RESEARCH ARTICLE

Chelonian Perivitelline Membrane-Bound Sperm Detection: A New Breeding Management Tool

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Perivitelline membrane (PVM)-bound sperm detection has recently been incorporated into avian breeding programs to assess egg fertility, confirm successful copulation, and to evaluate male reproductive status and pair compatibility. Due to the similarities between avian and chelonian egg structure and development, and because fertility determination in chelonian eggs lacking embryonic growth is equally challenging, PVM-bound sperm detection may also be a promising tool for the reproductive management of turtles and tortoises. This study is the first to successfully demonstrate the use of PVM-bound sperm detection in chelonian eggs. Recovered membranes were stained with Hoechst 33342 and examined for sperm presence using fluorescence microscopy. Sperm were positively identified for up to 206 days post-oviposition, following storage, diapause, and/or incubation, in 52 opportunistically collected eggs representing 12 species. However, advanced microbial infection frequently hindered the ability to detect membrane-bound sperm. Fertile Centrochelys sulcata, Manouria emys, and Stigmochelys pardalis eggs were used to evaluate the impact of incubation and storage on the ability to detect sperm. Storage at −20°C or in formalin were found to be the best methods for egg preservation prior to sperm detection. Additionally, sperm-derived mtDNA was isolated and PCR amplified from Astrochelys radiata, C. sulcata, and S. pardalis eggs. PVM-bound sperm detection has the potential to substantially improve studies of artificial incubation and sperm storage, and could be used to evaluate the success of artificial insemination in chelonian species. Mitochondrial DNA from PVM-bound sperm has applications for parentage analysis, the study of sperm competition, and potentially species identification. Zoo Biol. 35:95–103, 2016. © 2016 Wiley Periodicals, Inc.

Keywords: turtle; tortoise; conservation; fertility assessment; PVM; sperm; chelonian

INTRODUCTION

Turtles and tortoises are important targets for conservation programs, with many chelonian species facing the threat of extinction due to habitat destruction and excessive harvesting [Turtle Conservation Fund, 2002]. Conservation initiatives frequently include captive breeding programs for reintroductions and to serve as genetic assurance colonies. Therefore, any technique that can enhance a program’s success are valuable, as demonstrated by the Batagur baska conservation program, where a few successful clutches had a significant impact on the sustainability of the species [Weissenbacher et al., 2015].

Due to limited success with turtle and tortoise semen collection and cryopreservation [Browne et al., 2011], chelonian assisted reproductive techniques primarily involve pair management for maintenance of genetic diversity, artificial incubation, temperature manipulation for sex selection [Girondot et al., 1998], and protection of eggs from nest predation [Noel et al., 2012]. Eggs that fail to exhibit embryonic development during incubation can be a problem for pair/herd management because developmental failure can be caused by a variety of factors requiring

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different solutions. These include egg infertility due to copulatory organ deformity, lack of copulation, low-quality sperm in ejaculate, genetic incompatibility, inappropriate incubation parameters, or the persistence of embryonic diapause [Peters et al., 1994]. Although eggshell chalk formation and visualization of vasculature by candling indicate early embryonic development, incubated eggs lacking outward signs of development require necropsy to assess fertility. However, distinguishing between a blastoderm (fertile) and a germinal vesicle (infertile) during necropsy is difficult. Likewise, locating an early embryo is often challenging in eggs exhibiting extensive degradation or microbial infection following incubation (K. Croyle, personal observation). Yet, the ability to discern between fertile and infertile eggs is critical in troubleshooting clutch failures, especially in species with embryonic diapause, as eggs can remain arrested at the blastoderm stage following oviposition for extended periods of time [Miller and Dinkelacker, 2008].

There are currently no reliable methods to differentiate between infertility and early embryonic death (EED) in chelonian eggs. Previous studies of chelonian egg fertility and hatching rates are limited to categorizing eggs as undeveloped, without distinguishing between EED and true infertility [Miller, 1999]. In avian species, detection of sperm bound to the oocyte’s perivitelline membranes (PVM) has been used to study sperm-egg interaction [Birkhead et al., 1994; Bramwell and Howarth, 1992; Rabbani et al., 2006; Wishart, 1997], sperm competition [Birkhead et al., 1995], sperm storage [Birkhead et al., 1993; Wishart, 1987], estimate sperm production, and to infer egg fertility [Birkhead et al., 2008]. More recently, PVM-bound sperm detection was shown to be a valuable management tool for endangered avian breeding programs [Croyle et al., 2015; Hennings et al., 2012]. PVM-bound sperm detection can be used, in combination with behavioral observations and reproductive history, to assist in more timely breeding management decisions [Croyle et al., 2015].

Due to the similarities between avian and chelonian egg structure and pre-ovipositional embryonic development [Deeming and Ferguson, 1991], this study investigated the potential for PVM-bound sperm detection in turtle and tortoise eggs. The feasibility of using PVM-bound sperm staining for chelonian conservation was examined using opportunistically collected eggs. As avian PVM-bound sperm have been shown to degrade during incubation [Croyle et al., 2015; Small et al., 2000], we examined potential limitations of PVM-bound sperm detection in controlled conditions to evaluate the impact of incubation length, egg storage, and presence of microbial infection. PVM-bound sperm detection is a promising technique for chelonian conservation programs, future studies of chelonian reproduction, and is currently being used to manage chelonian breeding pairs and herds at the San Diego Zoo, Turtle Conservancy, and the Suzhou Zoo in China. To our knowledge, this study is the first to demonstrate the use of PVM-bound sperm detection in any reptile species.

MATERIALS AND METHODS

This study was reviewed and approved by the San Diego Zoo Global IACUC (assurance# A3675-01) in compliance with the guidelines for the use of animals in research [OLAW/ARENA, 2002]. The taxonomic classification, naming, and conservation status of turtles and tortoises is based on the IUCN Red List of Threatened Species [IUCN, 2014]. Unless otherwise noted, all chemicals were purchased from Fisher Scientific (Pittsburg, PA).

Eggs Collection

A total of 191 chelonian eggs representing 17 species were used for this study. Chelonian eggs were opportunistically collected from breeding facilities at San Diego Zoo Global (Galapagos giant tortoise [Chelonoidis nigra], Chinese three-striped box turtle [Cuora trifasciata], European pond turtle [Emys orbicularis], Burmese starred tortoise [Geochelone platynota], black-breasted hill turtle [Geoemyda spengleri], Burmese mountain tortoise [Manouria emys], spider tortoise [Pyxis arachnoides], leopard tortoise [Stigmochelys pardalis]), the Turtle Conservancy (radiated tortoise [Astrochelys radiata], ploughshare tortoise [Astrochelys yniphora], African spurred tortoise [Centrochelys sulcata], Chinese three-striped box turtle [C. trifasciata], Burmese starred tortoise [G. platynota], black spotted turtle [Geoclemys hamiltonii], sunburst turtle [Heosemys spinosa], serrated hinge-backed tortoise [Kinixys erosa], Burmese mountain tortoise [M. emys], spider tortoise [P. arachnoides], painted wood turtle [Rhinoclemmys pulcherrima]), the Santa Barbara Zoo (radiated tortoise [A. radiata]), Suzhou Zoo (Yangtze giant softshell turtle [Rafetus swinhoei]), and Dr. Brian Horne’s private collection (yellow-margin tortoise [Cuora flavomarginata], leopard tortoise [S. pardalis]). Eggs were either freshly laid (retrieved <24hr post-oviposition), removed following embryonic diapause cooling period at 17–20°C, removed from incubation following developmental failure, retrieved from the oviduct during necropsy (K. erosa), or stored at −20 or 4°C prior to examination or experimental treatment. Egg failure was determined by lack of visual embryonic growth upon candling and/or absence of eggshell chalking.

Egg Storage and Incubation

Incubation data for failed eggs were provided by each facility. Diapause and incubation lengths, as well as temperatures, varied greatly according to species and breeding program (Table 1). Incubation temperatures ranged from 25.5 to 33.3°C. Eggs were stored or incubated for up to 206 days post-oviposition prior to examination.

Freshly laid C. sulcata, S. pardalis, and M. emys eggs were kept at temperatures of −20, 4, 20-22, or 31°C for 30,
TABLE 1. One hundred and ninety-one eggs from 17 chelonian species were examined for this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Common name</th>
<th># of clutches</th>
<th># eggs examined</th>
<th>Membranes recovered</th>
<th>Positive eggs</th>
<th>Infected positive eggs</th>
<th>Max. # days sperm detected</th>
<th>Max. # sperm/fov</th>
<th>Conservation status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astrochelys radiata</td>
<td>Radiated tortoise</td>
<td>21</td>
<td>43</td>
<td>24</td>
<td>6</td>
<td>1</td>
<td>147 (D and I)</td>
<td>160</td>
<td>CR</td>
</tr>
<tr>
<td>Astrochelys yniphora</td>
<td>Ploughshare tortoise</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>CR</td>
</tr>
<tr>
<td>Centrochelys sulcata</td>
<td>African spurred tortoise</td>
<td>3</td>
<td>37</td>
<td>28</td>
<td>22</td>
<td>8</td>
<td>90 (I)</td>
<td>68</td>
<td>VU</td>
</tr>
<tr>
<td>Chelonoidis nigra</td>
<td>Galapagos giant tortoise</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>VU</td>
</tr>
<tr>
<td>Cuora flavomarginata</td>
<td>Yellow-margined box turtle</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0 (Fresh)</td>
<td>488</td>
<td>EN</td>
</tr>
<tr>
<td>Cuora trifasciata</td>
<td>Chinese three-striped box turtle</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>8 (I)</td>
<td>5</td>
<td>CR</td>
</tr>
<tr>
<td>Emys orbicularis</td>
<td>European pond turtle</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>NT</td>
</tr>
<tr>
<td>Geocheleone platynota</td>
<td>Burmese starred tortoise</td>
<td>7</td>
<td>15</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>120 (D and I)</td>
<td>3</td>
<td>CR</td>
</tr>
<tr>
<td>Geoclemys hamiltonii</td>
<td>Black spotted turtle</td>
<td>2</td>
<td>24</td>
<td>17</td>
<td>2</td>
<td>1</td>
<td>39 (I)</td>
<td>5</td>
<td>VU</td>
</tr>
<tr>
<td>Geoemyda spengleri</td>
<td>Black-breasted hill turtle</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>59 (I)</td>
<td>TMTC</td>
<td>EN</td>
</tr>
<tr>
<td>Heosemys spinosa</td>
<td>Sunburst turtle</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>EN</td>
</tr>
<tr>
<td>Kinixys erosa</td>
<td>Serrated hinge-backed turtle</td>
<td>1</td>
<td>7</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>81 (I)</td>
<td>2</td>
<td>DD</td>
</tr>
<tr>
<td>Manouria emys</td>
<td>Burmese mountain tortoise</td>
<td>4</td>
<td>15</td>
<td>11</td>
<td>5</td>
<td>3</td>
<td>70 (R)</td>
<td>19</td>
<td>EN</td>
</tr>
<tr>
<td>Pyxis arachnoides</td>
<td>Spider tortoise</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>206 (D and I)</td>
<td>12</td>
<td>CR</td>
</tr>
<tr>
<td>Rafetus swinhoei</td>
<td>Yangtze giant softshell</td>
<td>2</td>
<td>15</td>
<td>15</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>CR</td>
</tr>
<tr>
<td>Rhinoclemmys pulcherina</td>
<td>Painted wood turtle</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>69 (I)</td>
<td>40</td>
<td>NA</td>
</tr>
<tr>
<td>Stigmochelys pardalis</td>
<td>Leopard tortoise</td>
<td>3</td>
<td>12</td>
<td>9</td>
<td>6</td>
<td>0</td>
<td>60 (F)</td>
<td>492</td>
<td>NA</td>
</tr>
</tbody>
</table>

Total eggs: 191

D, diapause; I, incubated; R, refrigerated; F, frozen; CR, critically endangered; EN, endangered; VU, vulnerable; NT, near threatened; DD, data deficient; NA, not assessed; TMTC, too many to count. PVM-bound sperm were positively identified in 51 eggs, representing 11 species. Not all PVMs could be recovered due to degradation.
60, 70, 84, or 90 days to evaluate the effect of incubation and storage on PVM-bound sperm detection in a controlled environment (Table 2). At least one egg from each freshly laid clutch was tested prior to clutch use in time and temperature treatment groups to verify the presence of PVM-bound sperm. Due to a limited number of freshly laid eggs, it was assumed that the test egg was representative of all eggs within a clutch, at least in respect to the presence or absence of sperm. A total of 37 C. sulcata eggs were either opened immediately, incubated at 31°C in a Roll-X forced air incubator (Lyon’s, Chula Vista, CA) for up to 90 days following oviposition or 30 days of diapause, frozen at −20°C for 60 days, refrigerated at 4°C for 84 days, or stored at room temperature (20–22°C) in 100% ethanol or 5% formalin for 30 days (Table 2). Twelve S. pardalis eggs were opened immediately; incubated for 30 or 60 days; or frozen for 60 days. Five M. emys eggs were opened immediately or refrigerated for 70 days prior to examination. Experimental eggs were rotated frequently during the first 2 weeks of incubation to prevent embryonic development [Parmenter, 1980].

Microbial infection was determined by the presence of visible bacterial or fungal plaques upon egg breakout.

Microscopic Evaluation

PVMs were microscopically evaluated at 200× and 400× with a Zeiss AXIO Imager.A1 microscope with a UV florescence attachment (Zeiss, Thornwood, NY). Sperm heads were counted at 200× to determine the maximum number of sperm per field of view (fov). Sperm were counted in at least three separate fovs of high sperm concentration, when possible. Recovered membranes were thoroughly scanned prior to a determination of very low sperm counts or absence of sperm.

Photographs were taken at 200× for comparison of sperm counts and at 400× to document sperm head morphology.

Mitochondrial DNA PCR and Sequencing

PVMs from freshly laid C. sulcata, A. radiata, and S. pardalis eggs were frozen at −20°C following staining and sperm visualization for subsequent sperm-derived DNA isolation, amplification, and sequencing. Due to our inability to separate most of the thick albumin from the PVM, it was necessary to enzymatically digest the membranes overnight at 31°C (0.5% collagenase, 10 µL protease, and 0.05% trypsin in 1 mL PBS) prior to DNA isolation. Following enzymatic treatment, the membranes were macerated in 2.0 mL Tris, 2.5 mL de-ionized water, and 500 µL EDTA, using a Gentle MACS Dissociator (Miltenyi, Auburn, CA). DNA was phenol–chloroform extracted. Briefly, samples were diluted 1:1 with phenol:chloroform:isomyl alcohol (IAA; 25:24:1), vortexed, and centrifuged at 6,000 g for 5 min. The aqueous phase was diluted 1:1 with chloroform:IAA (24:1) centrifuged for 5 min, followed by precipitation of the aqueous phase by adding 0.1 M NaCl and two volumes of isopropanol. DNA was resuspended in 50 µL H2O. DNA was visualized using a gel electrophoresis system under UV illumination.

TABLE 2. Impact of incubation and storage on ability to detect PVM-bound sperm in C. sulcata, M. emys, S. pardalis eggs

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment (days)</th>
<th># eggs</th>
<th># positive</th>
<th># positive eggs infected</th>
<th>Max # sperm per fov (200×)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. sulcata</td>
<td>Fresh</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>17–20°C (30), 31°C (30)</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>31°C (7)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>31°C (30)</td>
<td>13</td>
<td>4</td>
<td>2</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>31°C (90)</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>4°C (84)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>−20°C (5)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>−20°C (30)</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Alcohol (30)</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Formalin (30)</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>M. emys</td>
<td>Fresh</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>4°C (70)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>S. pardalis</td>
<td>Fresh</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>492</td>
</tr>
<tr>
<td></td>
<td>−20°C (60)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>46</td>
<td>31</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

Zoo Biology
of 100% ethanol before storing at −80°C overnight. The samples were centrifuged at 21,000 g for 30 min at 4°C, followed by 100% and 75% ethanol washes and re-suspension in 50 μL Tris–EDTA buffer. When necessary, phenol–chloroform extraction was repeated.

Polymerase chain reactions were carried out using two mitochondrial primer sets designed using cytochrome b regions of the C. sulcata mitochondrial sequences from GenBank (CytB, #DQ497305.1; CytB, #AY678361.1): SMPF-8 (5′ – CCC CTT CCA CCC ATA TTT CT – 3′) and SMPR-8 (5′ – TGT GAG GAG GGG TTG ATA GG – 3′); SMPF-9 (5′ – TTC ATT GAC CTA CCA AGC CC – 3′), and SMPR-9 (5′ – CGT AGT AAA TTC CTC GGC CA – 3′). PCR amplifications were carried out in 16 μL reactions containing 13.0 μL Platinum Blue PCR SuperMix (22 U/mL Taq, 22 mM Tris–HCl, 55 mM KCl, 1.65 mM MgCl2, and 220 μM of each dNTP; Invitrogen Carlsbad, CA), 1 μL of each primer, and 1 μL of DNA. Polymerase chain reactions were performed on a GeneAmp 9700 thermo-cycler (Life Technologies, Grand Island, NY) in the following cycle conditions: 95°C (95°C, 55°C, 72°C), 31 72°C. PCR products were separated on 1.5% agarose gels and visualized on a Kodak Electrophoresis Documentation and Analysis System 120 (Kodak, Rochester, NY). The PCR bands were recovered using the Zymoclean Gel DNA Recovery Kit (Genesee Scientific, San Diego, CA), sequenced (Eton Bioscience, Inc., San Diego, CA), and aligned in GenBank to verify the species-specific sequences.

Statistical Analysis

Statistical analysis was not conducted for either sperm counts or time trials, due to the low number of available eggs from each species.

RESULTS

Sperm Staining

PVM-bound sperm were positively identified in 52 eggs, representing 12 species: A. radiata, C. sulcata, C. trifasciata, C. flavomarginata, G. platynota, G. hamiltonii, G. spengleri, K. erosa, M. emys, P. arachnoides, R. pulcherrima, and S. pardalis (Fig. 1). Of the 51 eggs exhibiting PVM-bound sperm, 16 were observed to have microbial infection (Table 1). Sperm were not detectable in freshly laid eggs from five species, presumably due to an

<table>
<thead>
<tr>
<th>Astrochelys radiata</th>
<th>Geochelone hamiltonii</th>
<th>Geochelone platynota</th>
<th>Manouria emys</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyxis arachnoides</td>
<td>Centrochelys sulcata</td>
<td>Kinixys erosa</td>
<td>Stigmochelys pardalis</td>
</tr>
<tr>
<td>Cuora trifasciata</td>
<td>Geoemyda spengleri</td>
<td>Rhinoclemmys pulcherrima</td>
<td>Cuora flavomarginata</td>
</tr>
</tbody>
</table>

Fig. 1. PVM-bound sperm head morphology in 12 chelonian species. Scale bar represents 10 μm.
absence of sperm in the infundibulum at ovulation. Known reproductive histories, including no record of previous offspring from the female *C. nigra* and a damaged penis on the *R. swinhoei* male, supported our assumption. *E. orbicularis* eggs were collected from an exhibit with multiple individuals, precluding information on individual reproductive history. However, as some nests produced fertile eggs, this suggests mate choice, or a female reproductive problem. *A. yniphora* and *H. spinosa* eggs underwent a lengthy incubation prior to examination, compromising egg integrity with microbial infection. Following egg examination, reproductive history revealed that the *H. spinosa* egg most likely would have contained identifiable sperm, had the egg been examined immediately following oviposition, as an egg from the same clutch contained a late stage embryo.

The number of PVM-bound sperm varied greatly between species and between individuals of the same species. For example, freshly laid eggs produced by two *S. pardalis* females housed with the same male exhibited noticeably different concentrations of sperm (Fig. 2). The maximum number of sperm per fov at 200× in positive eggs ranged from 2 to 492, across all 12 species (Table 1). In one *G. spengleri* egg, the concentration of sperm was too high to count. PVM-bound sperm head morphology was variable, most likely due to differences in sperm orientation within the membrane (Fig. 1). Storage conditions and length varied among eggs, with positive sperm detection ranging from <24 hr post-oviposition in multiple species to 206 days of combined diapause and incubation in a single non-infected *P. arachnoides* egg (Table 1). Sperm were identified in *A. radiata* and *G. platynota* eggs following 147 and 120 days of diapause and incubation, respectively.

Of the 37 *C. sulcata* eggs examined, 28 PVMs were recovered post-oviposition or following treatment, and 22 of the recovered membranes exhibited identifiable PVM-bound sperm (Table 2; Fig. 3). Maximum number of sperm per fov at 200× ranged from 1 to 75 sperm in *C. sulcata* eggs. *S. pardalis* and *C. sulcata* eggs that were frozen following positive identification of PVM-bound sperm in eggs from the same clutch all lacked microbial growth and exhibited detectable sperm during examination at 5, 30, or 60 days of storage at −20°C (Table 1). PVM-bound sperm were detectable following storage of *C. sulcata* eggs in formalin.

<table>
<thead>
<tr>
<th>Fresh</th>
<th>30 Day 31°C</th>
<th>30 Day 31°C (Infected)</th>
<th>30 Day -20°C</th>
<th>84 Day 4°C (Infected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
</tr>
</tbody>
</table>

Fig. 2. PVM-bound sperm nuclei numbers appear to vary greatly between individuals. Two female Stigmochelys pardalis in the same enclosure with one male exhibited a considerable difference in the sperm per microscopic field of view (200×). Eggs were collected fresh. Scale bar represents 20 µm; arrows, sperm nuclei; arrow heads, out of focus sperm nuclei.

Fig. 3. Visual detection of *Centrochelys sulcata* sperm nuclei becomes more difficult over time, as the sperm nuclei and egg contents degrade. Scale bar represents 20 µm; arrows, sperm head; arrow heads, out of focus sperm nuclei.
PVM-bound sperm were identified in all *M. emys* eggs stored at 4°C for 70 days, despite microbial infection (Table 1).

### Mitochondrial DNA PCR

Four primer sets (SMPF/R-6, SMPF/R-7, SMPF/R-8, SMPF/R-9) successfully amplified *C. sulcata* mtDNA from PVM-bound sperm. In addition, SMPF/R-7, -8, and -9 successfully amplified mtDNA from *A. radiata* PVM-bound sperm, and primer sets SMPF/R-8 and -9 successfully amplified *S. pardalis* mtDNA (Fig. 4). No DNA was detected by PCR in samples that did not contain detectable numbers of sperm.

### DISCUSSION

PVM-bound sperm detection is a valuable management tool currently used in avian breeding programs [Croyle et al., 2015]. This study demonstrates that PVM-bound sperm detection is both feasible and beneficial for chelonian conservation efforts.

Chelonian oocyte PVMs were easily identifiable in eggs with un-infected and intact yolks, such as those retrieved from freshly laid clutches. The PVM is very elastic, so even a small piece of membrane recovered from a broken oocyte represents a larger area of the intact oocyte (Fig. 5).

In this study, the impact on PVM-bound sperm degradation by incubation could not be differentiated from that of microbial infection, as they often coincide. The number of eggs exhibiting microbial infection was expected, as valuable eggs are only removed from incubation following long-term developmental failure. In this study, incubation and microbial infection did not preclude the ability to detect PVM-bound sperm, although the Hoechst does stain

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**Fig. 4.** PCR amplification of SMPF/R-9 (1–3) and SMPF/R-8 (5–7) using *C. sulcata* (1, 5), *S. pardalis* (2, 6), *A. radiata* (3, 7). Negative controls for SMPF/R 9 (4) and SMPF/R 8 (8).

**Fig. 5.** Albumin often forms a gelatinous coating surrounding the yolk, which must be carefully removed prior to staining (A). The PVM frequently contracts when removed from the yolk. YM, Yolk and PVM; Y, yolk; M, PVM; AL, albumin coat. Scale bar represents 1 cm. (A) *C. sulcata*, (B and C) *A. radiata*, (D) *R. swinhoei*. 
DNA in both fungus and bacteria often obscuring the sperm heads. Therefore, to minimize the effects of incubation and infection when PVM staining cannot be accomplished immediately following collection, we recommend that eggs be stored at −20°C until analyzed. Storage at −20°C did not visibly change sperm head morphology, or preclude PCR amplification of sperm DNA. Failure to detect sperm in incubated eggs from clutches exhibiting no development, should be verified by sacrificing (a) fresh egg(s) from the following clutch. This will help to eliminate management decisions influenced by false negative results due to sperm degradation or infection.

The ability to detect PVM-bound sperm in incubated chelonian eggs after 206 days of combined diapause (48 days) and incubation (158 days) greatly surpassed our expectations. In avian studies, PVM-bound sperm detection has only been demonstrated for up to 38 days following incubation [Croyle et al., 2015]. This difference could be explained if chelonians, unlike avian species [Stepinska and Olszanska, 2003], lack DNases in the PVM. A lack of PVM-bound DNAses raises interesting questions about the role of sperm-PVM interactions following fertilization in avian and chelonian species.

**Amplification and Sequencing**

Mitochondrial DNA from PVM-bound sperm was used to verify differences in length and sequence between *C. sulcata, A. radiata,* and *S. pardalis.* As amplified mtDNA varied in length and sequence, this may be a simple method for species identification of eggs. Our data using three species of Testudinidae suggests that the mtDNA primers SMPF/R-8 and -9 are good candidates for use within this taxa; however, it is unknown how well they will work in other chelonian taxa. Furthermore, maternal DNA contributions did not interfere with PCR analysis, as they were not detectable by PCR. This is most likely due to a loss of maternal mitochondria and oocyte nuclear DNA during the washing steps.

**Breeding Management**

Although sperm presence indicates successful copulation or use of stored sperm, and can, with some reservations, be used to infer oocyte fertilization. The absence of sperm in a fresh egg may indicate male infertility or subfertility, lack of copulation, unsuccessful copulation, or oviductal-sperm incompatibility. If, however, only a few sperm are detected, it may not represent a successful fertilization event. In zebra finches (*Taeniopygia guttata*) less than 20 sperm present on the PVM resulted in 50% egg fertility following artificial insemination [Birkhead and Fletcher, 1998]. The number of PVM-bound sperm was different in fowl [Wishart, 1987], suggesting that the number of sperm necessary for fertilization is species specific. However, it is impractical to conduct similar threshold level studies in chelonian species, which are capable of maintaining sperm in storage glands for months or in some cases for years. We, therefore, assume that if more than two sperm can be identified on a membrane, the male is functional, while several sperm throughout the PVM suggest that the egg was fertilized. In eggs lacking embryonic development following incubation, but containing PVM-bound sperm, embryonic loss may be due to genetic incompatibility or inappropriate incubation parameters.

In seasonal chelonians with limited opportunities for breeding, identifying the cause of reproductive failure for a particular individual can be a lengthy process. For species with unknown incubation parameters, especially those chelonians exhibiting embryonic diapause, it can take years to determine proper incubation temperatures and diapause timing for successful development. Although behavioral observations such as copulation offer important insights, large collections often do not have the resources to monitor breeding activity continually. For a breeding pair or herd of turtles with a history of non-developing eggs, PVM-bound sperm detection can provide managers with an additional tool and potentially help identify the cause of reproductive failures. For example, PVM-bound sperm detection was used to assess eggs from the 2014-breeding season of the last known breeding pair of *R. swinhoei* at the Suzhou Zoo in China. Following 7 years of egg production without any signs of embryonic development, we confirmed the absence of sperm in a subset of 12 fresh and 3 frozen eggs, collected from two clutches. These data were central in the decision to conduct a reproductive examination, including electro-ejaculation of the male, which produced viable sperm, followed by artificial insemination of the female. The reproductive examination of the male revealed an abnormal penis, likely causing an inability to successfully copulate with the female.

**Future Studies**

PVM-bound sperm detection has the potential to further our knowledge of chelonian reproduction and address specific breeding situations. For example, we found differences in the number of PVM-bound sperm in fresh *S. pardalis* eggs from two females housed together with access to the same males. This suggests that breeding choice, mate choice, and the use of stored sperm are complex issues that vary not only between species, but also between individuals. The ability to extract mitochondrial DNA from PVM-bound sperm could lead to in-depth analysis of reproductive dynamics surrounding sperm competition and multiple paternities, without the need to hatch, house, and care for offspring at research facilities with limited collection space.

Due to similarities between non-avian reptilian fertilization, egg structure, and embryonic development, it is reasonable to assume that PVM-bound sperm detection can be used in other reptiles that exhibit minimal oviductal embryonic development, including crocodilians. In reptiles with significant oviductal embryonic development (as...
REFERENCES

observed in many squamates), PVM-bound sperm detection is more difficult. Eggs lacking a visible embryo are often shriveled making PVM-bound sperm detection difficult (personal observation, K Croyle). This was tested using Jamaican iguana (Cyclura collier) and Cook Strait tuatara (Sphenodon punctatus) eggs lacking visible embryos at time of oviposition. However, no membranes for sperm staining could be recovered due to the congealed nature of the eggs (data not shown).

The detection of PVM-bound sperm is a promising tool for chelonian conservation in several ways. It may have an impact not only through the assessment of egg fertility for breeding management, but could, together with sperm-DNA isolation, be used in paternity, sperm competition, and sperm storage studies, as well as species identification of eggs.

CONCLUSIONS

1. Sperm trapped in the PVMs during fertilization of chelonian oocytes can be detected using the nucleic acid dye Hoechst 33342.
2. Eggs should be examined immediately post-oviposition or kept frozen until PVM-bound sperm detection, to prevent false negative results.
3. PVM-bound sperm DNA can be isolated and PCR amplified.
4. PVM-bound sperm detection can be incorporated into breeding management programs to optimize the production of endangered chelonian species in captivity.

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