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Sperm viability in spiders: a first approach using *Holocnemus pluchei* (Scopoli, 1763) (Synspermiata: Pholcidae)

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Abstract. Several studies have shown that sperm viability (SV) is a key trait during sexual competition. However, this has not yet been tested in spiders as no protocol has been developed to quantify SV. Here, we describe a methodology for estimating SV using the pholcid spider *Holocnemus pluchei* (Scopoli, 1763). In this method, male spermatozoa were released from the sperm ducts of copulatory bulbs in 50 µl saline solution and mixed with a vortexer. Finally, 10 µl of the sperm solution were combined with 1 µl of GelRed stain. We counted a total of 200 cells and calculated the percentage that was not stained. Males showed almost 100% of live sperm inside their genital bulbs. GelRed stain demonstrated a high effectiveness to distinguish between dead and living sperm cells in spiders and it is thus a more reliable option compared to the dyes typically used, such as propidium iodide and sybr14. The high SV in *H. pluchei* may be explained by the multiple mating nature of females, which suggests a selection for enhanced effectiveness during sperm competition within the female genital tract.

Keywords: Staining protocol, fluorescent assay, spermatozoa

Sperm viability (SV) is defined as the proportion of living sperm cells over the total number of sperm cells inside an ejaculate, which varies both intra- and inter-specifically (e.g., Hunter & Birkhead 2002; Gage et al. 2004; Rowe & Pruett-Jones 2011; Smith 2012). Such variation may be selected when spermatozoa of more than two males compete to fertilize female eggs, a process called sperm competition (Parker 1970). For example, Hunter & Birkhead (2002) showed that SV might covary with the strength of sperm competition so that a higher SV appears strongly selected in polyandrous species when compared to monandrous species.

Adult polyandrous spiders engage in multiple matings during a reproductive season (Schneider & Andrade 2011). Once mating occurs, males of most spider species transfer their sperm encapsulated by a proteinaceous sheath (Alberti 2000; Bukowski et al. 2001; Vöcking et al. 2013). Depending on the species, sperm cells can be individually encapsulated (cleistospermia) or encapsulated as conjugates (synspermia and coenospermia) (Alberti & Weinmann 1985; Eberhard 2004; Herberstein et al. 2011; Michalik & Ramírez 2014). Although different sperm traits have been widely studied in spiders, this has not been the case for SV. The only exception is the study of Archibald et al. (2014) who, nevertheless, encountered problems setting a laboratory protocol to determine SV in the Chilean rose tarantula *Grammostola rosea* (Walckenaer, 1837). These problems could include damage in the sperm plasma membrane caused during its mechanical release from the protein sheath, uncontrolled changes in osmolarity and the expiration of cells during the stain incubation period.

Here, we investigate and propose a simple methodology to quantify SV of individually encapsulated sperm in spiders and evaluate the percentage of SV in a cosmopolitan spider. We used the pholcid *Holocnemus pluchei* (Scopoli, 1763) which is a model species for studies of reproductive biology and mate competition in spiders (reviewed by Calbacho-Rosa & Peretti 2015). For the quantification of SV, we used GelRed dye and compared it with other established stains (CAM/EtDh-1).

METHODS

Collecting and rearing.—Juveniles were collected on the campus of the Universidad Nacional de Córdoba and the local zoological garden, in Córdoba, Argentina, from September 2016 to March 2017. Collected specimens were placed in cylindrical plastic containers (8 cm diameter x 15 cm high) covered inside with paper (to provide a surface for web building) and a piece of soaked cotton as a water supply. Juveniles were raised until they reached adulthood. Adult virgin males were maintained under 12:12 hrs light:dark photoperiod and fed weekly with *Drosophila melanogaster* adults. We used virgin males that had molted within 4 to 10 days. The analysed specimens were deposited in the spider collection of the Laboratorio de Biología Reproductiva y Evolución, IDEA, Universidad Nacional de Córdoba, Argentina.

Sperm preparation.—Virgin males (n = 16) were anesthetized, and both their genital bulbs removed from the pedipalps under a dissecting microscope (Nikon SMZ 1500). Each genital bulb was placed in a microcentrifuge tube accompanied with 50 µl of "spider saline solution" (3.26g NaCl, 0.13g KCl, 0.30g $CaCl_2 + 2H_2O$, 0.26g $MgCl_2 + 6H_2O$ and 250ml distilled water) (Albo & Peretti 2015). The genital bulb was gently crushed using fine tip tweezers to release the sperm into the solution.

Quantification of sperm viability using GelRed stain (Treatment 0).—The resulting sperm solutions were mixed in a vortex for 90 seconds. 10 µl of this sperm solution from each genital bulb was placed on a glass slide with 1 µl of GelRed (Biotium Inc., Hayward, CA, USA) (dilution 1/1000). This

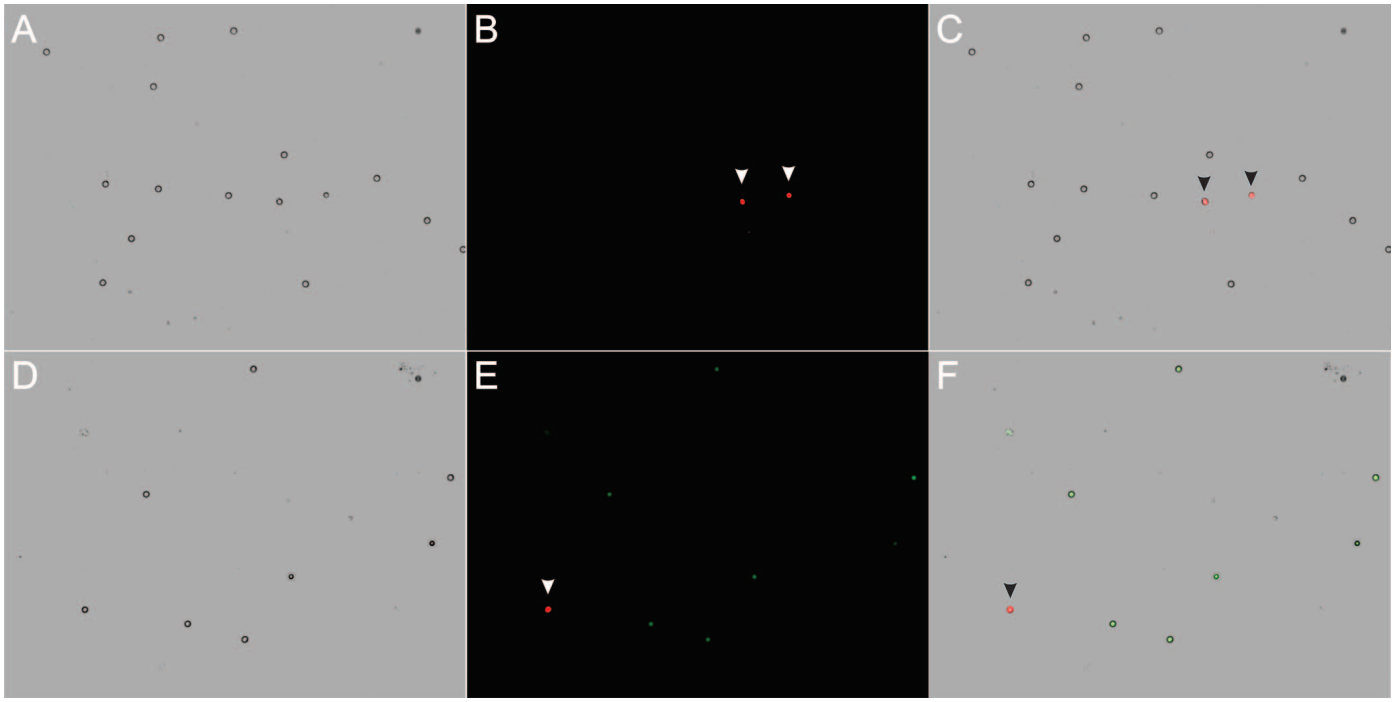

Figure 1.—A–C. *Holocnemus pluchei* spermatozoa stained with GelRed. A) Spermatozoa under light microscope. B) Spermatozoa under fluorescence filter—Dead spermatozoa stained in red (white arrows). C) Merged images of light microscopy overlaid with fluorescence signals. Notice that stained spermatozoa are dead (black arrows) while those not stained are alive. D–F. Spermatozoa stained with CAM/EthD-1. D) Spermatozoa under light microscope. E) Spermatozoa under fluorescence filter –Dead spermatozoa stained in red (white arrow) while those stained in green are alive. F) Merged images of light microscopy overlaid with fluorescence signals. Red spermatozoa are dead (black arrow) while those stained in green are alive.

DNA stain cannot penetrate living cells due to the selective-permeable property of the cell's membrane (Guzaev et al. 2017). The nature of this stain is that dead cells emit red fluorescence when GelRed stain penetrates them and binds with their DNA, whereas live cells do not emit such fluorescence. All samples were observed using an inverted epifluorescence microscope (Leica DiM8) (Filter cube RHODLP, excitation: 540/45, emission: 590). We calculated the proportion of live spermatozoa for each male bulb by using ImageJ image processing software (Schneider et al. 2012), counting a minimum of 200 cells. The percentage of SV was estimated as follows: % SV = (number of cells without staining/total number of cells counted) x 100.

Validation of staining procedure.—We performed a positive control using ethanol with three different concentrations (10%, n = 15; 20%, n = 15; and 50 %, n = 14; Treatments 10, 20 and 50, respectively) as ethanol effectively kills spermatozoa *in vitro* (Damiens et al. 2002; Pokharkar et al. 2015). Sperm cells were treated by incubating each sperm solution with each different ethanol concentrations for two minutes and then centrifuging samples at 5000 rpm for five minutes. The resulting pellet was resuspended in 10 µl of "spider saline solution" and stained with GelRed as described above.

Comparison with other established stains.—In order to confirm that our staining method is not overestimating the number of living cells in the samples (unstained cells), we compared SV of 11 males in the four treatments ("spider saline solution" and three alcohol treatments) with another vital staining technique (LIVE/DEAD viability/cytotoxicity kit) (Fig.1). In this case, the pellet of the resulting solutions was resuspended in 20 µl of "spider saline solution". The first 10 µl were stained with 1 µl of GelRed, while the other 10 µl were incubated for 30 minutes in the dark at room temperature (average = 26°C), and then stained with 1 µl of 20 µM calcein acetoxy methyl ester (CAM) and 1 µl of 40 µM ethidium homodimer-1 (EthD-1). CAM and EthD-1 are the components of the LIVE/DEAD viability/cytotoxicity kit (Thermo Fisher, Waltham, MA, USA). CAM is a lipophilic vital dye that once it enters inside viable cells, is converted to calcein by intracellular esterases. This conversion leads to an intense green fluorescence and is retained only by cells with an intact plasma membrane (Weston & Parish 1990; Bratosin et al. 2005). EthD-1 produces red fluorescence when it penetrates dead cells and conjugates with the DNA inside the nucleus (Kato et al. 2002). This method allowed us to differentiate living from dead cells without error, making it an excellent control to establish the effectiveness of the GelRed staining technique.

Statistical analyses.—In order to compare SV efficiency between GelRed and the CAM/EthD-1 methods, we used a paired t-test and Fisher-Pitman permutation test for each pair of the four treatments with both dyes. The Fisher-Pitman permutation test was used when the paired t-test was unable to

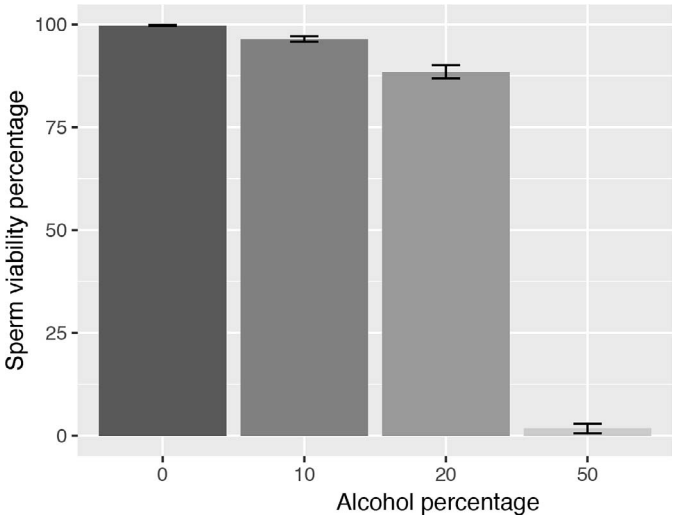

Figure 2.—Positive control of the sperm viability technique: Sperm viability (percentage of live spermatozoa) decreased while ethanol concentration increased.

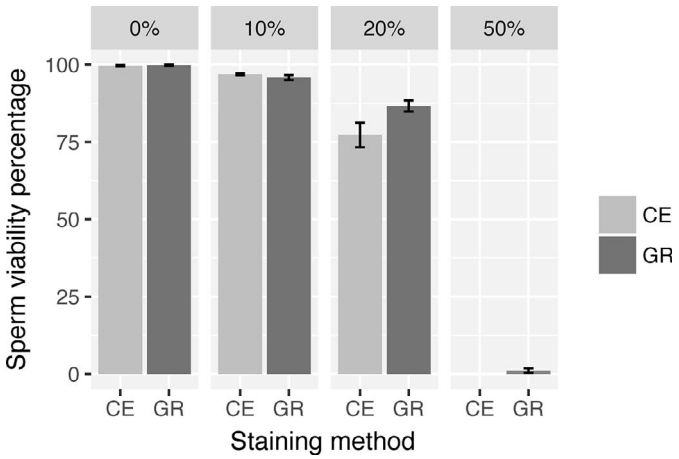

Figure 3.—Sperm viability percentage in three alcohol treatments (10%, 20%, 50%) and one control treatment with "spider saline solution" (0%) stained with two different dyes, GelRed (GR) and CAM/EthD-1 (CE).

compute an exact *P*-value due to ties in our data. This was needed for treatments 0 and 50. R Version 3.3.2 (R Core Team 2016) was used for all statistical analyses.

RESULTS

The SV percentage was high in both genital bulbs (right palp: 99.31%, left palp: 99.80%). In the positive controls with GelRed, the viability decreased as ethanol concentration increased, with almost 100% of cells dead (red stained cells) in the treatment with 50% ethanol (Fig. 2). In addition, we found no significant difference in the performance of the two staining methods (measured as percentage of SV) in each of the four treatments (Table 1) (Fig. 3).

DISCUSSION

A plausible explanation for the finding of high values of SV in *H. pluchei* is that sperm competition may positively select for SV because of the multiple-mating nature of *H. pluchei* females (Calbacho-Rosa & Peretti 2015). Given this female behavior, males may aim to maximize their fertilization success by increasing the number of viable sperm inside their ejaculates so that enough high quality sperm are transferred, as has been previously documented in insect species (Hunter & Birkhead 2002; García-González & Simmons 2005). An experiment to test this hypothesis in spiders should involve comparing species whose females vary in mating rate: males of monandrous species are expected to produce lower SV while males of polyandric species are expected to produce higher SV; a pattern already reported in insects (Hunter & Birkhead 2002). Securing such high viability might be related to the protein sheath surrounding the spermatozoa, which could play a protective role during sperm induction, sperm transfer, and/or inside the female sperm storage organs (Alberti 1990; Michalik & Lipke 2013). As mentioned before, sperm cells can be individually encapsulated or encapsulated as conjugates (Alberti & Weinmann 1985; Eberhard 2004; Herberstein et al. 2011; Michalik & Ramírez 2014). It is important to point out that *H. pluchei* falls within the cleistospermia type (individual encapsulation), while the species used by Archibald et al. (2014), presents coenospermia. Thus, it is possible that different types of sperm encapsulations contribute to or have an effect on the different patterns of sperm viability. A simple experiment should compare the sperm viability in spider species with different sperm encapsulation (cleistospermia, synspermia, and coenospermia) but a similar mating system. However, until more information is available, the discussion of this topic remains speculative.

The main salient result of our work is that we have demonstrated that GelRed is a reliable staining method of dead spider sperm cells and that the results obtained with this methodology were statistically similar to the live/dead technique. Thus, the GelRed staining technique seems a rapid and robust method for determining SV in spiders. From a laboratory safety point of view, our findings are of high relevance as studies in other species (mainly insects) have relied on hazardous SV assessment techniques such as CAM/EtDh-1 (Collins & Donoghue 1999; Hunter & Birkhead 2002) and Sybr14/propidium iodide (Damiens et al. 2002; Demary

Table 1.—Statistical comparison between the percentage of viable sperm in three alcohol treatments (10%, 20%, 50%) and in one control treatment with "spider saline solution" (0%) stained with two different dyes, GelRed (GR) and CAM/EthD-1 (CE).

Alcohol treatment	Test	Results
GR0%-CE0%	Fisher-Pitman permutation test	$z = -0.885$; $P = 0.440$
GR10%-CE10%	Paired t-test	$t = -1.222$; $P = 0.250$
GE20%-CE20%	Paired t-test	$t = -1.908$; $P = 0.089$
GR50%-CE50%	Fisher-Pitman permutation test	$z = -1.190$; $P = 0.201$

2005; Tarpy et al. 2012; Tarpy & Olivarez 2014; Schrempf et al. 2016). Propidium iodide and Syber14 (also used in the study of Archibald et al. 2014), can be carcinogenic for humans (material safety data sheet, Molecular Probes, 2005; safety data sheet, Life Technologies 2013). EthD-1 and CAM seem to not be mutagenic but have been shown to cause eye and skin irritation (safety data sheets, Life Technologies 2015a, b). In contrast, GelRed passed environmental safety tests (Guzaev et al. 2017). Given this, we suggest further evaluation of the GelRed technique for SV estimation in other spider and arachnid species.

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