Arch. Pol. Fish.	Archives of Polish Fisheries	Vol. 12	Fasc. 1	23-30	2004

DETERMINING THE VIABILITY OF ZEBRA MUSSEL (*DREISSENA POLYMORPHA*) SPERMATOZOA AND CHANGES IN THE INTEGRITY OF ITS PLASMA MEMBRANE USING THE FLUORESCENCE METHOD

Joanna Białkowska, Wiesław Demianowicz**, Jan Glogowski*,***

*Department of Evolutionary Ecology, University of Warmia and Mazury in Olsztyn, Poland
**Molecular Andrology Group, Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, Olszyn, Poland

ABSTRACT. The viability of zebra mussel (*Dreissena polymorpha*) sperm and changes in the integrity of its plasma membrane were examined using combined nucleic fluorescent stains SYBR-14 and propidium iodide. Approximately 97 ± 3% of the sperm in freshly ejaculated zebra mussel semen was stained with SYBR-14 (potentially viable). A statistically significant decrease in plasma membrane integrity was observed after 48 hours (8°C) when 42 ± 11% of the sperm stained as viable. Semen stored at room temperature (20°C) lost its plasma membrane integrity after 4 hours (0% viable). Motility declined from 60 ± 4% (0 time) to 30 ± 3% after 24 hours. The percentage of sperm stained with SYBR-14 was not correlated with sperm motility or the fertility test.

Keywords: ZEBRA MUSSEL (*DREISSENA POLYMORPHA*), SPERM, VITALITY, FLUORESCENCE

INTRODUCTION

The fluorescent stains SYBR-14 and propidium iodide (PI) have been demonstrated to be valuable tools for assessing membrane integrity and cell viability in the sperm of different species. SYBR-14 is a membrane-permeant stain that binds DNA of all the sperm, while PI is a membrane-impermeant stain that binds DNA in those sperm with leaky membranes (Garner et al. 1997). In other studies, the percentage of sperm stained as live has been correlated with the percentage of motile cells for bulls, honeybees, and porcine (Bochenek et. al. 1998, Chalah and Brillard 1998, Collins et al. 1999).

This paper proposes applying this technique for the first time as an indicator of sperm viability in *Dreissena polymorpha* (zebra mussel), an invasive European freshwater bivalve with external fertilization and free swimming larvae (veliger). The ani-

CORRESPONDING AUTHOR: Joanna Białkowska, Uniwersytet Warmińsko-Mazurski, Zakład Genetyki Ewolucyjnej, ul. Oczapowskiego 5, 10-957 Olsztyn, tel./fax: +48 89 5233754; e-mail: a.b@poczta.wprost.pl

mals are of opposite sex and release gametes into the water where the eggs are fertilized with sperm. One female can produce a few thousand oocytes; males ejaculate up to 10^6 spermatozoa ml^{-1} per cohort (Sprung 1987).

The objective of the present study was to apply fluorescent staining with SYBR-14/PI in order to determine sperm viability and check for changes in sperm plasma membrane integrity when it begins to lose motility.

MATERIAL AND METHODS

SEMEN AND EGG SAMPLES

The semen was obtained from nine males (from Lake Kortowo, Poland) following hormonal stimulation with 0.5 mM serotonine (5HT,5-hydroxytryptamine, t = 15 min), which was applied externally in the surrounding fresh water (FW) (Sprung 1987) as described in Ram et al. (1993). The number of sperm per milliliter FW was measured using a hemocytometer according to the standard method described by Bielański (1977). A concentration of 10^6-10^7 ml^{-1} was determined to be the best method for staining. Eggs used in the fertility test were collected in a pooled sample from a few females. A light microscope and a light/dark field were used to determine sperm motility.

STAINING

The SYBR-14 stain was prepared in dimethyl sulphoxide (DMSO) at a concentration of 1mg ml^{-1}. A working solution of SYBR-14 diluted 1:20 with DMSO was used for staining the sperm. The PI stain was dissolved in distilled water at a concentration of 4 mg ml^{-1}. A 0.5 ml aliquot of sperm was stained with 2 μl of the working solution of SYBR-14 and 1.5 μl of the PI stock solution. The samples were incubated at room temperature (20°C) for 15 minutes before examination (Donoghue et al. 1995). The semen were examined after incubation (0 time) and then after 24 and 48 h of storage at 8°C. The spermatozoa that fluoresced bright green with SYBR-14 were classified as being intact, while those stained red with PI were classified as damaged.

Two slides of samples were examined to determine the percentage of viable/nonviable sperm using microscopy and the proper filter. In order to count the live and dead sperm, the fluorescently stained sperm were photographed with an Olympus epifluorescent microscope equipped with a camera and the proper filter. For the quantitative assessment of plasma membrane integrity, at least 100 cells were

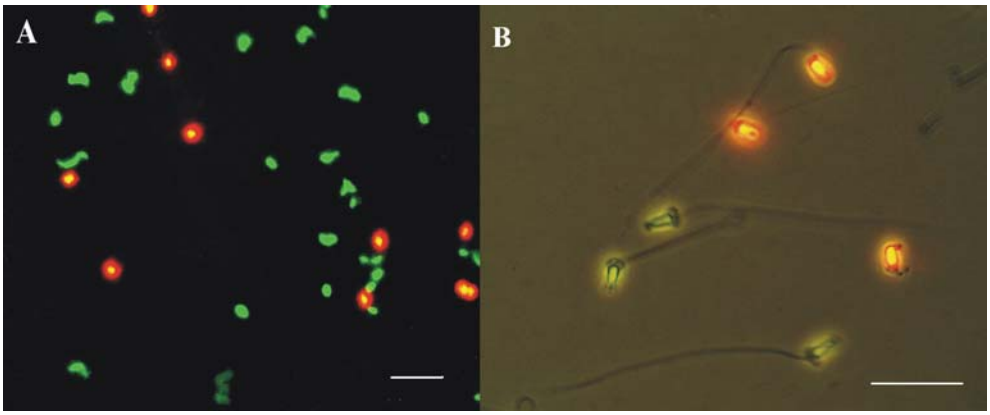

Photo 1. *Dreissena polymorpha* sperm stained with SYBR-14 and propidium iodide: A) epifluorescence and proper filter: spermatozoa that fluoresced bright green with SYBR-14 were classified as being intact, while those stained red with PI were classified as damaged (with a loss of plasma membrane integrity), B) fluorescence and phase contrast (sperm retained PI stain – abnormal and swollen, sperm retained SYBR-14 – potentially viable, morphologically normal), bar – 10 μm.

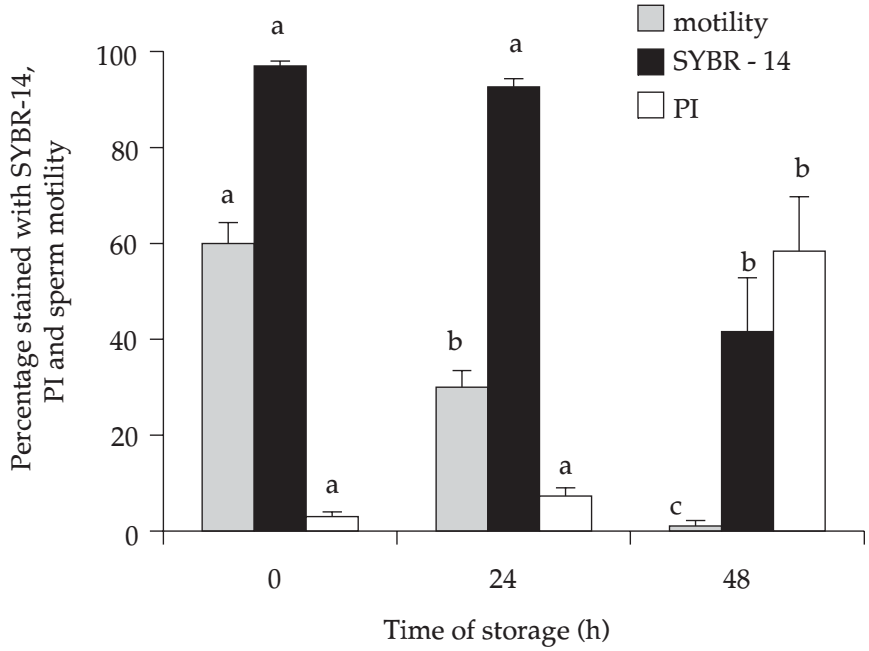

Fig. 1. Percentage of *Dreissena polymorpha* sperm stained with SYBR-14/PI and motility; a, b, c – statistically different values within all groups separately for motility, stained with SYBR-14 and stained with PI, $P \leq 0.05$.

counted in each 6 μl aliquot of stained samples. All of the preparations were analyzed by the same person.

FERTILITY TEST

The fertilization ability of zebra mussel spermatozoa were examined *in vitro* using fresh water (FW) as the medium. The sperm was mixed with oocytes at a ratio of 1:1. After three hours of incubation at room temperature (20°C), the percentage of fertilized eggs in all stages of development was determined and compared with the viability and percentage of motile sperm. To avoid the erroneous identification of weak-sperm fertilization ability that was actually caused by "old oocytes" (females react later than males in response to 5-HT), fresh oocytes, obtained by synchronizing the release of eggs with the spawning of males, were used in the experiment.

CHEMICALS

The chemicals used in the present experiment were purchased from Sigma Aldrich, and the Live/Dead Sperm Viability Kit was purchased from Molecular Probes.

STATISTICAL ANALYSIS

The motion characteristics, fertility percentage, and fluorescent measurements of the viability of the *D. polymorpha* sperm are presented as means ± SEM. Differences among the means were analyzed using one-way ANOVA, while the relation between the percentage of motility and fertility was tested with the Tukey *post-hoc* test and linear regression.

RESULTS

ASSESSMENT OF VIABILITY IN SEMEN SAMPLES

The green/red SYBR-14/PI-stained sperm were bright and easily distinguished when examined with a fluorescence microscope (Photo 1A). Vital sperm stained with SYBR-14 (green) were considered to be morphologically normal; however, immotile and dead sperm stained with PI were morphologically abnormal and swollen (red for PI, Photo 1B). The changes from green to red began at the posterior part of the head over the course of 30 seconds to one minute.

The motility for nine specimens varied from 40 - 80% with the concentration in the range of 10.5 - 60 × 10^6 sperm ml^{-1} FW when viability/membrane integrity was 97 ± 3% during the first 24 h (8°C). A statistically significant decrease in the number of cells

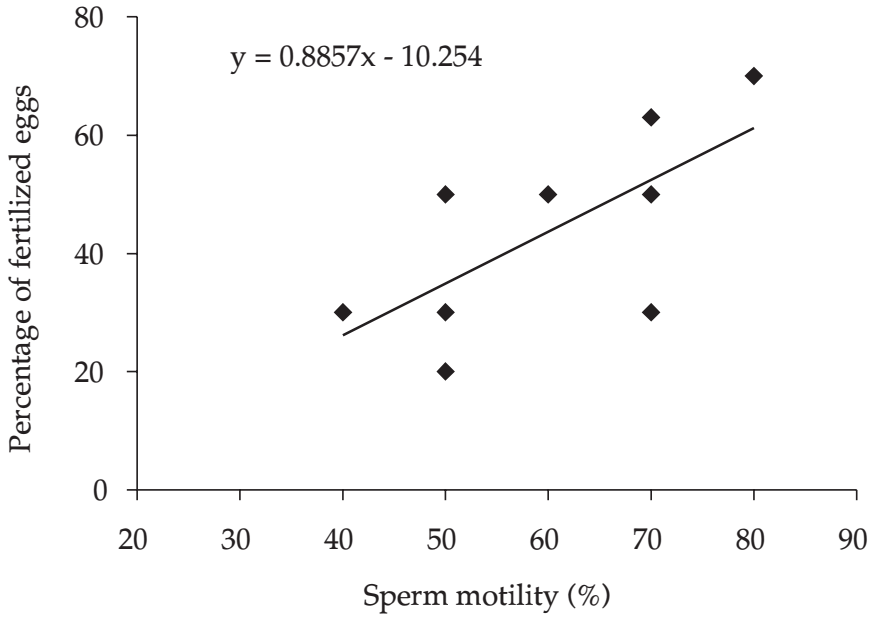

Fig. 2. Correlation between percentage of *Dreissena polymorpha* sperm motility and fertility ($n = 9$, $R^2 = 0.5$, $P \leq 0.05$).

stained as viable was observed after 48 h (8°C) at 42 ± 11%. Consequently, the percentage of sperm stained with PI increased to 58 ± 11% (Fig. 1). Statistical analysis showed that the number of sperm with an undamaged cell membrane is not correlated with the percentage of motile sperm at the initial time of the experiment and after storage. A significant decrease in motility compared to initial motility (60 ± 4%) was observed after 24 hours – 30 ± 4% ($P \leq 0.001$), whereas the percentage of sperm stained with SYBR-14 was still almost 93 ± 2%. The results from fresh semen examined at intervals of 0, 1, and 2 days stored at 8°C are shown in Fig. 1. Semen stored at 20°C lost membrane integrity after four hours when there were 0% live sperm expressed as the percentage stained with SYBR-14 (data not presented).

FERTILITY COMPARED TO VIABILITY

There was a significant correlation between the percentage of motility and fertility ($R^2 = 0.5$, $P \leq 0.05$) (Fig. 2), but no correlation between the percentage of fertility and viability ($P \geq 0.05$).

DISCUSSION

Studies on the integrity of the sperm plasma membrane may be important for evaluating the biological quality of semen, and the introduction of fluorescent DNA stains opens up new possibilities for evaluating the use of fluorescent microscopy (Bochenek et al. 1998). The change in SYBR-14 staining in relation to PI is evident because when sperm die they lose their ability to resist the influx of the membrane penetrating PI stain. It enters through pores in the nuclear membrane that are located in the diverticulum, or membrane folds, of the posterior region of the sperm head (Garner and Johnson 1995).

The motility of nine specimens of *D. polymorpha* varied from 40-80% when viability/membrane integrity assessed with SYBR-14 was 97 ± 3% (potentially viable) (Fig. 1). Until the twenty-fourth hour of the experiment, sperm stained with SYBR-14 represented the largest population. The change from green to red began at the posterior portion of the sperm head, proceeded towards the anterior, and was complete within one minute. Garner and Johnson (1995) observed the same activity in mammalian sperm.

The current experiment shows that not all immotile sperm lose their membrane integrity. Hong et al. (1988) compared human sperm motility with vitality and found that many vital sperm were immotile. This author also emphasized that the persistence of motility and the duration of vitality are not the same. Sperm may be vital but not motile. Another explanation may be that the maintenance of plasma membrane integrity in a media with a normal K^+ and Na^+ ratio needs a supply of intracellular ATP. So membrane damage measured by fluorescent probes may indicate prior metabolic failure as well as a concomitant loss of vital intracellular metabolites (i.e. adenine nucleotides) (Harrison and Vickers 1990).

There was a significant correlation in the semen of bulls, turkeys, boars, etc. between the sperm stained with SYBR-14 and motility (Garner and Johnson 1995, Garner et al. 1996). In the current study it was assumed that the SYBR-14/PI method would be reliable and exchangeable with motility estimation, but it was proven that the double-staining method using SYBR-14/PI is not good for examining this aspect of sperm quality. Differences occurred between vital sperm stained with SYBR-14 and motility (Fig. 1). There was no correlation between these two parameters.

Vetter et al. (1998) commented that membrane integrity is a necessary, although insufficient, criterion for predicting motility. However, Harrison and Vickers (1990)

used carboxyfluorescein diacetate and propidium iodide to assess membrane integrity in sperm populations from ram and boar and found that this technique appeared to provide a more reliable estimation of the percentage of functional cells than did motility estimations.

Changes in sperm quality – membrane integrity, etc. over time can provide valuable insight into reproductive biology, but the data shows the proportion of spermatozoa stained with SYBR-14 is also not correlated with fertility. There was a significant correlation for the percentage of fertilized eggs and percentage of motile sperm ($R^2 = 0.5$, $P \leq 0.05$, Fig. 2). This method cannot be used to evaluate semen quality with regard to the fertility aspect. Garner et al. (1997) reported that dual DNA staining of spermatozoa can be used to identify the ability of bull sperm to successfully undergo cryopreservation, but that the assessment of viability did not accurately measure fertility.

Zebra mussel sperm stored at 20°C rapidly became permeable to PI stain after four hours from the time the sperm is obtained (0% of live sperm expressed as the percentage stained with SYBR-14, data not presented). The storage of sperm samples at 8°C preserved membrane integrity and motility. A significant decrease in membrane integrity was observed after 48 hours when the percentage of PI-stained sperm increased to 58 ± 11%. A significant decrease in motility was observed after 24 h – 30 ± 3% ($P \leq 0.05$), whereas the percentage of sperm stained with SYBR-14 was still almost 93 ± 3% (Fig. 1). This demonstrated the sensitivity of the sperm plasma membrane to temperature conditions.

The other explanation may be that the temperature of 8-12°C is best for zebra mussel reproduction and rearing veligers (Sprung 1987, Borcherding 1991). The first 24 hours is probably the time when sperm are most likely to fertilize eggs *in situ* (after 24 h – 0% of recently obtained eggs were fertilized). It can be concluded that motility is the most reliable parameter for describing the biological quality of zebra mussel spermatozoa.

Dual DNA staining of *Dreissena polymorpha* spermatozoa can be used as an indicator of membrane integrity, but it is not as accurate or effective for evaluating biological or reproductive quality as it is with the major parameter – motility. The integrity of the plasma membrane of zebra mussel sperm changes more slowly than motility and sperm fertilizing ability.

REFERENCES

Bielański W. 1977 – Animal reproduction. Cattle, sheep, horses, swine – PWRiL, Warszawa (in Polish).

Bochenek M., Laszczka A., Sławiński J., Skrzyszowska M., Szczęśniak-Fabiańczyk B. 1998 – Integrity of bull sperm membranes stained with SYBR-14 and PI assessed by fluorescent microscopy and flow cytometry – 7th International Symposium "Molecular and physiological aspects of regulatory processes of the organism", Cracow.

Borcherding J. 1991 – The annual reproductive cycle of the freshwater mussel *Dreissena polymorpha* Pallas in lakes – Oecologia 87: 208-218.

Chalah T., Brillard J.P. 1998 – Comparison of assessment of fowl sperm viability by eosin- nigrosin and dual fluorescence (SYBR-14/PI) – Theriogenology 50(3): 487-493.

Collins A.M., Donoghue A.M. 1999 – Viability assessment of honey bee, *Apis mellifera* sperm using dual fluorescent staining – Theriogenology 51(3): 1513-1523.

Donoghue A.M., Garner D.J., Donoghue D.J., Johnson L.J. 1995 – Viability assessment of turkey sperm using fluorescent staining and flow cytometry – Poultry Sci. 74: 1191-1200.

Garner D.L., Johnson L.A. 1995 – Viability assessment of mammalian sperm using SYBR-14 and Propidium Iodide – Biol. Reprod. 53: 276-284.

Garner D.L., Johnson L.A., Allen C.H., Palencia D.D., Chambers C.S. 1996 – Comparison of seminal quality in Holstein bull as earling and as mature sires –Theriogenology 45(5): 923-934.

Garner D.L., Thomas C.A., Allen C.H., Senger P.L., Sasser R.G. 1997 – Effect of cryopreservation on bovine sperm viability as determined by dual DNA staining – Reprod. Dom. Anim. 35(6): 279-283.

Harrison R.A., Vickers S.E. 1990 – Use of fluorscent probes to assess membrane integrity in mammalian spermatozoa – J. Reprod. Fertil. 88(1): 343-352.

Hong C.Y., Huang J.J., Wu P., Lo S.J., Wei Y.H. 1988 – Fluorescence supravital stain of human sperm correlation with sperm motility measured by a transmembrane migration method – Andrologia 20(6): 516-520.

Ram J.L., Crawford G.W., Walker J.U., Mojares J.J., Patel N., Fong P.P., Kyozuka K. 1993 – Spawning in the zebra mussel (*Dreissena polymorpha*): activation by internal or external application of serotonin – J. Exp. Zool. 265: 587-598.

Sprung M. 1987 – Ecological requirements of developing *Dreissena polymorpha* eggs – Arch. Hydrobiol. 79: 69-86.

Vetter C.M., Millet J.E., Crawford L.M., Armstrong M.J., Clair J.H., Conner M.W., Wise L.D., Skopek T.R. 1998 – Comparison of motility and membrane integrity to asses sperm viability – Reprod. Toxicol. 12(2): 105-114.

STRESZCZENIE

WYKORZYSTANIE METODY FLUORESCENCYJNEGO BARWIENIA DO OKREŚLENIA PRZEŻYWALNOŚCI I ZMIAN W INTEGRALNOŚCI PLAZMOLEMY PLEMNIKÓW RACICZNICY ZMIENNEJ (*DREISSENA POLYMORPHA*)

Zastosowano fluorescencyjną metodę SYBR-14/jodek propydyny (tzw. metoda żywe/martwe) w celu określenia zmian w integralności plazmolemy plemników *Dreissena polymorpha*. W świeżo pozyskanym nasieniu odsetek plemników barwiących się jako żywe (SYBR-14) wynosił 97 ± 3% (rys. 1). Statystycznie istotny spadek integralności błony plazmatycznej stwierdzono po 48 h przechowywania w 8°C, podczas gdy w temperaturze 20°C już po 4 h 100% plemników wybarwiało się jako martwe (jodek propydyny). Nie stwierdzono zależności pomiędzy odsetkiem plemników barwiących się jako żywe a odsetkiem plemników ruchliwych i procentem zapłodnionych komórek jajowych.