REPRODUCTION
RESEARCH

Sperm quality assessments for endangered razorback suckers *Xyrauchen texanus*

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Abstract

Flow cytometry (FCM) and computer-assisted sperm motion analysis (CASA) methods were developed and validated for use with endangered razorback suckers *Xyrauchen texanus* collected ($n=64$) during the 2006 spawning season. Sperm motility could be activated within osmolality ranges noted during milt collections (here 167–343 mOsm/kg). We hypothesized that sperm quality of milt collected into isoosmotic (302 mOsm/kg) or hyperosmotic (500 mOsm/kg) Hanks' balanced salt solution would not differ. Pre-freeze viabilities were similar between osmolalities (79%±6 (S.E.M.) and 76%±7); however, post-thaw values were greater in hyperosmotic buffer (27%±3 and 12%±2; $P=0.0065$), as was mitochondrial membrane potential (33%±4 and 13%±2; $P=0.0048$). Visual estimates of pre-freeze motility correlated with total ($r=0.7589$; range 23–82%) and progressive motility ($r=0.7449$) by CASA and were associated with greater viability ($r=0.5985$; $P<0.0001$). Count (FCM) was negatively correlated with post-thaw viability ($r=-0.83$; $P=0.0116$) and mitochondrial function ($r=-0.91$; $P=0.0016$). By FCM-based assessments of DNA integrity, whereby increased fluorochrome binding indicated more fragmentation, higher levels were negatively correlated with count ($r=-0.77$; $P<0.0001$) and pre-freeze viabilities ($r=-0.66$; $P=0.0004$). Fragmentation was higher in isotonic buffer ($P=0.0234$). To increase reproductive capacity of natural populations, the strategy and protocols developed can serve as a template for use with other imperiled fish species, biomonitoring, and genome banking.

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Introduction

A primary focus of the propagation program for the Federally listed endangered razorback suckers *Xyrauchen texanus* (US Fish & Wildlife Service 1991) has been to slow the loss of biodiversity through improved genetic management by augmenting populations using either wild-born larvae from naturally spawning adults or hatchery fish (progeny produced by artificial cultivation with broodstock; US Fish & Wildlife Service 2002). This riverine catostomid in the order Cypriniformes was formerly abundant throughout the entire lower Colorado River drainage (Minckley *et al*. 1991), but the current range for the residual wild populations is primarily limited to Lake Mead, Lake Mohave (Mueller *et al*. 2000), and the middle Green River drainage (Modde *et al*. 1996, Cooke *et al*. 2005). Although the low levels of recruitment (replacement with younger individuals) have been directly linked to competition with, and predation by, non-native fish introduced for sportfishing (Minckley *et al*. 1991), the reduced availability of floodplain habitat (Modde *et al*. 1996) and the continued declines of flows into the Colorado River (Barnett & Pierce 2008) will likely exacerbate the predicted losses of remaining sustainable populations of razorback suckers. Selenium (Waddell & May 1995) and sublethal concentrations of contaminants (US Fish & Wildlife Service 2002) have also been implicated in population declines.

Strategies using assisted-reproductive techniques (ART) with endangered animals require methods targeted to species-specific reproductive biology (Comizzoli *et al*. 2000), and ART is often the final effort at maintaining genetic diversity in declining wildlife

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populations (Bawa *et al*. 1997). Facilitating the transfer of gametes from wild fish populations for use in hatcheries necessitates research into milt collection, storage methods, extender types, as well as into sperm cell quality. Thus, far, advances in ART with razorback sucker milt are consequences of integrating field studies with annual samplings (Carmichael *et al*. 1996) that have lead to sperm cryopreservation under hatchery conditions. In those studies, sperm motility estimates and fertilization trials comprised the evaluations of sperm cell quality (Tiersch *et al*. 1998). However, combinations of assays are necessary for selecting germplasm for immediate use and for building functional genetic resource banks for endangered species (Bainbridge & Jabbour 1998). Progress on sperm cryopreservation with other endangered fish species includes work with Formosan landlocked salmon *Oncorhynchus masou formosanus* (Gwo *et al*. 1999), pallid sturgeon *Scaphirhynchus albus* (Wayman 2003), and Tokyo bitterling *Tanakia tanago* (Ohta *et al*. 2001).

For spermatozoal competence, cell structures must be intact and functional, yet cryopreservation can alter cell composition and physiology to compromise fertilizing potential (Ohta *et al*. 2001). Steps involved in sperm processing and cryopreservation can damage organelles, membranes, and DNA (Drokin *et al*. 1998, Cabrita *et al*. 2001), and can reduce motility (Soler *et al*. 2005, Silva & Gadella 2006). Various tests have been used for predicting fertilization (Jenkins 2000), for successful freezing (Hernández *et al*. 2006), and for measuring cell damage induced by cryopreservation (cryoinjury; Terner 1986, Linhart *et al*. 2005, Soler *et al*. 2005). In aquatic species, a motility estimate made visually is the most frequent sperm quality assessment (Terner 1986), but as with mammals, it is not always correlated with fertilization (Ericsson *et al*. 1993) or with other sperm quality indicators (Soler *et al*. 2005). Computer-assisted sperm motion analysis (CASA), primarily used with domestic animals, humans, and some aquatic species (Rurangwa *et al*. 2004), offers rapid and objective quantification of sperm motility parameters. Because razorback sucker spermatozoa are motile for <1 min following activation by hypoosmotic pressure (Tiersch *et al*. 1997), CASA would improve accuracy of motility assessments with this species.

Prognostic evaluators recognized by the World Health Organization (WHO 1987) typically applied to human and domestic animal semen include sperm motility, sperm morphology, and sperm counts, which is a fundamentally important predictor of male fertility (Haubruge *et al*. 2000). The spermatocrit is often applied with aquatic species, and densities have been related to fertilization rates in Atlantic salmon *Salmo salar* (Aas *et al*. 1991). However, as with mammals, no single sperm count method is typical across aquatic species studies.

Functional analyses, including evaluations of sperm membrane integrity (viability), mitochondrial membrane potential (mitochondrial function), acrosomes, and chromatin are complementary to conventional andrology endpoints (Soler *et al*. 2005). For endangered aquatic species and their surrogates (Wayman 2003, Jenkins & Goodbred 2005, Jenkins & Draugelis-Dale 2006, Goodbred *et al*. 2007, Horváth *et al*. 2008), and for fish and shellfish (Segovia *et al*. 2000, Cabrita *et al*. 2001, Paniagua-Chavez *et al*. 2006, Tiersch *et al*. 2007, Horváth *et al*. 2008), viability and mitochondrial function assessments have proven useful. Mitochondrial respiration is responsible for ATP synthesis and sperm motility (Johnson *et al*. 1980), whereby mitochondrial membrane electrical potential has been correlated with mammalian spermatozoal normal morphology, concentration, and motility (Ericsson *et al*. 1993, Marchetti *et al*. 2002) and with motilities in Nile tilapia *Oreochromis niloticus* (Segovia *et al*. 2000) and in eastern oyster *Crassostrea virginica* (Paniagua-Chavez *et al*. 2006). Although mitochondrial membrane potential was the most sensitive test in determining human sperm quality (Marchetti *et al*. 2002) and likely provides insight with paddlefish *Polyodon spathula* (Horváth *et al*. 2008), sperm DNA integrity has been expected to yield superior diagnostic and prognostic information on fertility potential (Agarwal & Said 2003).

The integrity of paternal DNA is essential for the accurate transmission of genetic information (Ahmadi & Ng 1999, Ausió *et al*. 2009). Although defects in sperm chromatin have been negatively correlated with human fertility potential (Spanò *et al*. 2000) and golden hamster *Mesocricetus auratus* and human embryo development (Ahmadi & Ng 1999), the fertilization process may not be inhibited (Ahmadi & Ng 1999, Evenson *et al*. 2007). During milt storage and handling, degraded cellular and nuclear proteins can induce autolysis, DNA strand breaks, and decondensation, whereby chromatin becomes more vulnerable to nucleases and polymerases (Donnelly *et al*. 2001), and stainability is enhanced (Alanen *et al*. 1989). Milt contaminated with urine, as observed with razorback suckers (Tiersch *et al*. 2007) and marine fish species (Suquet *et al*. 2000), creates unfavorable osmotic conditions allowing sperm activation and consequent cell degradation. Cryopreservation-induced DNA damage has been shown in spermatozoa from sea bass *Dicentrarchus labrax* (Zilli *et al*. 2003), rainbow trout *Oncorhynchus mykiss* (Labbe *et al*. 2001), and infertile men (Donnelly *et al*. 2001).

Investigations for measuring sperm DNA damage have looked at DNA condensation, breaks and nicks, and nuclear fragmentation (Silva & Gadella 2006). Techniques have included single-cell gel electrophoresis or 'comet assay' (Singh *et al*. 1989), sperm chromatin structure assay (Evenson *et al*. 1999, Peris *et al*. 2004), chromatin dispersion (López-Fernández *et al*. 2009), and TUNEL (Sailer *et al*. 1995), yet protocols are not standardized among laboratories (Tice *et al*. 2000).

By flow cytometry (FCM) methodology, DNA strand breaks allow for enhanced binding of fluorescent dyes to DNA (Alanen *et al*. 1989), with greater dispersion of DNA indicated by increased coefficient of variation (CV; Shapiro 1995) or widening of the main histogram peak. After treatment with a variety of mutagens, nuclear peak widening has been observed (Dallas & Evans 1990), as with the 1C (haploid) peak of rat testicular cells (Bickham *et al*. 1992). By FCM, nuclear subpopulations with increased chromatin staining by fluorochromes may also be discernable by size (Zbieranowski *et al*. 1993). Advantages of FCM methods (Agarwal & Said 2003) are that sample sizes are typically at least ten times larger than those for comet assay (Potter *et al*. 2002), interpretation of results is less subjective, and data can be collected and analyzed within a few minutes (Shapiro 1995).

The necessity of non-invasive sampling procedures with the endangered razorback suckers and the typical long-distance sample shipment to laboratories for cell quality analyses and potential genome banking are dependent on improved protocols for milt handling and cell quality analyses (Tiersch *et al*. 2004). Objective assessment methods with FCM and CASA, nevertheless, require standardization and optimization per species to obtain useful results (Verstegen *et al*. 2002). This study was undertaken to develop and apply sperm quality estimators relevant for evaluating razorback sucker milt, addressing an overall hypothesis that sperm quality would be similar from milt collected into buffer extenders of either high or low osmolality. Specific objectives included the development and validation of impartial sperm quality analyses based on CASA for sperm motility and on FCM for sperm count, viability, mitochondrial function, and DNA integrity.

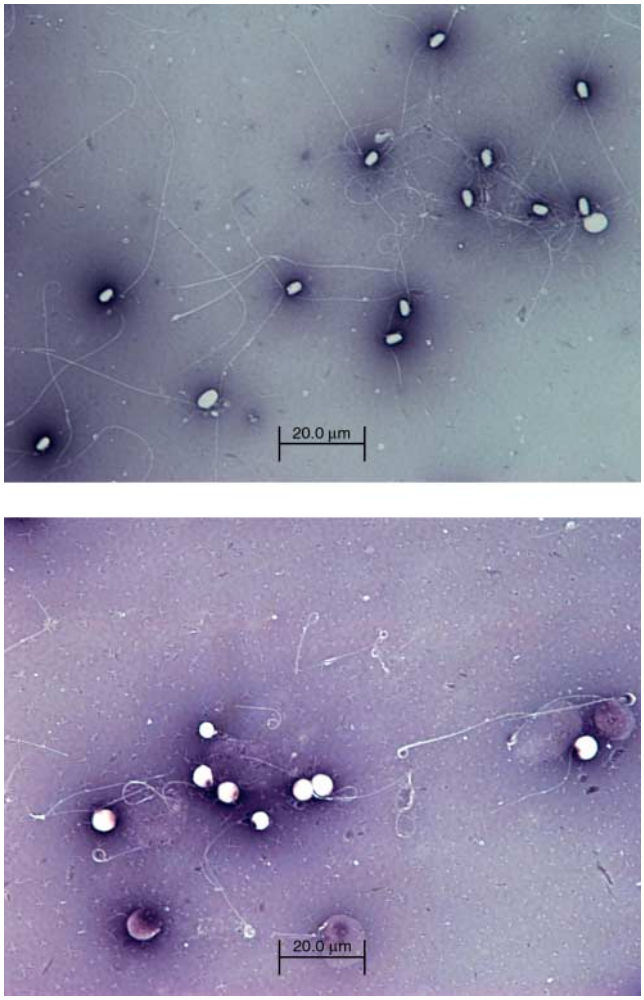

Figure 1 Eosin–nigrosin staining of razorback sucker spermatozoa imaged using light microscopy at 1000× (n=15). Milt was stored for 24 h in 302 mOsm/kg Hanks' balanced salt solution. The primary abnormality noted was macrocephalus, predominant in the lower image.

Results

Milt handling and morphology of spermatozoa

Individual males ranged from 1.3 to 2.2 kg, from 485 to 630 mm, and between 2 and 17 ml of milt were collected. Osmolalities of undiluted milt samples ranged from 167 to 343 mOsm/kg, and spermatocrits ranged from 1 to 24.5%. The primary abnormality observed was macrocephalus (Fig. 1). The percentage of abnormal cells that occurred in the low-osmolality buffer (5.4%±2.0) exceeded those in the high-osmolality buffer (1.9% ±0.5%), but the difference was not significant (P=0.0927). The percentage of the morphological abnormalities ranged from 1.2 to 15.8% and from 0.6 to 4.4% for low and high osmolalities respectively.

Motility

Visual estimates of pre-freeze sperm motility were correlated with total motility (r=0.7589) and progressive motility (r=0.7449) measured by CASA (n=13). Total motility by CASA (range 23–82%) had a slight positive linear association with viability (r=0.5234; P=0.0985; n=11) and a negative association with DNA fragmentation (r=−0.5152; P=0.0865; n=12). Higher visual motility estimates were associated with greater viability (r=0.5985; P<0.0001; n=26). Sperm motility measured by CASA exhibited more straight (r=0.636; y=0.146x+4.976) than curvilinear (r=0.259; y=0.088x+15.833) motion. In the short-term cold storage study (Fig. 2), no significant differences in motility were observed among the four buffers, but motilities in undiluted milt were significantly lower than those in milt stored in the four buffers (P<0.0001). Over time, the motilities at time 0 h were significantly higher than those measured at 12 h, and these were higher than values at both 24 and 33 h (P<0.0001). There was no significant interaction between buffers and time.

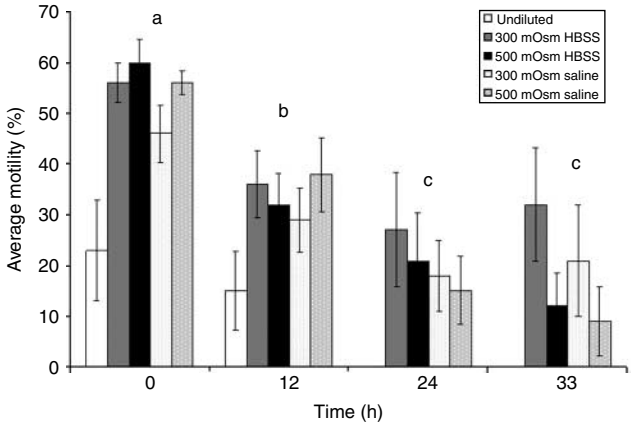

Figure 2 Estimates of motility made visually using razorback sucker milt collected into calcium-free Hanks' balanced salt solution (HBSS) or saline at either 302 or 500 mOsm/kg ($n=10$ milt samples from the same individuals per time period and treatment). No significant differences were observed in motility among the four buffers tested, but motilities at time 0 h were significantly higher than those measured at 12 h, and these were higher than values at 24 and 33 h ($P<0.0001$). The motility values of the undiluted samples were significantly lower at each time period than those stored in buffers ($P<0.0001$).

Viability and mitochondrial membrane potential

To validate assays for viability and mitochondrial membrane potential, comparisons were made between values of predetermined live and dead sperm cell ratios (predicted) and actual values measured by FCM. Regression curves were generated using duplicate samples from each of three individuals. Equations for viability were $y=24.873x-21.172$ ($R^2=0.9857$; fish #1), $y=27.332x-30.581$ ($R^2=0.9505$; fish #2), and $y=23.955x-11.786$ ($R^2=0.9304$; fish #3). The equations for membrane potential were $y=25.233x-20.802$ ($R^2=0.9708$; fish #1), $y=27.408x-42.713$ ($R^2=0.8539$; fish #2), and $y=23.513x-17.025$ ($R^2=0.9714$; fish #3). All these equations showed good fit between the predicted and actual values measured by FCM.

Ranges and averages of pre-freeze sperm viabilities were similar between the high- and low-osmolality buffer extenders used for milt collection (Table 1); however, post-thaw viability in the high-osmolality buffer was significantly greater ($P=0.0065$). Post-thaw mitochondrial membrane potential from cells collected in the high-osmolality buffer was also significantly higher ($P=0.0048$). The difference between pre-freeze and post-thaw viability values were greater for the low-osmolality buffer than for the high-osmolality buffer ($P=0.0089$).

Sperm counts

Sperm counts measured by hemocytometer and flow cytometer were correlated ($y=1.2188x+1\times10^8$; $R^2=0.8464$). The sperm count measured by FCM ranged from 1.7×10^6 to 7.8×10^9 cells/ml. Spermatocrits were correlated with sperm counts measured by FCM ($y=16.632-2\times10^{-9}x$; $R^2=0.3639$) and by hemocytometer ($y=7.1104+5\times10^{-9}x$; $R^2=0.4802$). Sperm cell count (by FCM) was negatively correlated with post-thaw viability ($r=-0.83$; $P=0.0116$) and mitochondrial function ($r=-0.91$; $P=0.0016$).

DNA integrity

Demonstrable DNA fragmentation was shown with razorback sucker spermatozoa exposed to hydrogen peroxide with nuclei outside the main population (NOMP) increasing from 1.55 to 12.24% using 0–100% hydrogen peroxide. The regression was significant ($P=0.0004$) with $r=0.8572$ using a no-intercept model. The exposed control (common carp *Cyprinus carpio*) spermatozoa stained for DNA integrity immediately and following fixation showed increased CV with hydrogen peroxide concentration, with both regression models being no-intercept. For nuclei that were immediately stained, $r=0.8446$ ($P=0.0344$), and for fixed nuclei, $r=0.9573$ ($P=0.0105$). For NOMP, the regression models using fixed and live cells were no-intercept, with $r=0.9154$ ($P=0.0291$) and $r=0.8609$ ($P=0.0277$) respectively. The CV of the shipped razorback sucker samples were positively correlated with NOMP ($r=0.8121$; $P<0.0001$) by a linear with no-intercept

Table 1 Percentage viability and mitochondrial function (mean ± S.E.M.) of razorback sucker spermatozoa prior to and following cryopreservation of milt collected into buffers* at 302 and 500 mOsm/kg.

	Pre-freeze		Post-thaw	
	302 mOsm/kg	500 mOsm/kg	302 mOsm/kg	500 mOsm/kg
Viability (%)	79.0 (6.3)[a]	76.1 (7.1)[a]	12.1 (1.9)[a]	27.0 (2.6)[b]
Range (%)	20.3–99.2	21.4–98.6	3.7–16.2	18.0–37.7
Sample size	14	11	6	6
Mitochondrial function (%)	94.3 (4.5)[a]	33.9 (0.0)[a,†]	13.0 (2.0)[a]	32.7 (4.0)[b]
Range	80.9–98.9	32.1–37.7	4.3–17.2	23.5–46.3
Sample size	4	2	6	6

*Hanks' balanced salt solution, calcium free. [a,b]Different letters along rows within either the pre-freeze or post-thaw categories indicate significant statistical differences. [†]Because of the small sample size resulting from technical difficulties, statistics regarding this pre-freeze value are not discussed in the text.

model. No significant difference was found between CV and NOMP analysis methods for assessing DNA fragmentation.

In razorback sucker samples, no differences among individual fish ($n=30$) were observed in the percentage of cells with fragmented DNA, but there were significantly higher percentages from the low-osmolality buffer ($P=0.0234$; $n=30$). The percentage of cells with fragmented DNA was negatively correlated with sperm cell count (by FCM; $r=-0.77$; $P<0.0001$) and with pre-freeze viabilities ($r=-0.66$; $P=0.0004$). When samples with over 10% fragmented nuclei were removed from statistical analysis, a strong negative correlation was also observed with spermatocrit ($r=-0.96$; $P=0.0006$).

Discussion

Non-game fish, such as the catostomids, are not considered of direct economic or recreational benefit to humans and generally are not the subject of conservation efforts until they are imperiled (Cooke *et al.* 2005). One focal point for recovery of the endangered razorback suckers is to identify and maintain genetic diversity (US Fish & Wildlife Service 2002). Owing to their endangered status, non-destructive and non-invasive techniques are crucial. Therefore, this study was designed to verify preferable conditions for milt handling in the field that would support meaningful gamete quality measurements in the laboratory and to validate a panel of sperm cell biomarkers for use with hatchery broodfish or wild-caught fish for immediate milt use in ART or in environmental assessments, as well as for evaluations following long-term storage by cryopreservation.

Limited sample sizes are inherent in studies with endangered animals. The technically sound methods and practical approach developed in this study with razorback suckers (Fig. 3) could be applied for use with other species offering similar challenges. Generally, ART for endangered mammalian species have been adapted from technologies developed in domestic species, and as with this study, methods need to be tailored to accommodate work under field conditions (Comizzoli *et al.* 2000). The results of this study support the collection of razorback sucker milt directly into a hyperosmotic extender to counteract potential damage caused by unavoidable and variable urine contamination.

Osmolality is lowered when urine is inadvertently collected along with palpated milt. With the threshold activation value at 264 mOsm/kg (Tiersch *et al.* 1997), in this study, 78% ($n=27$) of undiluted milt samples were below isotonicity and subject to premature activation. The apparent macrocephalus abnormality (Fig. 1) indicated cellular swelling and hypoosmotic stress, conditions that lessen chromatin integrity. In fact, a significantly higher number of cells with fragmented DNA was found in the low-osmolality buffer, which is

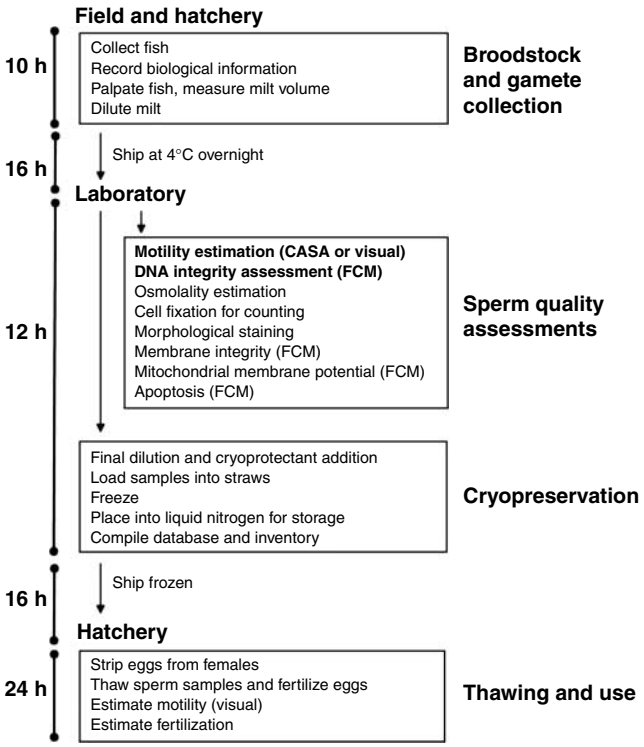

Figure 3 Overview and chronology of activities in the field, hatcheries, and laboratories for assessing sperm quality and cryopreservation of razorback sucker *Xyrauchen texanus*. The DNA integrity and motility estimations (bold) could be first in a tiered approach to assessing sperm quality.

less capable at counteracting the osmotic effects of urine than the high-osmolality buffer. In the short-term storage study, however, the visual estimates of razorback sucker sperm motilities were not dissimilar between the low- and high-osmolality buffers, but were different over time (Fig. 2). A follow-up study using objective CASA methods may provide useful data.

In a study of semen traits in natural wildlife populations of red deer *Cervus elaphus hispanicus*, sperm viability was proposed to not be directly associated with male fertility because viability is indispensable for fertilization, and males with low values have been intensively selected (Malo *et al.* 2005). However, membrane integrity assessments are integral for optimizing sperm handling and cryopreservation processes. Similar to an avian study (Blanco *et al.* 2000), attempts were made here to increase osmotic pressure by milt collection into higher osmolality (500 mOsm/kg) buffer, and this proved particularly useful for maintaining post-thaw viability. No differences were observed in pre-freeze viability between the low- and high-osmolality buffers, yet significantly higher post-thaw values for viability and mitochondrial function were observed in the high-osmolality buffer (Table 1).

Sublethal cryopreservation damage to membranes is especially important for freshwater fish spermatozoa, whereby the ability to fertilize requires dilution

in a hypoosmotic environment (Cabrita *et al*. 2001). Integrity of sperm plasma membranes is required for fertilization (Graham *et al*. 1990), yet cryopreservation reduced the percentage of membrane-intact spermatozoa (Cabrita *et al*. 2001) and impacted membrane structures in rainbow trout (Drokin *et al*. 1998). The lack of accord observed between fertilization rates and standard semen parameters or functional assessments (Linhart *et al*. 2005) may be partially explained by sublethal damage. In a study with sturgeon spermatozoa, various combinations of extenders and cryoprotectants yielding different osmolalities resulted in a variety of fertilization rates, even when post-thaw sperm motility and viability were similar to pre-freeze values (Horváth *et al*. 2008). The diverse biological properties of spermatozoa and dissimilarities among species underscore the use of a combination of sperm quality assessments, particularly in the less well-studied animals.

For CASA evaluations to be replicated, standardizations of instrument settings and protocols are needed for the species under study. Brief motilities of fish spermatozoa may be due to the relatively low mitochondrial number (Christen *et al*. 1987). In this study, spermatozoal movement occurred for <30 s and was more linear than curvilinear. In mammals, linear movement has been attributed to mature spermatozoa (Syntin & Robaire 2001) and to low intracellular calcium concentrations in European perch *Perca fluviatilis* (Alavi *et al*. 2007). Studies with non-threatened catostomid species may help in gaining insight into factors important to razorback sucker sperm motility (Syntin & Robaire 2001), including aging, which is relevant in this long-lived species (e.g. 40 years; Minckley 1983).

Accuracy in estimating sperm number is important in ART (Evenson *et al*. 1993, Hansen *et al*. 2002), in the standardization of cryopreservation methods (Dong *et al*. 2007), for studies of environmental chemical impacts to reproductive health (Haubruge *et al*. 2000, Eustache *et al*. 2001), as well as in predicting fertility (Nadir *et al*. 1993) and post-thaw survival (Centola *et al*. 1992). No single counting method is uniformly preferred in andrology or research laboratories. Counting by FCM can lessen variability and decrease errors by discriminating debris and particulates from nuclei (Evenson *et al*. 1993, Eustache *et al*. 2001, Hansen *et al*. 2002), and it has been used with fish species (Jenkins & Draugelis-Dale 2006). As in this study, positive linear relationships have been shown between spermatocrits and sperm density estimates (Tvedt *et al*. 2001). In this study, counts by FCM provided a complementary assay to DNA fragmentation and viability, being negatively correlated with increased levels of fragmentation and with post-thaw viability.

Investigations of DNA integrity are used in cancer biology, toxicology, environmental health studies (Potter *et al*. 2002), and in ART including IVF and ICSI (Donnelly *et al*. 2001). Proposed causes of DNA damage in human spermatozoa – defective sperm chromatin packaging, apoptosis, and oxidative stresses (Agarwal & Said 2003) – can be extended to other species. Endogenous breaks may indicate anomalies during spermiogenesis and incomplete maturation (Manicardi *et al*. 1995). Rates of genomic DNA alterations or fragmentations ranged from 0.1% in ejaculates of fertile donors to 10% in infertile men (Ahmadi & Ng 1999); another study showed that when >30% of sperm DNA was damaged, natural pregnancy in mice was not possible (Evenson *et al*. 1999). In male mice *Mus musculus*, when the degree of DNA damage was <8%, oocyte-mediated mechanisms could repair it, yet, regardless of the degree of DNA damage, spermatozoa were capable of fertilization (Ahmadi & Ng 1999). In ponds with higher concentrations of radionucleotides and genotoxicants, fecundity of western mosquitofish *Gambusia affinis* was negatively correlated with levels of double-stranded DNA breaks in spermatozoa as well as with the number of malformed embryos (Theodorakis *et al*. 1997).

Cryopreservation and osmotic stress can exacerbate DNA defects related to partially decondensed chromatin or DNA strand breakage (Peris *et al*. 2004, Silva & Gadella 2006). Disparities in the levels of DNA damage between fertile and infertile men were shown following semen cryopreservation, where significantly more damage was observed in spermatozoa from the subfertile group (Hammadeh *et al*. 1999, Donnelly *et al*. 2001). DNA fragmentation assessments provided good correlation with bull fertility (Garcia-Macias *et al*. 2007). The structural integrity of DNA is particularly important for fish that employ external fertilization because of the osmotic challenge to spermatozoa.

Sperm chromatin of razorback suckers retains somatic-like histones for compaction, but not protamines or protamine-like sperm nuclear basic protein (SNBP) types (Frehlick 2009). The SNBPs decrease head volume, providing better protection against externally damaging agents. Identifying the SNBP types has implications for cryopreservation techniques, especially important for endangered species (Ausió *et al*. 2009). In this study, 20% of the razorback sucker milt samples before cryopreservation (pre-freeze) had nuclear DNA damage exceeding 10% of the population of nuclei. Because alterations in the primary structure of DNA in germ cells can result in heritable damage, such a FCM assay for DNA integrity with the endangered razorback suckers could be considered to be a quality control screening assay, as in a tiered testing approach to be performed before further assessments of sperm quality (Fig. 3). The NOMP method provides an additional means by which spermatozoal haplotypic DNA damage can be used in broodstock selection or for cryopreservation, and in examining between-male variation, as with a sperm chromatin dispersion assay used with tench *Tinca tinca* (López-Fernández *et al*. 2009). In fact, sperm chromatin has been considered an independent measure of sperm quality apart from standard sperm parameters (Agarwal & Said 2003).

Data on DNA integrity, supplementary to the typical sperm prognostic evaluators, can be informative in predictions of the response of spermatozoa to freezing (Hernández *et al*. 2006). Dietary ascorbic acid has been shown to maintain genetic integrity of spermatozoa by preventing oxidative damage to DNA in rainbow trout (Dabrowski & Ciereszko 1996) and humans (Fraga *et al*. 1991), and its addition may be practical in extenders for razorback sucker milt. Generation of reactive oxygen species in human spermatozoa was shown to be related to DNA damage (Irvine *et al*. 2000). When performed following cryopreservation, the measure of chromatin condensation has been recommended as a sensitive parameter for sperm quality assessments (Hammadeh *et al*. 1999). In this study, discrepancies in the percentage of viability and mitochondrial membrane potential between the high- and low-osmolality buffers became apparent after thawing (Table 1). Similarly, differences were observed between pre- and post- thaw values of sperm motility and fertility with zebrafish *Danio rerio* (Yang *et al*. 2007).

Strategies for field collection and laboratory sperm quality assessments were developed for use with endangered razorback suckers, and the approach could be modified and adapted for use with other fish species. Collection of razorback sucker milt into a hypertonic buffer will enhance success in field collections of milt for meaningful analyses. The gamete quality assays developed for razorback suckers could be applied in selecting males for use in artificial spawning, for gamete cryopreservation, and for assessment of reproductive capacity in natural populations.

Materials and Methods

Fish collection

During the Lake Mohave annual spawning of razorback suckers in March, 2006, samples were collected by palpation from wild-caught individuals ($n=64$ samples) and processed at Willow Beach National Fish Hatchery (WB), Willow Beach, Arizona, located 17 river km downstream from Hoover Dam. Fish were tagged with passive integrated transponders for identifying individuals (Biomark, Inc., Boise, ID, USA), weighed, and total lengths were measured. Milt samples were express-mailed at 5 °C for arrival at the Aquaculture Research Station (ARS), Louisiana State University Agricultural Center, Baton Rouge, Louisiana, USA. Sperm quality analyses were performed immediately upon receipt, during short-term cold storage for 33 h, or following cryopreservation or fixation as described below.

Collection and handling of spermatozoa

Milt was allowed to flow freely for 1–2 s (Tiersch *et al*. 1997) before collection into sterile 50-ml screw cap tubes. Because the activation of sperm motility in razorback suckers occurs at osmolalities below 270 mOsm/kg, and the range of milt osmolalities at collection is typically wide due to contamination by urine (Tiersch *et al*. 1998), this study was designed to address such variability. Samples were collected into a 1:1 dilution with calcium-free Hanks' balanced salt solution (HBSS) at either 302 (just above isoosmotic conditions) or 500 mOsm/kg (hyperosmotic to account for potential dilution) at pH 7.4. Milt collected into different buffer treatments, as well as undiluted, was used in a short-term storage study of sperm motility at WB (see below). After centrifugation at 350 ***g*** for 10 min, spermatocrits ($n=27$) were obtained. Upon receipt at ARS, osmolalities were measured with a vapor pressure osmometer (Wescor Corp., Logan, UT, USA). Cells were frozen at 2×10^6/ml in a computer-controlled freezer with 0.5-ml French straws (IMV International, Maple Grove, MN, USA) and 10% dimethyl sulfoxide as a cryoprotectant, with 15 min equilibration, −8 °C/min cooling rate, and thawing at 40 °C for 10 s. For cell counts and DNA integrity analyses by FCM, subsamples were fixed 1:1 by volume in 10% buffered formalin, stored at 8 °C, and analyzed 3 months later.

Morphology of spermatozoa

Sperm morphologies were assessed by diluting one part of the shipped sample to three parts HBSS, and one part of this suspension was mixed with seven parts of eosin–nigrosin (Lane Manufacturing, Inc., Denver, CO, USA) and smeared onto a slide by using the edge of a precleaned microscope slide. Preparations were air-dried and examined by using a light microscope (Leitz Diaplan, Wetzlar, Germany) at 1000× total magnification under oil immersion. Between 200 and 500 spermatozoa were evaluated per slide ($n=15$) in accordance with the World Health Organization protocols (WHO 1987), whereby the two most commonly observed abnormalities (macrocephalic and cytoplasmic droplets) were scored in relation to apparent normal sperm morphologies.

Motility of spermatozoa

Spermatozoal motility was subjectively assessed visually, where one part of the shipped sample was diluted into two parts HBSS, and 0.25 µl was diluted into 25 µl deionized water (18 mOsm/kg) on a microscope slide to activate flagellar motion. The percentage of progressively motile cells was estimated using dark-field microscopy at 100× magnification. In a short-term cold storage study up to 33 h, motility was recorded using undiluted milt and milt diluted 1:1 in saline or HBSS at low (302 mOsm/kg) and high osmolality (500 mOsm/kg) held at 8 °C. Motility values at each of the four time periods and for each of the five treatments were generated from the same ten individuals.

Motility was assessed with CASA by using a total of 25 µl activated milt (as above; $n=13$ males) in a chambered slide (Leja 20 SC20-010040-B; Leja Products, Nieuw-Vennep, The Netherlands) and viewed with phase microscopy (Olympus BX41; Olympus America, Inc., Center Valley, PA, USA) at 200× magnification. Owing to the short-lived motility (∼30 s), one visual field per sample was electronically captured and analyzed (SpermVision, Version 3.0; Minitube of America, Verona, WI, USA). Software settings included area of cell identification 8×30 µm^2; immotile at average

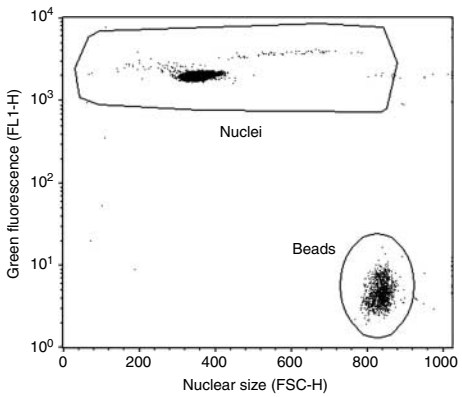

Figure 4 Flow cytometric dot plot illustrating the unknown concentration of razorback sucker sperm nuclei compared with a known number of fluorescent microbeads in one sample ($n=32$ fish). Particle size is on the *x*-axis (forward scatter or FSC-H) and green fluorescence of stained nucleic acids and microspheres is on the *y*-axis (fluorescence detector FL1-H).

orientation of head (μm; AOC)+distance straight line (μm; DSL) <25 and DSL <1; local motile at DSL <4; hyperactive velocity curved line (μm/s; VCL) >80 and linearity (LIN; as VSL/velocity average path) <0.65 and amplitude of lateral head displacement (μm; ALH) >6.5; linear straightness (STR; as VSL/VCL) >0.7 and LIN >0.7; non-linear STR <0.9 and LIN <0.5; and curvilinear at DAP/radius >3 and LIN <0.5. Total and progressive motilities, DSL, and distance curved line (DCL) parameters were statistically analyzed.

Sperm counts

Spermatozoa were manually counted ($n=9$) with a Neubauer hemocytometer using the formula: count=sperm number in five small squares/80×(4.0×10^6)×(dilution factor). Spermatozoa were enumerated ($n=32$) in duplicate with a commercial reagent (Bacteria Counting Kit, Molecular Probes, Eugene, OR, USA), whereby a fluorescent nucleic acid stain (SYTO BC) and a known concentration of fluorescent polystyrene microspheres were added to 10-μl sample aliquots with 990 μl HBSS prior to analysis with a FACSCalibur (Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA, USA; Fig. 4).

Sperm membrane integrity and mitochondrial membrane potential

To validate flow cytometric assays for sperm membrane integrity and mitochondrial membrane potential, the shipped milt was used from each of three fish exhibiting 100% sperm motility by visual observations. Subsamples of 1×10^6 cells/ml in 3 ml HBSS heated at 70 °C for 10 min and exhibiting no motility were considered non-viable and non-functional. Viable cells at the same concentration were combined with heat-treated cells in ratios of 100:0, 75:25, 50:50, 25:75, and 0:100. These live–dead cell combinations were used to generate regression lines between the expected viable (or functional) spermatozoa and the actual, measured values for membrane integrity and mitochondrial membrane potential.

Fluorescent stains used for membrane integrity were SYBR-14 and propidium iodide (PI; Live/Dead Sperm Viability kit, Molecular Probes), and rhodamine 123 (R123; Molecular Probes) and PI for mitochondrial membrane potential (Segovia *et al.* 2000, Silva & Gadella 2006). After assay validation, the shipped samples were analyzed before (pre-freeze) and after (post-thaw) cryopreservation by FCM (see Table 1) as before, with an excitation wavelength of 488 nm following instrument calibration using FACSComp software (BDIS). Triplicate samples were analyzed, and 10 000 cells per replicate were acquired and analyzed with CellQuest software (BDIS).

DNA integrity

To validate DNA integrity analyses, the shipped milt was used from razorback suckers and from common carp *Cyprinus carpio* induced by pituitary extract injection (Rottmann *et al.* 1991). Razorback sucker spermatozoa were diluted to 4×10^6 cells/ml and subjected for 4 h to freshly opened hydrogen peroxide

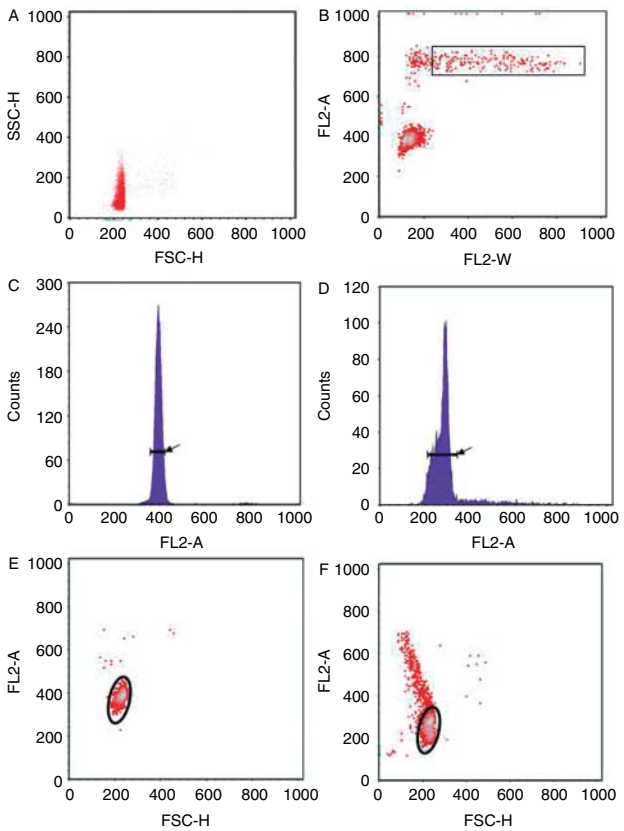

Figure 5 Flow cytometric plots showing nuclei of razorback sucker spermatozoa stained with propidium iodide. (A) The main population of nuclei is shown with size (FSC-H) on the *x*-axis and scatter (SSC-H) on the *y*-axis. (B) Nuclear aggregates are removed (rectangular gate) from further analysis by using doublet discrimination mode with width (FL2-W) and area (FL2-A). (C) A histogram of razorback sucker #3 showing the primary spermatozoal haploid peak with coefficient of variation (CV)=3.15 (arrow), and (D), the CV (arrow) from razorback sucker #15 is 10.01, showing a wider dispersion of nuclear fluorescence. (E) The percentage of nuclei from razorback sucker #3 outside the main population (outside the gated circle; NOMP) is equal to 0.3%, and (F) with razorback sucker #15, NOMP is 10.5%, showing fragmented DNA primarily having higher fluorescence (FL2-A) and smaller size (FSC-H) than the main population of nuclei (circle gate).

(Sigma–Aldrich; 150 mM in HBSS) dilutions (0–100% at 10% intervals) to damage DNA (Donnelly 1999, Termini 2000, Potter *et al*. 2002). Carp milt was similarly diluted and exposed to 0, 75, 85, 95, and 100% hydrogen peroxide and analyzed immediately and after subsample fixation with 2% paraformaldehyde.

The shipped fixed razorback sucker milt ($n=32$) was diluted to 2×10^6 cells/ml and stained with PI (25 µg/ml), DNase-free RNase A at 1 µg/ml, and 0.1% (v/v) Triton X-100 in sodium citrate (Sigma–Aldrich) for 30 min at 24 °C in the darkness (Crissman & Steinkamp 1973). Stained nuclei were filtered through 30-µm nylon mesh (Small Parts, Miami Lakes, FL, USA) prior to analysis in duplicate by FCM. Nuclei were analyzed at 1×10^6/ml at a rate of fewer than 300 per s, and 10 000 events per sample in duplicate were collected by using a 1024-channel FL2 parameter measured at 585 nm. Cytograms were generated using CellQuest (BDIS) upon linear analysis of nuclei in forward scatter (FSC-H) (size) versus side scatter (SSC; Fig. 5A). Aggregates were gated out from analysis using doublet discrimination mode with FL2 width (FL2W) versus FL2 area (FL2A; Fig. 5B). CV were measured from the main histogram (haploid) peak (Fig. 5C and D) and nuclei with fluorescence outside the main population (NOMP) were denoted in dot plots generated by FSC versus FL2A (Fig. 5E and F), where larger values for each CV and NOMP were indicative of higher levels of DNA fragmentation.

Statistical analysis

Assay results were not generated for each shipped sample of razorback sucker milt because of limited fish numbers, constraints in field collections, shipping, and instrument performance. Differences were tested among sperm quality parameters from milt collected in 302 or 500 mOsm/kg HBSS. Arcsine (sqrt) transformations were applied to proportions (percentage) data, and cell counts were log transformed. Unequal variances in morphology and DNA fragmentation data necessitated the use of two parametric *t*-tests, whereas one-way ANOVA was performed with other data. A two-way ANOVA was performed for the analysis of motility during cold storage. Correlations between sperm quality results were examined. Models for the best fit regression of hydrogen peroxide-exposed spermatozoa with fresh carp and razorback sucker sperm and fixed carp sperm were generated for NOMP and CV. Differences in DNA fragmentation among fish were tested by ANOVA. The level of significance was $\alpha=0.05$, and all statistical analyses were performed using SAS (SAS Institute 1999).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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