Cryopreservation of Frog (Rana Pipiens) Sperm Cells Collected By Non-Methathetical Methods
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ABSTRACT
There are few reported studies of cryopreservation of frog spermatozoa. None have used non-methathetical methods for collection, which involves a simple IP injection, followed by collection of sperm from the urinogenital sinus. Our study compared post-thaw survival of spermatozoa frozen using three different diluents (Salamon’s semen diluent (SSD), our modification of Salamon’s semen diluent (MSSD), and a diluent reported to be currently the most successful, for freezing frog sperm: Browne’s frog diluent (BFD)). Sperm were divided and combined with diluents in 1.5 mL microcentrifuge tubes. Tubes were suspended in liquid N2. Upon thawing, pellets were removed from liquid N2 and left to freeze for 3 min. Frozen sperm were washed into liquid N2. Upon thawing, pellets were removed from liquid N2 and dropped into 200 µL of thawing solution at 21 °C while gently vortexing. Spermatozoa were examined using live/dead cell staining and fluorescent microscopy. Percentages of live sperm were assessed by comparing cell counts before and after the freezing and thawing process. All samples were evaluated by light microscopy, epi-fluorescent microscopy, and mechanical staining procedures. We discovered that our method of cryopreservation resulted in significantly higher numbers of live sperm compared to either SSD and BFD.

INTRODUCTION
The purpose of this project is to test a method for cryopreservation of spermatozoa collected by non-methathetical methods. We used a commercially available method of cryopreservation for ram semen and compared the methods successfully used for preservation of frog spermatozoa. Salamon’s ram semen diluent (SSD), modifed Salamon’s semen diluent (MSSD), and a diluent reported to be currently the most successful for freezing frog sperm: Browne’s frog diluent (BFD). Sperm were divided and combined with diluents in 1.5 mL microcentrifuge tubes. Tubes were suspended in liquid N2. Upon thawing, pellets were removed from liquid N2 and left to freeze for 3 min. Frozen sperm were washed into liquid N2. Upon thawing, pellets were removed from liquid N2 and dropped into 200 µL of thawing solution at 21 °C while gently vortexing. Spermatozoa were examined using live/dead cell staining and fluorescent microscopy. Percentages of live sperm were assessed by comparing cell counts before and after the freezing and thawing process. All samples were evaluated by light microscopy, epi-fluorescent microscopy, and mechanical staining procedures. We discovered that our method of cryopreservation resulted in significantly higher numbers of live sperm compared to either SSD and BFD.

METHODS
Animals
Rana pipiens purchased from the Carolina Biological Supply were housed in 100 gal tanks filled to create a habitat that is half aquatic and half terrestrial. The lighting schedule was maintained at 12 hours light and 12 hours dark. Frogs were fed a diet of crickets twice a week and tanks were cleaned daily.

Cryopreservation Diluents
Three cryoprotective solutions were compared in this study: Salamon’s ram semen diluent (SSD), and Browne’s frog diluent (BFD). SSD contains components from Table 1. MSSD includes all the components from Table 1 with the addition of 13.4 m/L of glycerol and 13.4% egg yolk. Egg yolk is known to aid sperm storage in the testes and is fresh immediately before mixing with sperm and freezing. BFD is fresh immediately before mixing with sperm and freezing. BFD contains components from Table 1.

METHODS CONTINUED

RESULTS
The percentage of live cells in SSD resulted in significantly higher numbers of live sperm compared to either SSD and BFD. Sperm frozen in SSD resulted in significantly higher numbers of viable sperm (F<0.0001). The estimate for post-thaw survival of live sperm was 30% (44.1% intact), 58.5% live (53.9% intact); SDD, 40% (58.5% live (53.9% intact); BFD, 34.4% intact (18.1% live X 73.3% intact). The percentage of live cells that did not live were considerably lower. Although a significantly higher percentage of live cells were in BFD when compared to SSD, the data suggest that ultimately you get a greater number of viable cells using SSD compared to BFD. Overall, SSD produces far greater percentage of viable cells following cryopreservation of Rana pipiens sperm.