Effects of freezing and activation on membrane quality and DNA damage in *Xenopus tropicalis* and *Xenopus laevis* spermatozoa

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Abstract. There is growing concern over the effect of sperm cryopreservation on DNA integrity and the subsequent development of offspring generated from this cryopreserved material. In the present study, membrane integrity and DNA stability of *Xenopus laevis* and *Xenopus tropicalis* spermatozoa were evaluated in response to cryopreservation with or without activation, a process that happens upon exposure to water to spermatozoa of some aquatic species. A dye exclusion assay revealed that sperm plasma membrane integrity in both species decreased after freezing, more so for *X. laevis* than *X. tropicalis* spermatozoa. The sperm chromatin dispersion (SCD) test showed that for both *X. tropicalis* and *X. laevis*, activated frozen spermatozoa produced the highest levels of DNA fragmentation compared with all fresh samples and frozen non-activated samples ($P < 0.05$). Understanding the nature of DNA and membrane damage that occurs in cryopreserved spermatozoa from *Xenopus* species represents the first step in exploiting these powerful model organisms to understand the developmental consequences of fertilising with cryopreservation-damaged spermatozoa.

Additional keywords: cryopreservation, DNA fragmentation, sperm chromatin dispersion.

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Introduction

In the face of the current global amphibian extinction crisis, sperm cryopreservation represents a key potential strategy for supporting threatened populations and enabling the future re-establishment of recently extinct species (Clulow *et al.* 2014). There is also increasing interest in the use of sperm cryopreservation for storing genetically altered lines of amphibians, especially *Xenopus laevis* and *Xenopus tropicalis*, given their growing importance as biomedical models (Khokha 2012; O'Neill and Ricardo 2013; Pratt and Khakhalin 2013; Schmitt *et al.* 2014). Such storage strategies enhance animal welfare by reducing the number of animals held and have a positive effect on the cost-effectiveness of biomedical research. Although cryopreserved amphibian spermatozoa recover motility and can fertilise eggs *in vitro* (Beesley *et al.* 1998; Browne *et al.* 1998; Michael and Jones 2004; Sargent and Mohun 2005), success rates are variable and species dependent.

Previous studies have reported that between 5% and 10% of *X. laevis* spermatozoa are potentially motile following the freeze–thaw process and show fertility rates ranging from 36% to 81%. Despite the decrease in post-thaw survival,

sufficient spermatozoa were recovered to generate mutant genotypes (Sargent and Mohun 2005). This is increasingly important because a wide range of mutants is now available in this model organism (e.g. Fish *et al.* 2014; Nakayama *et al.* 2015; Shi *et al.* 2015) that provides cost-effective access to a very wide range of experimental approaches while sharing much of the genome structure of humans (Hellsten *et al.* 2010). More recent efforts to optimise cryopreservation of *X. laevis* spermatozoa based on a matrix devised to evaluate the various stages of the freezing protocol yielded encouraging results (Mansour *et al.* 2009). However, there was a reduction in fertilisation rate (from 82% to 60%) and hatching rate (from 60% to 48%) from fresh to frozen samples. These decreases strongly suggest that cryopreserved amphibian spermatozoa have undergone damage that reduces the success of embryonic development; despite this, little is known about the nature and extent of such damage.

DNA fragmentation is an inherent feature of DNA condensation during spermiogenesis (Meistrich *et al.* 2003), but it can also be caused by external factors (González-Marín *et al.* 2012) (Wright *et al.* 2014). There is increasing evidence that the processes involved in sperm cryopreservation, which include

exposure to various chemicals as well as temperature and osmotic excursions, induce sperm DNA fragmentation (Kopeika *et al.* 2015). Therefore, the consequences for genome integrity as a result of the freeze–thaw process are of great concern given the importance of an intact genome for successful embryonic development. For example, inhibition of DNA repair in trout embryos derived from DNA-damaged spermatozoa revealed differential expression of genes involved in growth and repair, nervous system development, morphogenesis and cell differentiation (Fernández-Díez *et al.* 2015). Furthermore, trout embryos obtained from frozen spermatozoa had an increased number of deaths and altered mRNA levels of genes involved in growth and morphogenesis, including insulin growth factor receptor 1a (*Igfr1a*), growth factor 1 (*Gh1*) and insulin 1 (*Ins1*), suggesting a direct effect of fertilising with DNA cryodamaged spermatozoa (Pérez-Cerezales *et al.* 2011). In the loach, fertilisation with cryopreserved spermatozoa in the presence of a DNA repair inhibitor also revealed extensive embryonic death and axial defects in the surviving embryos (Kopeika *et al.* 2004). It is therefore clear that cryopreservation of spermatozoa can directly affect development, but in order to understand the mechanisms affected by sperm cryopreservation in detail it is necessary to work in a well-defined embryo model system.

The processes and mechanisms that govern embryonic development in *Xenopus* are well understood and the usefulness of this model has improved since the genomes of both species have become available (Hellsten *et al.* 2010; Karpinka *et al.* 2015). *Xenopus* are particularly suited to these studies because of the large numbers of embryos that can be produced synchronously and develop externally, and because they are readily analysed at various stages of development both phenotypically and by high-throughput biochemical analysis (Harland and Grainger 2011). More studies are urgently needed to shed light on the developmental consequences of cryoinduced DNA damage and *Xenopus* provides an ideal platform to do so. The present study represents an important first step towards this objective.

The aim of the present study was to test, in *Xenopus*, how sperm freezing and subsequent activation for IVF modify both the integrity of the sperm plasma membrane and the quality of the DNA. We hypothesised not only that cooling and freezing would induce DNA damage, but also that the extent of DNA damage would be exacerbated by motility activation. The practical use of frozen–thawed spermatozoa at the European *Xenopus* Research Centre (EXRC) has suggested that the effectiveness of cryopreservation differs between *X. laevis* and *X. tropicalis*, and one of the objectives of the present study was therefore to investigate and document these differences systematically. Species differences in sperm cryopreservation success occur frequently and sometimes provide useful insights into the mechanisms of cryoinjury (Holt 2000).

Materials and methods

Animals and recovery of spermatozoa

All the experiments described herein were performed under the appropriate national legislation. *Xenopus* were maintained in a recirculating aquarium system (Tecniplast) and fed at least once daily with high-protein trout pellets; *X. laevis* were maintained

at 18°C and *X. tropicalis* were maintained at 25°C. Four male *X. tropicalis* and four male *X. laevis* sourced from the EXRC were killed by terminal anaesthesia in ethyl-m-aminobenzoate (Sigma Aldrich) followed by destruction of the brain. Testes were removed and placed in $1.0\times$ modified Barth's saline (MBS; Gurdon 1977). To obtain fresh sperm samples from an *X. tropicalis* male, a single testis was macerated in $250\,\mu L$ of $0.1\times$ MBS and diluted with an equal volume of either $0.1\times$ MBS or distilled water (Sigma-Aldrich), for non-activated or activated samples respectively, to a final concentration of approximately 10×10^6 spermatozoa mL^{-1}. To obtain fresh sperm samples from an *X. laevis* male, a quarter of a testis was used in the same way but was diluted with $1.0\times$ or $0.1\times$ MBS for non-activated or activated samples respectively.

Experimental design

Analyses of sperm plasma membrane integrity and DNA fragmentation were performed within 2 min of sperm collection, using activated and non-activated sperm samples from each male (T0). Four biological replicates were performed side by side using a random sampling order and 300 spermatozoa were analysed on each slide. Analyses were repeated after incubation for 4 h (T4) or 24 h (T24) at room temperature. From each male, a separate portion of testis was removed, spermatozoa were extracted and then cryopreserved (see Sperm Freezing and Thawing section below) and the same analyses were performed.

Cryoprotective solution

Cryoprotective solution was made by first dispersing one chicken egg yolk ($\sim$15 mL) in an equal volume of distilled water and then diluting to 20% v/v in solution containing 0.4 M sucrose, 10 mM $NaHCO_3$ and 2 mM pentoxyfylline. Aliquots of cryoprotective solution were then centrifuged at $10\,000g$ for 20 min at 10°C in an Eppendorf 5810R centrifuge with an F-34-6-38 rotor. The pellets were discarded and the supernatants were divided into 500-μL aliquots then frozen and stored at −20°C (method adapted from the Harland Laboratory; http://tropicalis.berkeley.edu/home/obtaining_embryos/sperm-freezing/sperm-freeze.html, accessed 1 October 2013).

Sperm freezing and thawing

A single *X. tropicalis* testis or approximately one-quarter of an *X. laevis* testis was transferred to an Eppendorf tube in 0.5 mL Leibovitz-15 (L-15) medium (Sigma-Aldrich) supplemented with 10% calf serum (Sigma-Aldrich) and 2 mM L-glutamine; it was then dissociated by gentle application of an Eppendorf pestle. Ice-cold cryoprotective solution was added to ice-cold sperm macerates in a ratio of 1 : 1 and gently mixed, and 250 μL samples were pipetted into 0.5-mL thin-walled polypropylene Eppendorf tubes (Fisher Scientific). The tubes were placed in a polystyrene box covered with aluminium foil and placed directly into the −80°C freezer for at least 24 h. This was the most effective of the three published methods (Sargent and Mohun 2005; Mansour *et al.* 2009; Harland lab protocol, above) we have tested when measured by healthy tadpole production. After a minimum of 24 h, sperm samples were removed from the −80°C freezer and held by hand to defrost them. *X. tropicalis*

samples were activated with 250 µL sterile nuclease-free water and *X. laevis* samples were activated with 250 µL of $0.1\times$ MBS before analysis (method adapted from the Harland Laboratory; http://tropicalis.berkeley.edu/home/obtaining_embryos/sperm-freezing/sperm-freeze.html, accessed 1 October 2013).

Plasma membrane integrity

Sperm plasma membrane integrity was assessed using propidium iodide (PI; Yániz *et al.* 2013). Each sample was diluted to 1.6×10^6 cells mL^{-1}; then, 8 µL was pipetted onto the surface of a glass slide and mixed with 1 µL acridine orange (Sigma Aldrich) and PI (Sigma Aldrich), each at a stock concentration of 1 mg mL^{-1}. Acridine orange, which has an emission maximum of 525 nm (green) when bound to DNA, permeates all spermatozoa. However, this is displaced by PI in spermatozoa with damaged plasma membranes, which then fluoresce red (emission maximum 617 nm). Three hundred spermatozoa were manually counted per sample using a Leica DMRB epifluorescence microscope (Leica Microsystems) equipped with single-band fluorescence block filter for green (fluorescein isothiocyanate (FITC) equivalent) and red (Cy3 equivalent) fluorescence. The proportion of spermatozoa stained green was then calculated.

Sperm chromatin dispersion test

In the present study the sperm chromatin dispersion (SCD) test was performed using the Sperm-Halomax kit (Halotech) adapted for *Xenopus*, with the following protocol. Molten 50-µL aliquots of low melting point agarose maintained at 35°C were mixed with 20-µL portions of sperm suspension. Aliquots (10-µL) of the sperm–agarose mixture were then pipetted onto the surface of a pretreated glass slide (provided in the Sperm-Halomax kit) and covered with a glass coverslip (18×18 mm). Gentle pressure was applied to the coverslip to ensure the formation of a thin, even microgel. The slides were then placed onto a prechilled (4°C) metallic tray and placed in the refrigerator at 4°C for at least 5 min. The slides were then taken out of the refrigerator, the coverslips removed and the slides placed horizontally into a bath of lysing solution provided in the Sperm-Halomax kit for 5 min to lyse the spermatozoa and deproteinise the DNA. The slides were then removed from the lysis solution and placed in a bath of distilled water for 5 min, followed by subsequent washes in 70% and 100% ethanol for 2 min each time to dehydrate the microgels. The slides were then finally left to air dry. Equal parts of SYBR green (Sigma Aldrich) and VectaShield mounting medium (Vector Laboratories) were added to the slides, which were visualised using fluorescent microscopy. Three hundred spermatozoa were counted per sample using a Leica DMRB epifluorescence microscope (Leica Microsystems) equipped with single-band fluorescence block filter for green (FITC equivalent) and red (Cy3 equivalent) fluorescence. The maximum excitation wavelength of SYBR green is 497 nm and the fluorescence emission of SYBR green-stained DNA is 520nm. The proportion of spermatozoa showing DNA fragmentation was calculated.

In situ nick translation

For *in situ* nick translation (ISNT), spermatozoa were diluted to 10×10^6 cells mL^{-1} and embedded into an agarose microgel on a pretreated slide as described above for the SCD test using slides and lysis solution from the Sperm-Halomax kit. Once the slides had been dehydrated in the ethanol series and dried, they were washed in phosphate-buffered saline (PBS) for 5 min and then incubated in DNA polymerase reaction buffer (10 mM Tris-HCl, 5 mM MgCl$_2$, 7.5 mM dithiothreitol, pH 7.5) for 10 min at 37°C. Then, 100 µL reaction buffer containing 25 units DNA-polymerase I Large (Klenow) Fragment (Invitrogen) and 20 µM of each nucleotide, including biotin-16-dUTP, was pipetted onto the slide, covered with a plastic coverslip and incubated in a moist chamber for 30 min at 37°C. As a negative control, the same procedure was repeated on another microgel containing spermatozoa from the same male but without the DNA polymerase I. The slides were then washed three times in PBS for 5 min each time and then dehydrated in sequential 70%, 90% and 100% ethanol baths and air dried. To detect the incorporation of biotin-16-dUTP, slides were incubated with streptavidin Alexa Fluor 488 conjugate (Life Technologies) diluted 1 : 500 for 30 min at 21°C and then washed three times in PBS to remove excess streptavidin Alexa 488. The maximum excitation wavelength of Alexa Fluor 488 is 493 nm, with a fluorescence emission of 519 nm. Slides were then counterstained with PI (2 µg mL^{-1}). When PI is bound to nucleic acids, the fluorescence excitation maximum is 535 nm and the emission maximum is 617 nm. Fluorescence images were captured using a Zeiss Axiomager ZI fluorescence microscope equipped with a Hamamatsu digital camera (C4742-95) with single-band fluorescence block filters for green (FITC equivalent) and red (Cy3 equivalent) to detect fluorescence. Areas of interest were captured using Velocity 4 software (Improvision; PerkinElmer). Three hundred spermatozoa were assayed and counted for each treatment.

Statistical analysis

Statistical analyses were performed using STATISTICA for Windows (Statsoft UK). Effects were examined using analysis of variance (ANOVA) and specific contrasts were examined within the ANOVA using orthogonal polynomial coefficients. Where data did not exhibit normal distribution, the non-parametric Kruskal–Wallis test was used for statistical analysis. Relationships between membrane integrity, DNA fragmentation and sperm freezing were examined using analysis of covariance (ANCOVA; vassarstats.net, accessed 21 May 2014). All data are shown as the mean $\pm$ s.e.m.

Results

Assessment of sperm DNA quality in response to stressors requires the dynamic evaluation of DNA damage. This approach of assessing DNA damage over time to create a 'profile' indicative of DNA stability can reveal damage that is cryptic and/or occurring at levels not easily detectable at the time the spermatozoa are extracted or thawed (Gosálvez *et al.* 2009, 2011*a*). Thus, more reliable comparisons between individuals or groups can be drawn and this approach was adopted throughout.

Plasma membrane integrity

The dye exclusion test was able to distinguish effectively between spermatozoa with intact or damaged membranes in
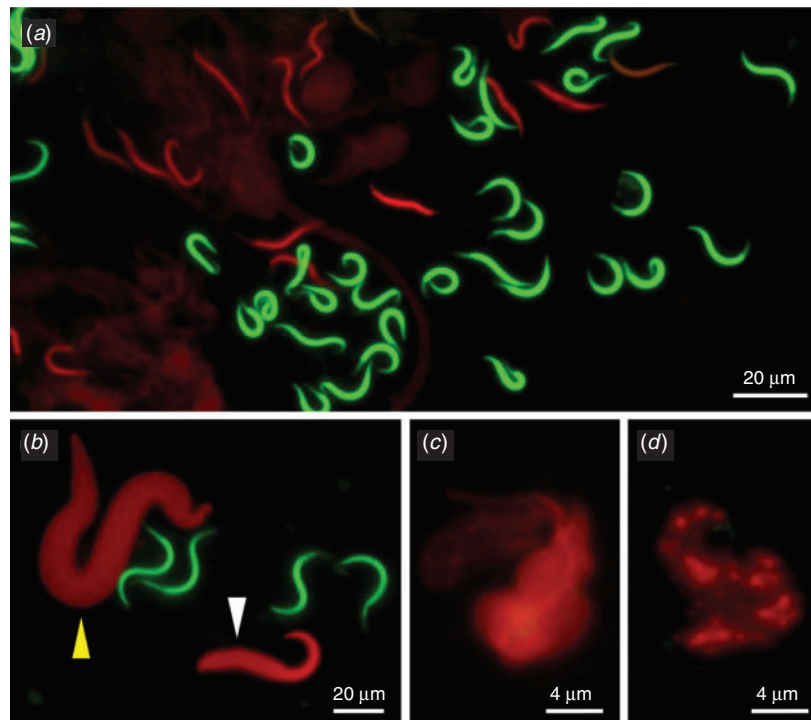
Fig. 1. Spermatozoa from *Xenopus tropicalis* were frozen–thawed, stained to reveal plasma membrane damage and then photographed under fluorescence microscopy. (*a*) *X. tropicalis* spermatozoa with intact membranes stained green with acridine orange, whereas spermatozoa with damaged membranes stained red after acridine orange had been displaced by propidium iodide. (*b*) Spermatozoa with damaged membranes have sperm heads that exhibit significant swelling and are larger than spermatozoa with intact membranes (white arrowhead). A spermatozoon with macrocephaly is also shown (yellow arrowhead). (*c*) Progressively more diffuse staining is noticeable in spermatozoa with damaged membranes and (*d*) persistent clusters of intense fluorescence may represent apoptotic bodies. *Xenopus laevis* sperm morphology is the same (data not shown).

X. tropicalis (Fig. 1*a*). Spermatozoa with damaged membranes also showed swelling (e.g. Fig. 1*b*, white arrow). We also detected a morphological abnormality in the form of macrocephaly, suggesting a polyploid spermatozoon (e.g. Fig. 1*b*, yellow arrow). More significant progressive swelling and diffuse staining was observed in spermatozoa stained red (Fig. 1*c, d*). Although the number of spermatozoa with poor membrane integrity was higher in frozen and activated samples (see below) than in controls, there were no clear morphological differences between the samples or between species (data not shown).

Sperm activation status had little or no effect on the detrimental effects of freezing and thawing ($F_{1,36} = 3.77$; $P > 0.05$) and, at T0, the extent of freezing-induced plasma membrane damage was significant, regardless of activation status ($F_{1,18} = 27.95$ ($P < 0.001$) and $F_{1,18} = 15.01$ ($P = 0.001$) for non-activated and activated spermatozoa respectively). Therefore, activation status was disregarded for the purposes of further data analysis.

Sperm plasma membrane integrity was significantly reduced in *X. tropicalis* after freezing and thawing (Fig. 2); mean values at T0 for fresh and frozen–thawed spermatozoa were $68.8 \pm 4.1\%$ and $40.4 \pm 1.1\%$ respectively ($F_{1,8} = 370.7$;

$P < 0.001$). Equivalent mean values for the entire 24 h period were $52.3 \pm 3.7\%$ and $23.7 \pm 3.7\%$ for fresh and frozen–thawed spermatozoa respectively ($F_{1,46} = 20.9$; $P < 0.001$). Membrane integrity of fresh and frozen–thawed spermatozoa declined at equivalent rates over the first 4 h of incubation, as indicated by the lack of significant interaction between percentage membrane integrity and freezing during that period ($F_{1,28} = 1.62$; $P > 0.05$), but after 24 h all the frozen–thawed spermatozoa exhibited plasma membrane damage.

Plasma membrane integrity of *X. laevis* spermatozoa was also reduced by freezing and thawing (Fig. 3). At T0 the proportion of frozen–thawed spermatozoa with an intact plasma membrane (median 10%) was nearly one-seventh that of fresh spermatozoa (median 68.5%; Kruskal–Wallis $H = 11.31$, d.f. $= 1$, $n = 16$; $P < 0.001$). A similar difference was also observed at T4 (median percentage of fresh and frozen–thawed spermatozoa with an intact plasma membrane 52.5% and 7.5% respectively; Kruskal–Wallis $H = 11.31$, d.f. $= 1$, $n = 16$; $P < 0.001$). No statistically significant difference in the effects of freeze–thawing was observed between activated and non-activated spermatozoa.
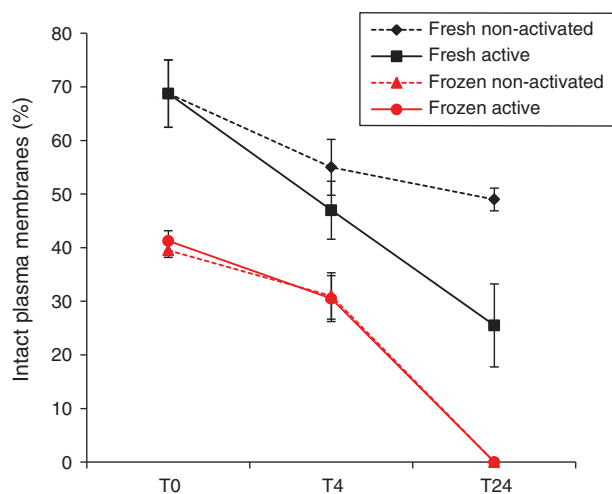
Fig. 2. *Xenopus tropicalis* ($n = 4$) spermatozoa from fresh samples or frozen–thawed samples that were either activated or non-activated were stained to reveal plasma membrane damage and then counted using fluorescence microscopy. The mean percentage of spermatozoa with intact membranes was counted at the times shown following incubation at room temperature and plotted. T0, within 2 min of sperm collection; T4 after 4 h incubation; T24, after 24 h incubation. The percentage of spermatozoa with intact plasma membranes is higher in fresh than frozen–thawed samples at all time points ($P < 0.001$), whereas there is only a significant difference between non-activated and activated samples at T24 ($P < 0.05$). Data are the mean ± s.e.m.
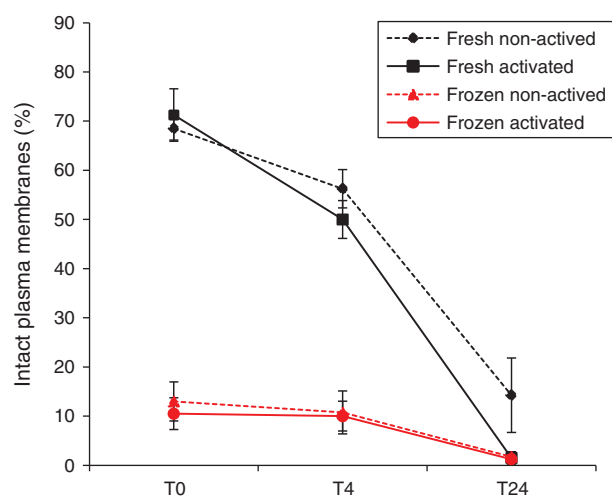

Fig. 3. *Xenopus laevis* ($n = 4$) spermatozoa from fresh samples or frozen–thawed samples that were either activated or non-activated were stained to reveal plasma membrane damage and then counted using fluorescence microscopy. The mean percentage of spermatozoa with intact membranes was counted at the times shown following incubation at room temperature and plotted. T0, within 2 min of sperm collection; T4 after 4 h incubation; T24, after 24 h incubation. The percentage of spermatozoa with intact plasma membranes is higher in fresh than frozen–thawed samples at all time points ($P < 0.001$). There is no significant difference between activated and non-activated samples. Data are the mean ± s.e.m.

Validation of the SCD test through ISNT

ISNT was used to label the ends of DNA strand breaks and therefore to confirm the presence of DNA fragmentation observed in the SCD test. Incorporated nucleotides bound to streptavidin-labelled fluorescein incorporated at the 3′-OH ends of single-strand breaks (SSBs) and double-strand breaks (DSBs) produce green fluorescence and, when merged with images showing the same sample stained with PI, frozen–thawed spermatozoa exhibiting fragmented DNA can be identified by their green–yellow halo (Fig. 4*a–f, a′–f′*). As a negative control, DNA polymerase I was omitted from the reaction, resulting in no incorporation of labelled nucleotides, as shown by a lack of green fluorescence (Fig. 4*g–l*). Notably, green fluorescence can be detected in the nuclear core of spermatozoa that do not exhibit a halo of fragmented DNA (Fig. 4 *e, f, e′, f′*).

DNA fragmentation and sperm morphology

Sperm DNA fragmentation assessment through the SCD test revealed four main morphotypes. Sperm morphotypes (SM) 1 and 2 (Fig. 5*a, b*) showed no DNA fragmentation, but loss of the classic elongated crescent core of the sperm head was observed in SM-2. The chromatin surrounding the sperm head was still highly organised and restricted to the immediate periphery. SM-3 showed a low level of DNA fragmentation, with the chromatin dispersing further, thus creating a larger halo (Fig. 5*c*). The final morphotype, SM-4, shows a high level of DNA fragmentation with complete loss of the chromatin ring and with highly dispersed fragments of DNA (Fig. 5*d*). Omission of DNA polymerase I as a control prevented the incorporation of biotin (Fig. 5*g–l*). In some cases, the halos produced in the SM-4 morphotype were difficult to identify because of the highly dispersed fragments of DNA and reduced size of the nuclear core (Fig. 6; cf. cells indicated by yellow and white arrows). This may have produced underestimates of the number of spermatozoa with highly damaged DNA at later time points.

At T0, the activation status of spermatozoa did not affect the rate of DNA fragmentation. However, after 4 h incubation, activated spermatozoa showed a significantly higher DNA fragmentation rate than non-activated spermatozoa (regardless of the freeze–thawing treatment; $F_{1,12} = 8.69$; $P = 0.012$). An elevated DNA fragmentation rate due to freeze–thawing was also detectable after 4 h incubation ($F_{1,12} = 10.08$; $P = 0.008$), but no significant effects of freeze–thawing on DNA fragmentation were observed at either T0 or T24 (Figs 7, 8).

The data showed significant interactions between the effects of freezing and sperm activation at all the time points examined. Activated spermatozoa always exhibited higher proportions of DNA fragmentation. Freeze–thawing induced significant increases in percentage DNA fragmentation at T0 ($F_{1,12} = 18.11$; $P = 0.001$) and T4 ($F_{1,12} = 61.9$; $P < 0.001$), but paradoxically the reverse was observed at T24. This T24 result is considered to be an artefact (see Discussion).

Combined effect of freezing on membrane integrity and DNA quality

Data for *X. laevis* ($r^2 = 0.7$; $P < 0.001$) and *X. tropicalis* ($r^2 = 0.48$; $P = 0.014$) revealed significant negative correlations
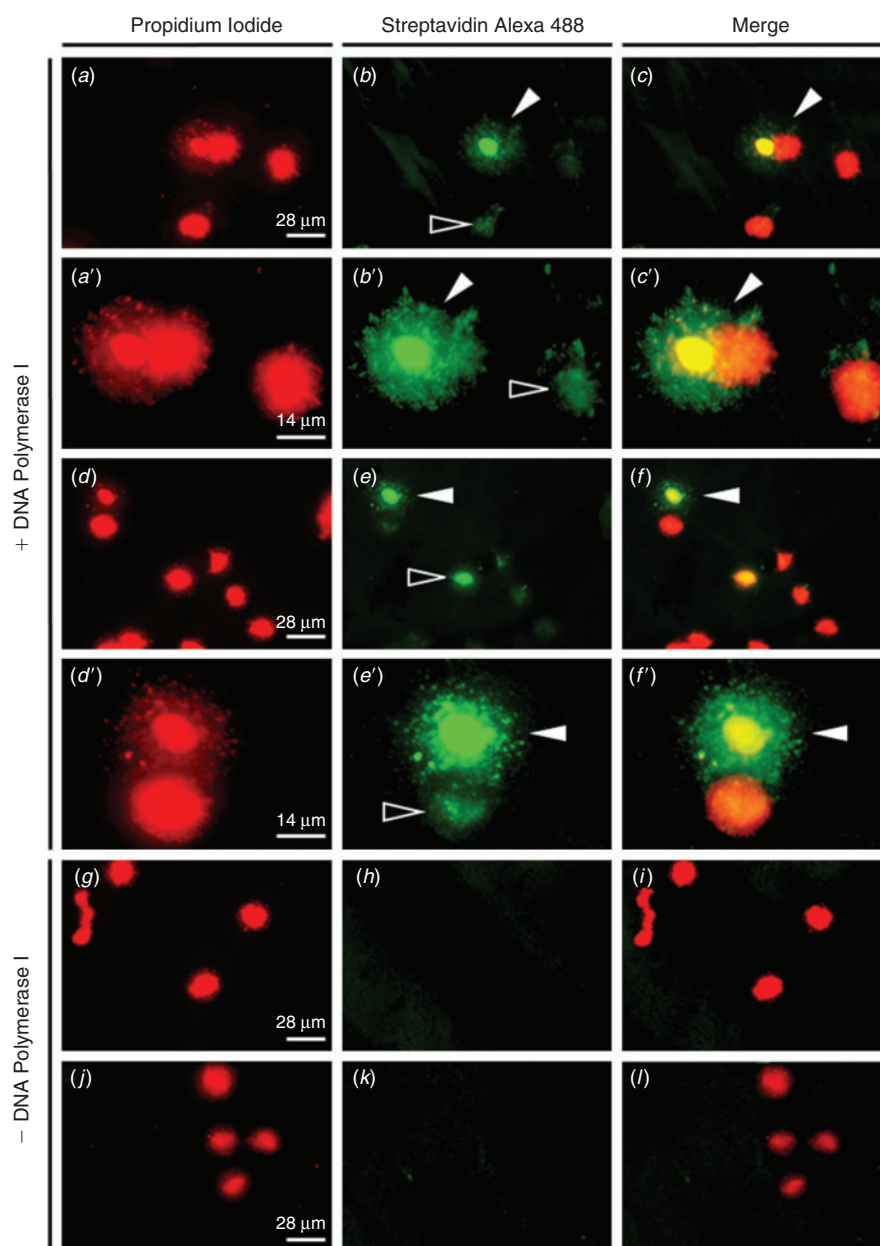
Propidium Iodide	Streptavidin Alexa 488	Merge

Fig. 4. Spermatozoa from one *Xenopus tropicalis* were frozen–thawed and DNA breakage was then assayed using *in situ* nick translation. (*a*, *a'*, *d*, *d'*) Spermatozoa stained with propidium iodide show the presence of fragmented DNA in the form of a halo surrounding the nuclear core. (*b*, *b'*, *e*, *e'*) Incorporation of biotin-16-dUTP onto the strand breaks, as indicated by the green staining from bound streptavidin Alexa 488 (white arrowheads). Some green fluorescence in the nuclear core of spermatozoa that did not show the characteristic halo of fragmented DNA was also detected (clear arrowhead). (*c*, *c'*, *f*, *f'*) Merged images. The halo of fragmented DNA stained by propidium iodide corresponds to the incorporation of biotin-16-dUTP onto the strand breaks shown by the green staining from bound streptavidin Alexa 488 (white arrowheads). As controls, incubations were performed without DNA polymerase I; no biotin-16-UTP incorporation was detected (*g*–*l*).

between DNA fragmentation and sperm plasma membrane integrity. These data are the mean of the activated and non-activated samples, which showed no difference from one another. Spermatozoa with plasma membrane damage showed higher levels of DNA fragmentation, as indicated by the regression lines in Fig. 9. One-way ANCOVA for the combined effect of freezing on membrane integrity and DNA fragmentation showed that the regression lines for fresh and frozen
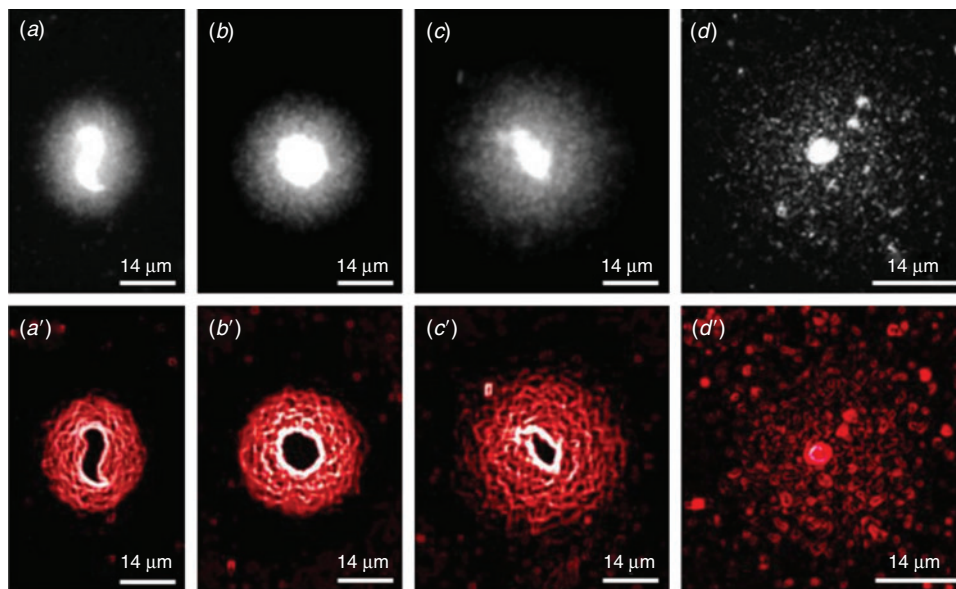
Fig. 5. Spermatozoa from one *Xenopus tropicalis* were frozen–thawed and DNA breakage was then assayed using the sperm chromatin dispersion test, which produces four spermatozoa morphotypes (SM) following lysis treatment to allow DNA to diffuse out of the sperm head. (*a*) Spermatozoa with undamaged DNA show a chromatin halo that is highly compact and restricted to the periphery of the sperm head (SM-1). (*b*) Undamaged spermatozoa can also show swelling where the sperm head adopts a more rounded shape and loses the classic corkscrew shape. The halo is still organised and compact (SM-2). (*c*) A low level of DNA fragmentation is shown when the halo begins to expand and disperse, and fragments can be seen around the halo (SM-3). (*d*) A high level of DNA fragmentation is seen when the halo is completely disorganised with fragments of DNA spread around the sperm head (SM-4). (*a′–d′*) Images were filtered to enhance these differences in red. The morphologies of the spermatozoa were similar between fresh and frozen–thawed samples, but the proportion of spermatozoa showing the various morphologies did change.

spermatozoa did not depart significantly from being parallel in either species.

Discussion

In the present study we demonstrated detrimental effects on DNA quality in the spermatozoa of *X. tropicalis* and *X. laevis* caused by two treatments: cryopreservation and activation. A simple dye exclusion test revealed that the freeze–thaw process causes significantly more damage in *X. laevis* than *X. tropicalis* spermatozoa at T0. The plasma membrane integrity of both fresh and frozen samples from both species continued to decrease over time, with frozen samples reaching a point of complete loss of viable spermatozoa by 24 h. Throughout the time course we did not observe a significant effect of activation on membrane integrity. Because even minor sperm damage is revealed during prolonged incubation (Johnston *et al.* 2012), there is therefore no evidence that activation contributes to sperm death within the time it takes for fertilisation to occur. However, cryopreservation caused very significant damage: a 30% decrease in plasma membrane integrity for *X. tropicalis* and a 60% decrease for *X. laevis*. The *X. laevis* data in the present study are very similar to those obtained by Mansour *et al.* (2009), with both studies reporting approximately 70% spermatozoa with intact membranes from fresh samples and the maximum 20% for frozen samples in the present study falling into the range reported by Mansour *et al.* (2009) of 7–50%. To our knowledge, this is the

first description of sperm plasma membrane integrity measurement for *X. tropicalis*.

In addition to detecting damaging effects on plasma membrane integrity, the present study has shown a significant detrimental effect of cryopreservation on DNA quality in both *Xenopus* species. The sperm DNA fragmentation index was determined by the SCD test, which, in turn, was validated by ISNT through incorporation of fluorescently labelled nucleotides into SSBs and DSBs. At T0, the most relevant for fertilisation use, there is a significant decrease in DNA integrity in frozen activated *X. laevis* samples compared with all other treatments. Because this is the sample that would be used for fertilisation, it is clear that the DNA damage observed is relevant to the use of such frozen–thawed spermatozoa. Although in this case we were able to detect DNA damage at T0, DNA damage levels can sometimes be very low or cryptic at T0, but potentially significant nonetheless. Effects may not be seen until 4 h or later; hence, we measured DNA stability over time. Overall, a cryopreservation and activation-induced increase in DNA damage was observed over the 24-h period for both species. For *X. laevis* at T24, activation alone significantly enhanced DNA damage in fresh sperm samples. Although there was no significant DNA damage enhancement detected at T0 for *X. tropicalis*, cryptic damage was detected at later times; thus, for both species, there is strong evidence that when frozen–thawed and activated spermatozoa are used to fertilise eggs, they contain damaged DNA. Although
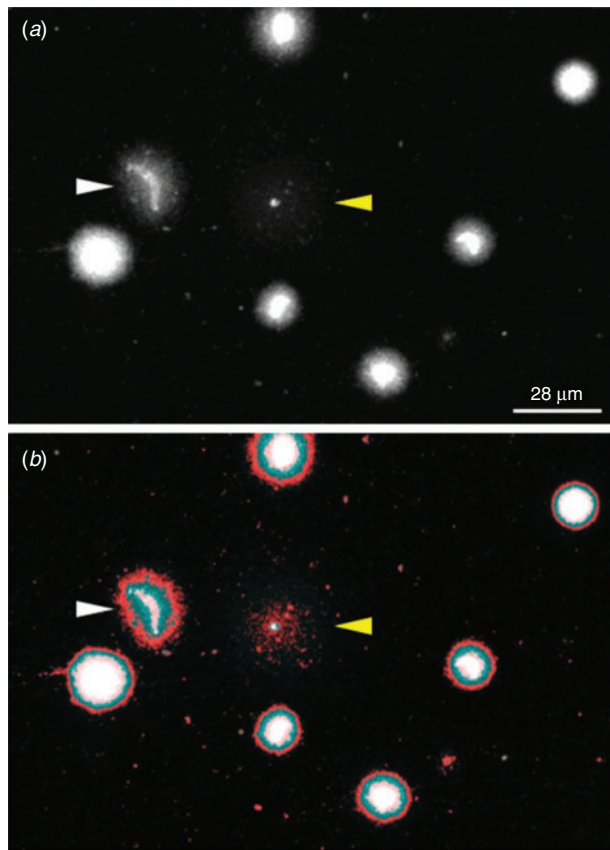
Fig. 6. Spermatozoa from one *Xenopus tropicalis* were frozen–thawed and DNA breakage was then assayed using the sperm chromatin dispersion (SCD) test. (*a*) Typical example of the field of view when observing halos from the SCD test. (*b*) Digital enhancement highlighting the different morphotypes. White arrowheads point to a spermatozoon with a low level of DNA damage and the yellow arrowheads point to a spermatozoon with a high level of DNA damage.

Fig. 7. *Xenopus tropicalis* ($n = 4$) spermatozoa from fresh samples or frozen–thawed samples that were either activated or non-activated were treated with lysis solution, stained to reveal DNA fragmentation and then counted using fluorescence microscopy. The mean percentage of spermatozoa with fragmented DNA was counted at the times shown following incubation at room temperature and plotted. T0, within 2 min of sperm collection; T4 after 4 h incubation; T24, after 24 h incubation. DNA fragmentation was higher in the frozen–thawed than fresh samples at T4 and T24 ($P < 0.05$) and in the activated than activated samples ($P < 0.05$). Data are the mean $\pm$ s.e.m.

the SCD assay has already been validated in *X. laevis* (Pollock *et al.* 2015), we can find no evidence of data from either *Xenopus* or other amphibia with which to compare the data reported here.

Although the exact mechanisms that underlie the process of cryoinduced DNA damage are not fully understood, reactive oxygen species (ROS) formed during cryopreservation have been widely implicated in inducing DNA damage, both in fish (Li *et al.* 2010) and in mammalian spermatozoa (Lopes *et al.* 1998; Bennetts and Aitken 2005; Gosálvez *et al.* 2014). An alternative explanation to the oxidative stress mechanism is the enhancement of pre-existing DNA damage, defects in DNA repair enzymes (Zribi *et al.* 2010) or activation of caspase and apoptosis (Paasch *et al.* 2004; Said *et al.* 2010). Because the delay between the sperm extraction process and necessary preparation steps for analysis was kept to a minimum and did not exceed 2 min, the likely explanations for the enhancement of DNA damage described here are limited.

The molecular differences that underpin the response of spermatozoa to cryopreservation largely remain to be explored, but spermatozoa of species with low DNA protamination have
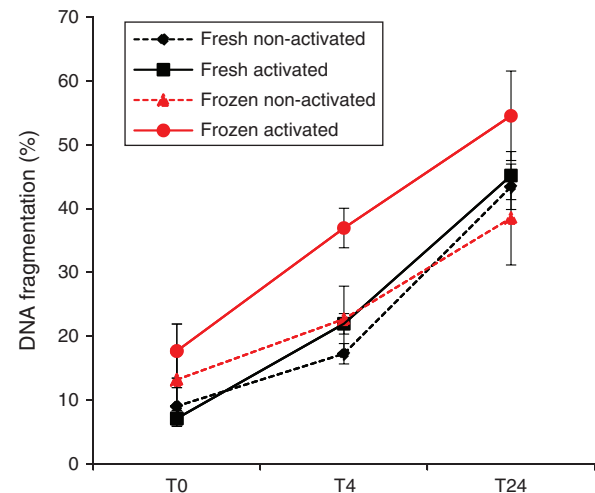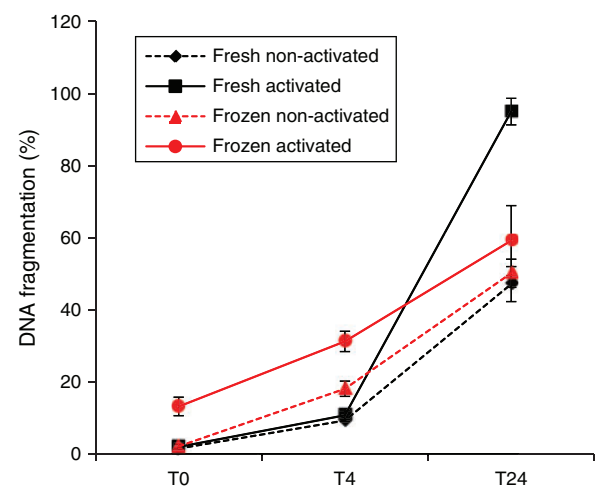

Fig. 8. *Xenopus laevis* ($n = 4$) spermatozoa from fresh samples or frozen–thawed samples that were either activated or non-activated were treated with lysis solution, stained to reveal DNA fragmentation and then counted using fluorescence microscopy. The mean percentage of spermatozoa with fragmented DNA was counted at the times shown following incubation at room temperature and plotted. T0, within 2 min of sperm collection; T4 after 4 h incubation; T24, after 24 h incubation. DNA fragmentation was higher in the frozen–thawed than fresh samples at T0 and T4 ($P < 0.01$) and in the activated than activated samples at T0 and T24 ($P < 0.05$). Data are the mean $\pm$ s.e.m.
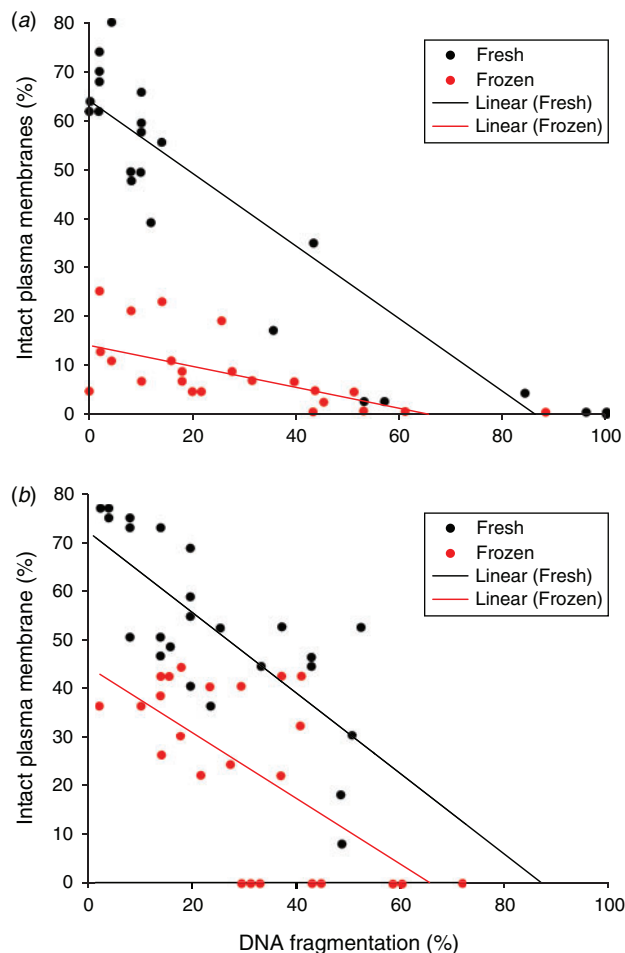
Fig. 9. Combined scatter plots showing the correlation between the proportion of spermatozoa with intact membranes and the proportion of DNA fragmentation in fresh and frozen–thawed samples for (*a*) *Xenopus laevis* ($r^2 = 0.7$; $P < 0.0001$) and (*b*) *Xenopus tropicalis* ($r^2 = 0.48$; $P < 0.014$). Data are the mean $\pm$ s.e.m.

increased vulnerability to oxidative damage (Gosálvez *et al.* 2011*b*). There is growing consideration for the developmental consequences that could potentially arise from such scenarios. For example, genome regions that are not associated with protamines may permit 'easy' access to transcriptional machinery after fertilisation, but may also confer an increased predisposition to iatrogenic assault resulting in DNA fragmentation of key embryonic genes. Indeed, it has been demonstrated in trout spermatozoa that positional differences are a key factor in determining sensitivity to oxidative damage and that certain genes can function as good biomarkers for cryoinduced damage, such as *Hox* genes (González-Rojo *et al.* 2014).

X. laevis lack protamine disulfide bonds but adopt an alternative chromatin-stabilising mechanism where alkali labile sites (ALS) produce stretches of highly repetitive and highly sensitive single-stranded DNA that heavily condenses the chromatin during spermatogenesis (Cortés-Gutiérrez *et al.* 2008). Because *Xenopus* are external fertilizers, it is thought that this serves as a key mechanism for ensuring that the integrity of

Xenopus spermatozoa is not compromised during transit to the eggs. ALS may explain SM-2, where the distinct corkscrew shape of the sperm head is lost but no DNA fragmentation is detected. This was also apparent in the ISNT assay, where spermatozoa that did not exhibit morphotypes showing halos of DNA fragments had still incorporated fluorescent-labelled nucleotides, signifying the incorporation of nucleotides within the nuclear core at SSBs in ALS (Pollock *et al.* 2015). Indeed, from a functional and evolutionary standpoint, it is not surprising that activation of spermatozoa and the subsequent increase in DNA breakage following their release into the surrounding aquatic environment before fertilisation may diminish the quality of the DNA. It is possible that this serves as a mechanism to ensure that only spermatozoa that have spent a minimal amount of time exposed to the external and potentially detrimental environment can fertilise eggs.

This interpretation is dependent on the underlying assumption that spermatozoa with high DNA fragmentation are likely to have low fertility rates or less capacity to fertilise. Indeed, for *X. laevis* there is a strong correlation between high DNA fragmentation and poor membrane integrity. This therefore represents a strong selection pressure on reproductive performance. Interestingly, the data for *X. tropicalis* show that there is a population of sperm samples that has a higher proportion of intact plasma membranes than *X. laevis*. Furthermore, these sperm samples show levels of DNA fragmentation that exceed 30%. This suggests that frozen *X. tropicalis* spermatozoa, which have fragmented DNA, better retain their capacity to fertilise compared with *X. laevis* spermatozoa and that there is an increased probability of DNA-fragmented *X. tropicalis* spermatozoa fertilising an egg. Indeed, even fertilisation by spermatozoa with DNA fragmentation levels lower than 30% and with intact membranes could still have significant developmental consequences. It may not necessarily be the quantity of DNA damage alone, but also the nature of the DNA damage (i.e. SSB or DSB) and location within the genome that determines any detrimental effect on embryo development. For example, DNA damage does not affect normal development in intracytoplasmic sperm injection-derived pig embryos (Men *et al.* 2013), but there are reports that the opposite is true in humans (for a review, see Zini *et al.* 2009).

In conclusion, the present study has shown that the freeze–thaw process induces significant reductions in sperm membrane and DNA quality in both species of *Xenopus* and that activation of frozen spermatozoa enhances the detrimental effects on DNA integrity. In future, a combination of high-throughput transcriptome analysis and more classical embryological techniques applied to *Xenopus* embryos produced using cryopreserved spermatozoa would determine the possible existence of DNA damage hotspots and begin to identify molecular pathways compromised in such embryos.

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