importance of this phenomenon in a
21 new major taxon, but also for understanding the impact of changing environmental factors
22 and disease on amphibian spermatogenesis and for developing improvements for use of the
23 sperm in assisted reproduction technologies (ART), used in the captive propagation of
24 threatened and endangered species. For example, *in vitro* fertilisation (IVF) in amphibians
25 is becoming an increasingly useful protocol as part of species propagation used to support

1 the genetic management and reproductive output of amphibian breeding programs (Kouba
2 and Vance 2009).

3

4 Recent studies have demonstrated that sperm DNA fragmentation is in fact a highly
5 dynamic process and typically increases from the time of ejaculation or thawing following
6 cryopreservation up until the time of fertilisation (Gosálvez *et al.* 2011b). This dynamic
7 loss of DNA integrity is particularly relevant for externally fertilising vertebrates such as
8 teleost fish (*Tinca tinca*; López-Fernández *et al.* 2009), where spawning in fresh or
9 seawater osmotically initiates sperm activation in order for external fertilisation to occur. It
10 is likely that once activation has commenced that there could also be a consequent rapid
11 loss of DNA integrity; this phenomenon has never been examined but is highly relevant
12 when developing and refining protocols for sperm conservation. The first step towards
13 using the SCD procedure as an investigative tool of amphibian sperm biology is the co-
14 validation of the technique with other methods of assessing DNA damage. Therefore, the
15 aim of this study was to apply the SCD test to an amphibian sperm model (African clawed
16 frog; *Xenopus laevis*) and then to validate these results up against DNA specific molecular
17 probes and the comet assay.

18

19 **MATERIALS AND METHODS**

20

21 *Animals, sperm recovery and experimental design*

22 For this study, three male wild-type *Xenopus laevis* were sourced from a breeding
23 facility maintained at the European *Xenopus* Resource Centre (EXRC; Portsmouth, United
24 Kingdom) with controlled conditions for housing, photoperiod, temperature, water quality
25 and feeding. All animals were healthy, with a history of reproductive success. Sperm

1 samples were recovered according to EXRC protocol (EXRC 2012). Briefly, animals were
2 euthanased by an overdose of Tricaine/MS222 (Sigma Aldrich, Dorset, England, UK) and
3 the whole testes dissected into 1.0 M MBS (Modified Barth's Saline) at room temperature.
4 A portion of the testis was macerated by gentle application of an Eppendorf pestle in 200
5 μL of chilled ($4\text{ }^{\circ}\text{C}$) 0.1 M MBS to achieve sperm activation and attain a final
6 concentration of approximately 10×10^6 spermatozoa mL^{-1} . Identification of sperm
7 motility under $\times 400$ magnification confirmed activation. Analysis of sperm DNA
8 fragmentation for each sperm sample was conducted immediately following activation
9 (T0) and again after one hour (T1) and 24 hours (T24) of incubation at room temperature.
10 The restricted incubation period reflects the rapid decline in the fertilisation capacity and
11 motility of *Xenopus laevis* spermatozoa following activation (Wolf and Hedrick 1971;
12 Sargent and Mohun 2005). The prepared slides were then transported to the AUM, Madrid,
13 Spain for microscopic analysis.

14

15 *The Sperm chromatin dispersion test*

16 The extent of DNA fragmentation in each sample was assessed with a prototype kit
17 (Halomax Proto-Xenopus) adapted from the Halomax[®] kit (Halotech SL, Madrid, Spain)
18 using the following method. Aliquots of 50 μL of low point melting agarose in Eppendorf
19 Safe Lock Tubes[®] (Eppendorf, Hamburg, Germany) were suspended on a float in a water
20 bath at 90-100 $^{\circ}\text{C}$ for five minutes to melt the agarose. The temperature of the agarose was
21 then equilibrated to 35 $^{\circ}\text{C}$ in a second water bath before 25 μL of the extended sperm
22 suspension was added and gently mixed. Aliquots (10 μL) of the sperm-agarose mixture
23 were pipetted onto pre-treated glass slides (provided in the Sperm-Halomax[®] kit) and
24 covered with a glass coverslip (18 x 18 mm). The slides were then placed on a chilled
25 metallic plate ($4\text{ }^{\circ}\text{C}$) and left to solidify into a thin microgel for five minutes in a

1 refrigerator at 4 °C. The coverslips were gently removed and the slides promptly
2 introduced (maintained at a horizontal position) into a plastic bath of lysing solution
3 provided in the Sperm-Halomax[®] kit for five minutes to deprotenise the sperm DNA. The
4 slides were removed from the lysing solution and washed in a bath containing distilled
5 water for five minutes and then sequentially dehydrated in increasing concentrations of
6 ethanol (70, 90 and 100%) for two minutes each and finally left to air-dry. For
7 visualisation under fluorescence microscopy, the slides were mounted with equal parts
8 SYBR[®] Green (Sigma Aldrich, Madrid, Spain) and VectaShield[®] mounting medium
9 (Vector Laboratories, Burlingame, California, USA) to inhibit fluorochrome fading. A
10 total of 300 spermatozoa were counted per sample using a Leica DMRB epifluorescence
11 microscope (Leica Microsystems, Barcelona, Spain) equipped with single-band
12 fluorescence block filters for green (FITC equivalent) and red (Cy3 equivalent)
13 fluorescence. The percentage of spermatozoa with fragmented DNA was calculated.

14

15 *In situ nick translation (ISNT)*

16 For ISNT, sperm microgel slides were prepared at T0 as described previously for
17 the SCD test and then subsequently processed for ISNT (López-Fernández *et al.* 2009).
18 After protein lysis, the slides were washed four times in phosphate buffered saline (PBS)
19 for five minutes and then incubated in reaction buffer for DNA polymerase I (10 mM Tris-
20 HCL, 5 mM MgCl₂ and 7.5 mM DTT; pH 7.5) for five minutes. Next, 100 µL of reaction
21 buffer containing 25 units of DNA polymerase I and digoxigenin-16-dUTP were pipetted
22 onto the slide, covered with a glass coverslip (18 x 18 mm) and incubated in a moist
23 chamber at 37 °C for 30 minutes. The slides were then washed in TBE buffer (89 mM
24 boric acid and 2.5 mM EDTA; pH 8.3) and finally dehydrated in sequential ethanol baths
25 (70, 90 and 100%) and air-dried. The incorporated nucleotide was detected by incubation

1 (30 minutes) with anti-digoxigenin-FITC (Roche, Madrid, Spain). Spermatozoa were
2 visualised using propidium iodide (2 $\mu\text{g}/\text{mL}$) in VectaShield[®] mounting medium (Vector
3 Laboratories, Burlingame, California, USA). All images were captured with a CCD
4 camera (Leica DFC350 FX, Leica Microsystems, Barcelona, Spain).

5

6 *Comet assay*

7 In the present study, a two-directional or ‘double’ comet assay was used to
8 characterise both DSB and SSB simultaneously in the same spermatozoon (Enciso 2009).
9 This approach enabled the visualisation of comets that are run perpendicular to each other
10 (DSB; horizontal direction and SSB; vertical direction). Sperm microgel slides were
11 prepared as described previously for the SCD test above. For the initial neutral
12 electrophoresis step, the slide was treated with the lysis solution provided in the Sperm-
13 Halomax[®] kit for five minutes to deprotenise the sperm DNA and then removed and
14 washed in 1 X Tris-borate-EDTA (TBE) buffer solution (0.089 M Tris, 0.089 M boric acid
15 and 0.002 M EDTA) for 5 minutes. The slides were then electrophoresed (8 minutes; 20
16 V) in 1 X TBE buffer to allow the DNA fragments resulting from DSB to migrate away
17 from the spermatozoon nucleus towards the anode during application of the electrical
18 current. At the completion of electrophoresis, the slide was removed and placed in 0.9%
19 NaCl solution for two minutes. The slide was then transferred to a chilled (4 °C) alkaline
20 solution (0.03 M NaOH and 1 M NaCl; pH 12.5-13) for two and a half minutes to cleave
21 the alkali-labile sites of the DNA. The slide was introduced into a fresh alkaline buffer
22 (0.03 M NaOH; pH 12.5-13) at room temperature for the second electrophoresis (four
23 minutes; 20 V) having been repositioned 90 ° clockwise from its original position during
24 the first electrophoresis step. This resulted in the migration of DNA fragments arising from
25 SSB towards the anode trode while the DNA fragments from DSB remained in their

1 original positions. Finally, the slide was washed with neutralising buffer (0.4 M Tris-HCL;
2 pH 7.5) for five minutes, followed by two minutes in 1 X TBE buffer and subsequent
3 dehydration in a series of 70, 90 and 100% ethanol baths for two minutes each. Comets of
4 fragmented sperm DNA were visualised using GelRed[®] (Biotium, Hayward, California,
5 USA).

6

7 *Statistical analysis*

8 A Pearson's correlation was performed between the percentage of SDF revealed by
9 the SCDt and comet assay using the StatPlus:mac – statistical analysis program for Mac
10 OS Version 2009 (See www.analysis.com/en/).

11

12 **RESULTS**

13

14 *Sperm morphology after the SCD test*

15 Sperm samples processed with the SCD test for the visualisation of DNA
16 fragmentation revealed three primary sperm morphotypes. Amphibian sperm morphotype
17 type 1 (ASM-1) maintained the normal slightly coiled filiform shape of the sperm head,
18 showing only a small, dense halo of chromatin dispersion which corresponded to
19 spermatozoa with intact DNA (Figure 1a). ASM-2 was similar to that of type 1 in terms of
20 chromatin dispersion but the original morphology of the nucleus had swollen or
21 decondensed into a more spherical appearance (Figure 1b); while there was clearly some
22 structural change to the nucleus, the DNA of these nuclei were typically not fragmented.
23 At T0, immediately following activation, most of the spermatozoa displayed an elongated
24 coiled core, but after 1h of incubation the proportion of spermatozoa with rounded
25 morphologies increased (compare Figure 1a and 1b). In contrast to ASM-1 and ASM-2,

1 ASM-3 displayed a large, stellar halo of dispersed chromatin with multiple fragments of
2 DNA (Figure 1a – arrow). Spermatozoa containing damaged DNA typically showed less
3 fluorescence than non-fragmented DNA as the DNA fragments were more widely
4 dispersed in the microgel; i.e. the nuclear core decreased in size as the DNA halo
5 expanded. In some cases, the halo became progressively difficult to visualise as the
6 chromatin dispersed further from the nuclear core; the nuclear core also became
7 progressively smaller in size with greater DNA damage (Figure 1b – arrow).

8

9 *In situ DNA labelling of DNA strand breaks*

10 ISNT was employed to confirm whether the different halo morphologies of
11 chromatin dispersion identified by the SCD test occurred in conjunction with DNA strand
12 breaks. *In situ* labelling involved the direct incorporation of labelled nucleotides to the free
13 3'-OH ends resulting from SSB or DSB. After DNA labelling, all sperm nuclei were
14 positively labelled with green fluorescence, but with varying intensities (Figure 1c).

15

16 *Double comet assay*

17 The double comet assay revealed three distinct comet morphologies indicating
18 different degrees of severity in DNA damage (Figure 2). The first comet morphology
19 (ACM-1) was revealed only after the alkaline electrophoresis and manifested as comets
20 with a consistent nuclear core and tail size indicative of SSB (Figure 2a and b); these
21 comets were observed in almost all spermatozoa and were interpreted as a constitutional
22 structural SSB arising from alkali labile sites (ALS), which are sensitive DNA regions that
23 produce structural SSB following denaturation. The second comet morphology (ACM-2)
24 was characterised by the migration of DNA fragments in the same direction as structural
25 SSB, however, the comets possessed notably longer comet tails and a much reduced

1 nuclear core (Figure 2e). We propose that these SSB comets are likely to contain more
2 single-stranded DNA breaks than what would normally be regarded as structural SSB. The
3 third comet morphology (ACM-3) was only revealed following application of the neutral
4 electrophoresis whereby, DNA fragments migrated in the direction perpendicular to that of
5 SSB comets, producing comets of DSB (Figure 2c and d). ACM-2 and ACM-3 were
6 regarded as sperm with fragmented DNA. The incidence of fragmented sperm nuclei
7 revealed by the SCD test (ASM-3) and double comet assay (ACM-2 and ACM-3) is
8 reported in Tables 1 and 2, respectively. A correlation analysis to compare the results of
9 estimates of the total SDF (ASM-3) from the SCD and the double comet assay (ACM-2 +
10 ACM-3), revealed a strong positive correlation ($R = 0.972$, $P = 0.00001$; Figure 4),
11 although the double comet assay typically gave slightly higher values of total SDF than the
12 SCD test.

13

14 **DISCUSSION**

15

16 The present study has demonstrated a strong positive correlation between the levels
17 of sperm DNA fragmentation revealed by the SCD test (ASM-3) and the double comet
18 assay (ACM-2 and ACM-3). While differentiation between single and double-stranded
19 DNA breaks was possible using the double comet assay; rarely did SDF as revealed by
20 SSB and DSB occur separately but occurred concurrently. ISNT was used to further
21 confirm that ASM-3 morphology was associated with SDF damage. We are therefore
22 confident that the SCD procedure validated in this study can now subsequently be used to
23 investigate the behaviour of amphibian sperm DNA to range of endogenous and
24 exogenous stressors. We have already seen in the current study that SDF increases with

1 incubation at room temperature, but the effect of cryopreservation and subsequent
2 incubation is likely to be even more pronounced.

3

4 The SCD procedure resulted in three distinctive morphotypes. ASM-3 was the
5 result of massive chromatin dispersal away from nucleus and is clearly associated with
6 severely damaged DNA; this was also evidenced by the fact that nucleoid core had
7 coincidentally reduced with increased levels of DNA dispersal. Equally, the morphology of
8 ASM-1 was also relatively straightforward to interpret, with the sperm nucleus retaining its
9 general morphology, but only showing evidence of a small halo of DNA migration; this
10 phenomenon is indicative of an ordered chromatin relaxing and corresponds to the
11 structural comets observed with the comet assay. However, the morphology of ASM-2 is
12 somewhat more difficult to explain, as these sperm nuclei showed no evidence of massive
13 DNA migration or reduction of the nuclear core, but a characteristic change in shape of the
14 nucleus from a spiral elongated form to rounded head morphology; interestingly a shift in
15 frequency from ASM-1 to ASM-2 was also observed with *ex vivo* room temperature
16 storage, indicating that this change is likely to be a consequence of the activated
17 spermatozoa being subject to induced iatrogenic damage.

18

19 The mechanism leading to the loss of the original nuclear morphology may be
20 similar to the typical “chromatin swelling or relaxation” that has been observed in other
21 species devoid of disulphide bonds in their protamines. In these species it is possible that
22 the sperm’s nuclear basic proteins render the DNA more susceptible to protein depletion
23 (Zee *et al.* 2009). The chromatin swelling phenomenon observed here is similar to that
24 described in the koala (*Phascolarctos cinereus*; Johnston *et al.* 2006, 2007, 2012),
25 common wombat (*Vombatus ursinus*; Johnston *et al.* 2006) and teleost fish (*Tinca tinca*;

1 López-Fernández *et al.* 2009). Chromatin swelling in *Xenopus laevis* spermatozoa without
2 DNA fragmentation may be due to the extensive presence of alkali labile sites (ALS);
3 highly sensitive DNA motifs (regions of specific repetitive sequences) or baseline DNA
4 lesions that are produced when the sperm is processed in vitro; these DNA motifs become
5 “hot-spots” producing stretches of single-stranded DNA following alkaline denaturation
6 (Cortés-Gutiérrez *et al.* 2007; Cortés-Gutiérrez *et al.* 2014a, in press). This phenomenon
7 would also explain the pervasiveness of structural SSB revealed in sperm nuclei by the
8 alkaline step of the double comet assay and suggests that ALS are a constitutive feature of
9 the *Xenopus laevis* sperm genome. This explains why all spermatozoa, including those
10 exhibiting small compact chromatin halos of undamaged DNA, expressed positive *in situ*
11 nick translation labelling, given that the DNA nucleotide is incorporated into the free ends
12 of single-stranded DNA. These sites are proposed as an alternative DNA configuration that
13 functions in addition to/in the absence of protamine-mediated disulphide bonds to produce
14 the highly condensed state of the chromatin during spermiogenesis (Cortés-Gutiérrez *et al.*
15 2009). Cortés-Gutiérrez *et al.* (2009) suggests that ALS are likely to be universally present
16 in the mature spermatozoa of all mammalian spermatozoa and accordingly, structural SSB
17 have been observed in a number of mammalian species (Enciso *et al.* 2009, Cortés-
18 Gutiérrez *et al.* 2007), however, persist more extensively in disulphide bond-deficient
19 species such as the koala (*Phascolarctos cinereus*; Zee *et al.* 2009), short-beaked echidna
20 (Johnston *et al.* 2009) and teleost fish (*Tinca tinca*; López-Fernández *et al.* 2009). Thus,
21 the prevalence of ALS in the mature spermatozoa of *Xenopus laevis* is likely to be related
22 to the absence of protamines, the cysteine residues of which are essential for the formation
23 of disulphide bonds (Yokota *et al.* 1991).

24

1 Interestingly, the absence of protamines/cysteine residues and associated disulphide
2 bonds in *Xenopus laevis* spermatozoa did not appear to make the affected spermatozoa
3 more susceptible to post-testicular assault by processes such as reactive oxygen species
4 and denaturation (Sakkas and Alvarez 2010). Given the lack of disulphide bond-forming
5 protamines in the spermatozoa of *Xenopus laevis*, an underlying assumption of the study
6 was that DNA fragmentation would proceed in a rapid manner consistent with that of other
7 protamine-deficient species. However, in the three males examined, the SDF did not
8 exceed 61% in activated sperm samples over the duration of the 24 hour incubation period.
9 This phenomenon is in direct contrast to that observed in teleost fish, which also lack
10 cysteine residues and therefore disulphide bonding. For the bony fish *Tinca tinca*, an
11 increase in SDF occurs only a few minutes after sperm activation (López-Fernández *et al.*
12 2009). The rapid rate of DNA fragmentation observed in fish has not been reported in the
13 sperm of mammalian species that contain the capacity for disulphide bonding and thus,
14 may be due to the combination of osmotic stress imposed on the spermatozoa following
15 activation in addition to a chromatin structure lacking disulphide bonds (López-Fernández
16 *et al.* 2009). It is, therefore, interesting to note that *Xenopus laevis* that utilises the same
17 strategy of external fertilisation and osmotically induced activation and possesses a
18 chromatin structure devoid of disulphide bonds does not exhibit a similarly rapid decay of
19 DNA quality. This may indicate that another mechanism of chromatin stabilisation
20 functions in *Xenopus laevis* spermatozoa and highlights the importance of considering
21 inter-species differences in sperm chromatin structure when applying the SCD test to a
22 novel species.

23
24 This study supports that the SCD test can be used to successfully identify DNA
25 damage in *Xenopus laevis* spermatozoa and produces results concordant with other

1 standard DNA fragmentation assays. The unique situation with *Xenopus laevis* sperm
2 nuclear basic proteins reinforces the importance of developing species-specific DNA
3 fragmentation assays. This is particularly the case for the SCD test where visualisation of a
4 differential chromatin dispersion pattern and thus, an accurate assessment of DNA damage
5 is dependent on sufficient protein removal and exposure of the DNA molecule. As the first
6 amphibian protocol for the assessment of SDF, this protocol has the potential to benefit
7 assisted breeding programs using ART to support amphibian conservation efforts. The
8 structural integrity of sperm DNA is accepted as a potential indicator of an individual's
9 fertility. The ability to identify males whose sperm DNA is compromised or particularly
10 sensitive to fragmentation following activation will optimise mate pairing, minimise
11 wastage of female gametes and may aid the reproductive output of ART. Given that
12 gamete cryopreservation is a fundamental aspect of ART, it would be prudent to assess the
13 impact of freeze-thawing on the structural integrity of amphibian sperm DNA. Despite
14 successful protocols established for a number of amphibian species (Browne *et al.* 1998;
15 2002ab; Sargent and Mohun 2005; Michael and Jones 2004; Beesley *et al.* 1998), the
16 process of cryopreservation inherently promotes cellular damage that can compromise the
17 stability of the sperm chromatin. Furthermore, the information presented in this study has
18 the potential to provide an insight into the mechanisms of amphibian declines in the wild.
19 Given their unique anatomy, amphibians are particularly sensitive to fluctuations in
20 environmental conditions; their exposed and permeable skin coupled with a dependence on
21 water for breeding means they often react adversely to changes in the environment before
22 that of higher order vertebrates (Burlibasa and Gavrilina 2011). Accordingly, amphibians
23 have long been recognised as a sentinel species for modelling the effects of climate change
24 and environmental damage. The ability to evaluate the effect of climate

1 change/environmental phenomena on sperm DNA stability may prove valuable for the
2 conservation of threatened and endangered amphibians and their environment.

3

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FIGURE LEGENDS

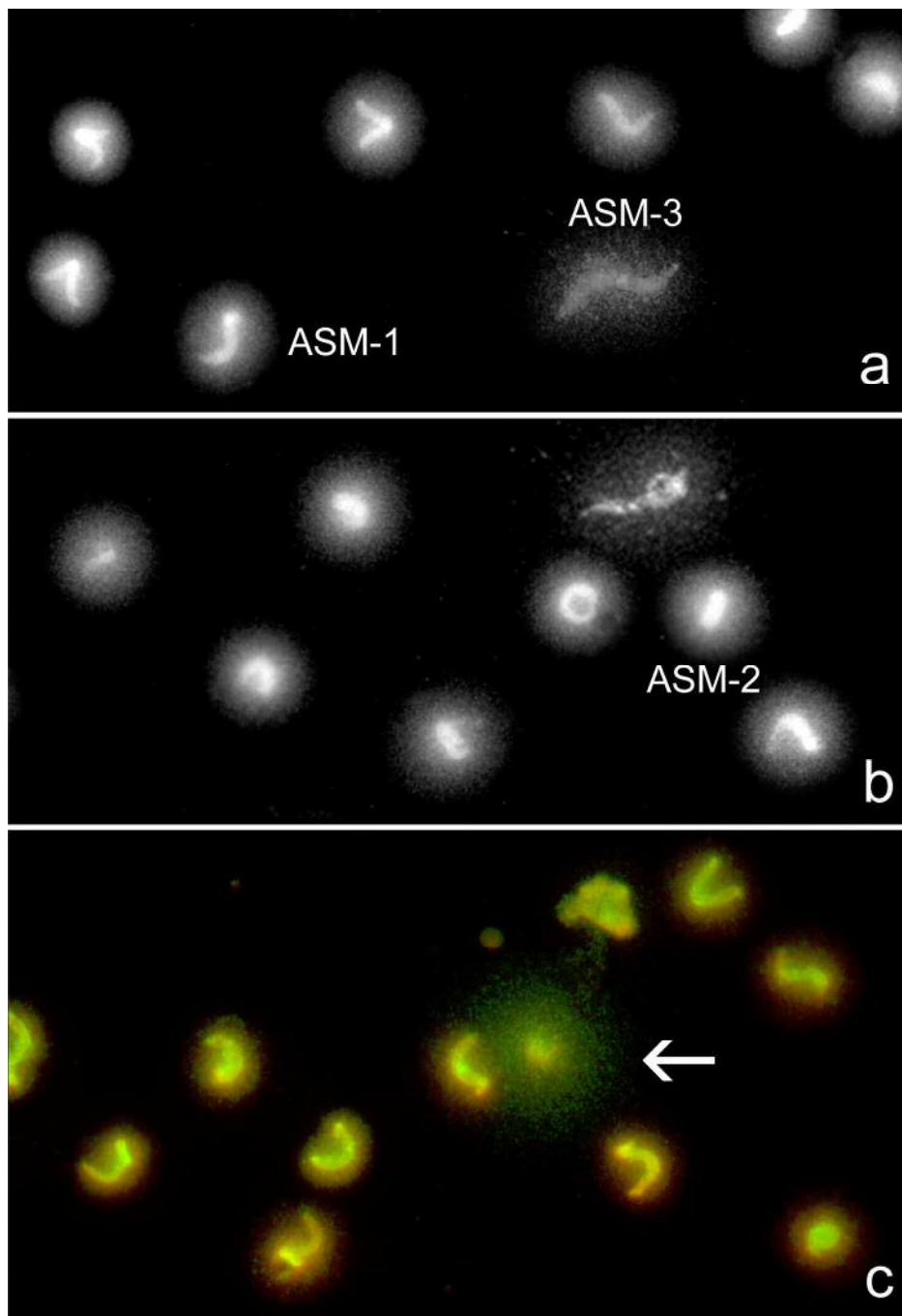
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3 Figure 1: *Xenopus laevis* sperm nuclear morphotypes after the SCD test. Arrows indicate
4 spermatozoa with large, stellar halos of dispersed chromatin corresponding to fragmented
5 DNA. a) Spermatozoa observed at T0 with coiled cores and small, compact halos of
6 dispersed chromatin representing non-fragmented DNA. The spermatozoon containing
7 fragmented DNA has retained a defined core; b) A higher proportion of spermatozoa with
8 round and slightly swollen cores was observed following one hour of incubation at room
9 temperature. Note the diminished core and halo of the fragmented spermatozoon. c) *In situ*
10 nick translation showing the direct incorporation of labelled nucleotides (green signal) in
11 all *Xenopus laevis* spermatozoa processed with the SCD test including a single sperm with
12 fragmented DNA (arrow).

13
14 Figure 2: Evidence of DNA fragmentation in *Xenopus laevis* spermatozoa following the
15 double (neutral + alkaline) comet assay at T0 immediately following activation; (a and b)
16 sperm nuclei with large nuclear cores and compact comet tails representing possessed
17 structural SSB only – ACM-1; (c) sperm nucleus with severely damaged DNA fragments
18 indicated by the presence of comets comprised of both SSB and DSB (ACM-3); (d) sperm
19 nucleus showing a comet comprised of structural SSB and DSB and (e) sperm nucleus
20 showing a high degree of SSB as evidence by a long comet tail and much reduced nuclear
21 core (ACM-2).

22
23 Figure 3: Correlation between the percent of SDF identified in three male *Xenopus laevis*
24 immediately following activation as estimated by SCD test (ASM-3) and double comet
25 assay (ACM-2 + ACM-3).

1

Figure 1



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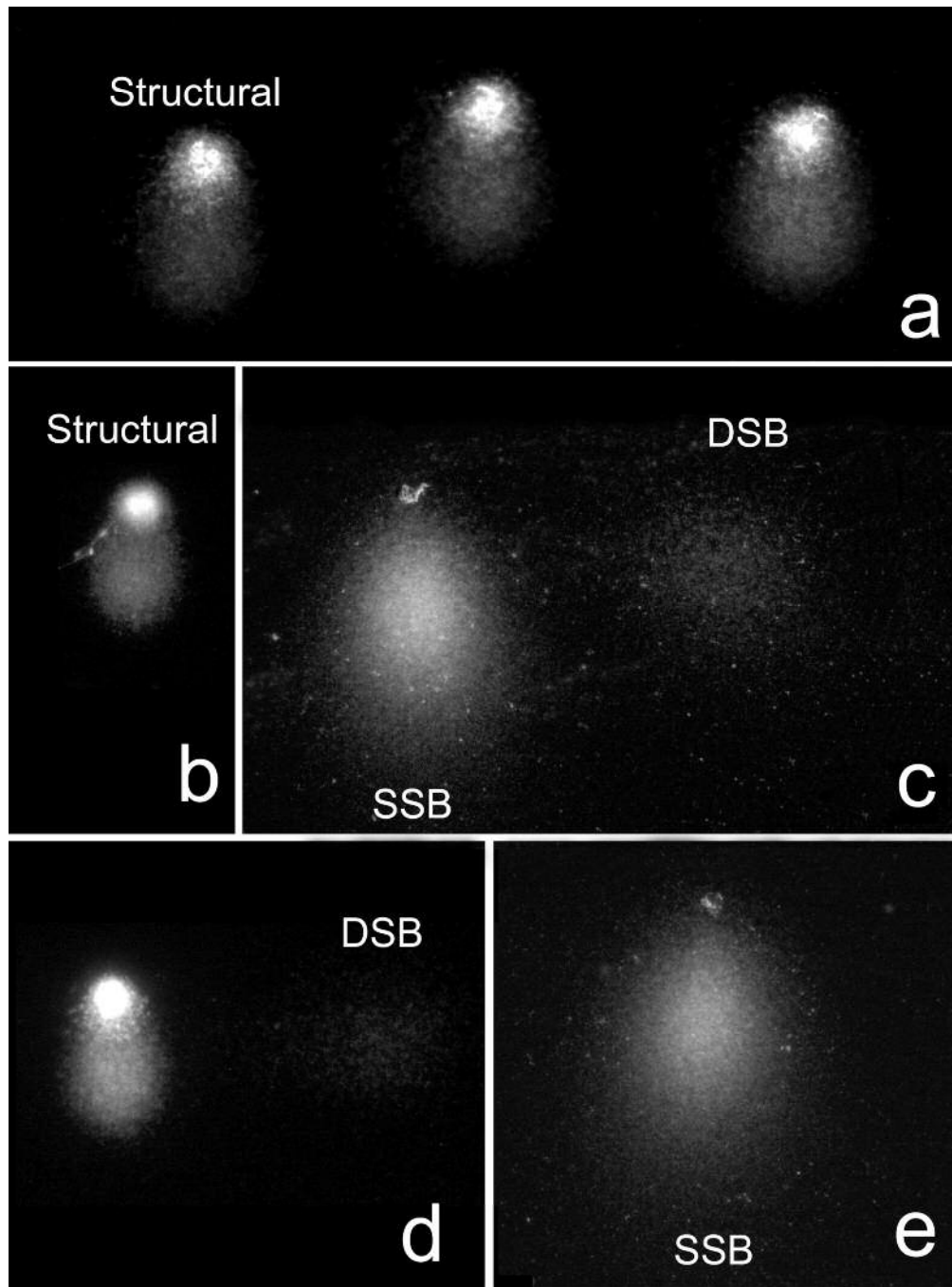
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Figure 2



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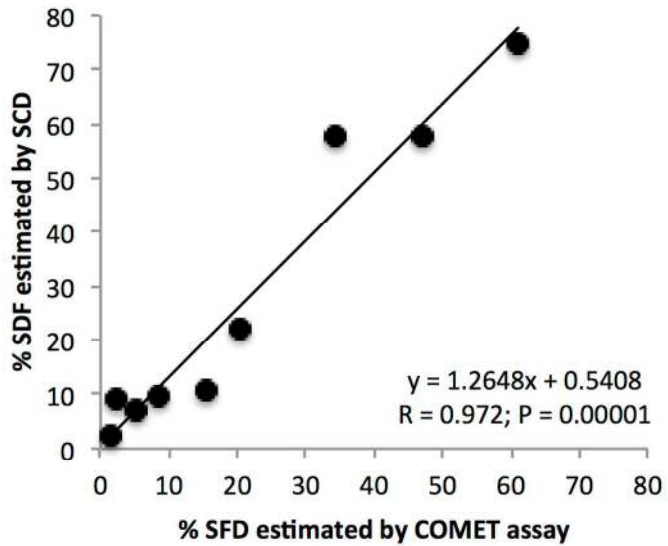
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Figure 3



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Review Only

1 Table 1: Sperm DNA fragmentation as assessed by the SCD in fresh activated *Xenopus*
 2 *laevis* sperm in three frogs incubated over 24h at 25°C.

3

Male	Time (T)	ASM-1	ASM-2	ASM-3	Total SDF (ASM-3)
1	T0	85.7	12	2.3	2.3
	T1	68.7	16	15.3	15.3
	T24	12.0	41	47	47
2	T0	92.7	2	5.3	5.3
	T1	55.7	22	20.3	20.3
	T24	4	35	61	61
3	T0	97.7	1	1.3	1.3
	T1	68.3	23	8.7	8.7
	T24	8.7	57	34.3	34.3

4

5 Table 2: Sperm DNA fragmentation as assessed by the double comet assay in fresh
 6 activated *Xenopus laevis* sperm in three frogs incubated over 24h at 25°C.

7

Male	Time (T)	ACM-1	ACM-2	ACM-3	ACM-2+3	Total SDF
1	T0	91.1	0.9	0.6	7.3	8.9
	T1	82.1	2.3	7.3	8.3	10.6
	T24	40	4	2	54	58
2	T0	91	1	2	6	7
	T1	64.7	15	13	7.3	22.3
	T24	20	7	5	68	75
3	T0	92.4	0.6	6.5	2	2.6
	T1	83.1	2.3	7.3	7.3	9.6
	T24	46	4	2	54	58

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9