6-1997

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Proc 8th Int Coral Reef Sym 2:1341–1344. 1997

USE OF FLUORESCENCE MICROSCOPY IN AN ASSAY OF SPERM DENSITY FOR THE GORGONIAN CORAL, *PLEXAURA KUNA*

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ABSTRACT

The density of sperm in the water column during the broadcast spawning events of marine invertebrates is often undetermined or reported in terms of fertilization potential. The density of sperm during such events can be determined by directly counting numbers of spermatozoa using a modification of the acridine orange direct count (AODC) technique for enumerating bacteria. A number of variables in the handling and processing of samples may bias estimates. Sample collection in glass and rapid fixation and filtration are necessary for reproducible estimates. Once filtered, samples are stable for many months, and counts on filters that were poorly stained in the field can be enhanced by additional staining with 4'6-diamidino-2-phenylindole (DAPI). The AODC technique was employed to examine the effects of distance from a male colony on density of sperm for the gorgonian coral *Plexaura kuna*. *In situ* sperm densities ranged from 0 to 1,000 sperm/ml. Sperm densities in 18 l aquaria containing 20 cm long *P. kuna* explants reached 100,000 sperm/ml.

INTRODUCTION

A wide variety of marine taxa have reproductive systems in which gametes are cast into the water column. Reproductive success among these species is commonly reported in terms of female reproductive success, i.e., numbers of eggs that are produced and fertilized (Levitan 1991; Babcock and Mundy 1992; Brazeau and Lasker 1992; Levitan et al. 1992; Lasker et al 1996). Female reproductive success is a function of the density of sperm in the water column and of sperm to egg ratios (Vogel et al. 1982; Lavation et al. 1991; Babcock et al. 1994; Benzie and Dixon 1994). Therefore, analyses of reproductive success should ideally incorporate data on sperm density. The lack of accurate techniques for the measurement of *in situ* sperm concentrations have forced most studies to either ignore sperm density or rely on indirect assays, such as the fertilization potential of field collected water samples (Oliver and Babcock 1992; Lasker et al. 1996). An accurate technique for the enumeration of *in situ* sperm densities is essential for studies of reproductive biology and analyses of the reproductive output of broadcast spawning taxa.

We describe the use of a direct count technique for determining the density of sperm in field collected water samples. Shapiro et al. (1994) have previously used a direct count technique to measure in situ sperm density of the wrasse *Thalassoma bifasciatum*. However, their procedures are not effective for all taxa (Lasker, unpubl. data). We introduce an adaptation of the acridine orange direct count (AODC) method for enumeration of bacteria (Hobbie et al. 1977). This method has been widely accepted as the most reliable and inexpensive procedure for the enumeration of bacteria (Kirchman et al. 1982), and the procedure can be used to stain and enumerate any cells that contain DNA. We discuss some of the collection and preparation effects that can bias sperm density estimates and report laboratory and *in situ* measurements of sperm densities found during spawning by the Caribbean gorgonian *Plexaura kuna*.

MATERIAL AND METHODS

The technique consists of the following steps: a water sample is collected, preserved with formalin (5%), stained with 0.02% acridine orange (AO), filtered onto a 0.45 μm pore size polycarbonate membrane filter, and the sperm were counted using an epifluorescence microscope. The AO concentration and incubation period in our protocol are greater than that suggested by Hobbie et al. (1977), this enhanced the quality of slide preparations and minimized fluor quenching during microscopic examination.

Detailed methodology

Water samples were collected in 125 ml glass jars with Teflon-lined polypropylene caps; 20 ml of each sample was transferred to a glass scintillation vial with 1 ml of formalin (yielding a 5% formalin solution). Samples were stored 10 to 15 h prior to processing. Prior to filtration, the water sample contained in the scintillation vial was vigorously agitated to assure complete suspension of sperm cells. The entire sample was decanted into a 250 ml glass filter apparatus. In situations where the suspected sperm concentration was very high (i.e., $>10^5$ sperm/ml, such as in water samples collected from aquaria containing male colonies), only 1 ml of the sample was utilized. Samples were vacuum filtered through a moistened irgalin black pre-stained polycarbonate membrane filter (0.45 μm pore size, 25 mm diameter, Poretics®, Livermore, CA). Filtering was performed under a constant vacuum (50 KPa) until 1 ml of the sample remained in the funnel. 0.2 ml of 0.1% AO was added to this remaining volume to create a final concentration of approximately 0.02%. The sample was incubated for 5 min, after which filtering was resumed.

After all of the sample had been drawn through the polycarbonate membrane, the vacuum was maintained while the funnel was removed to eliminate the moisture trapped between the funnel and the base. The filter was left in place under suction for an additional 30 s to remove excess moisture from the filter. The membrane filter was mounted on a glass slide with a drop of low fluorescence immersion oil. A cover slip was placed over the filter, and the slide was stored in the dark until viewing.

Irgalin black stained filters can be produced by staining filters for a minimum of 24 h in irgalin black (2 g in 1 L of 2% acetic acid) prior to use. We used pre-stained filters (Poretics®, Livermore, CA), which saved time without affecting the observed density. Because AO in solution is light and temperature sensitive, we prepared AO solutions no more than 12 hours prior to use. The 0.1% AO solution was prepared by adding distilled water to AO powder (AO is stable when dehydrated). Immediately prior to staining of sperm samples the AO solution was filtered through a 0.45 μm pore size syringe filter to remove insoluble solids.

After staining, filters were viewed under blue excitation (450-490 nm) with a Zeiss epifluorescence microscope at 160 x. To determine sperm concentration of a water sample, 10 fields on each filter were counted. The average number of sperm per field was adjusted for the area of the field under the microscope and the effective area of the filter. That number was divided by the volume of the sample to yield the concentration of sperm.

Sources of bias

In order to test the accuracy and reproducibility of this technique, a series of laboratory experiments were performed using sperm collected from the sea urchin, *Stongylocentrotus purpuratus*. Sea urchins were induced to release sperm by injection with 1-5 ml of 0.5 M KCl. The "dry" sperm was collected and diluted with 100 ml of filtered (0.45 μm pore size filter) seawater. Samples were preserved with formalin 1 h after collection, then stained and filtered 12 h later. This protocol approximates the timing of sample collection and processing that we employed when estimating *Plexaura kuna* sperm concentrations in the field.

1. Container effect. Developmental studies generally employ glass containers for sample collection. The effects of collection, preservation, and initial storage (24 h) in a glass container were assessed by collecting dry sperm, diluting them and decanting either directly into the filtration apparatus or into a glass scintillation vial. Formalin preservation and 24 h storage was performed as described above.

In the field, the use of less fragile containers is often advantageous. Four different containers were tested to see if their use biased sperm counts: i) 20 ml glass scintillation vial (G-SV); ii) 120 ml polypropylene specimen cup (PP-SC); iii) 50 ml polypropylene centrifuge tube (PP-CT); and iv) 50 ml polystyrene centrifuge tube (PS-CT). The sea urchin sperm solution was transferred into each of the four container types with the entire experiment repeated 3 times using a separate stock sperm solution in each experiment. Because the initial sperm concentration in each of the three repeated experiments was different, sperm counts within each experiment were normalized relative to the maximum density in that experiment. The data were arcsine-transformed prior to an analysis of variance (ANOVA) to compare the effects of using different containers on sperm counts.

2. Sperm concentration effect. Water samples obtained in the field are likely to vary in sperm concentration. To examine the accuracy of sperm counts over a range of

densities a series of 10^3, 10^4, and 10^5 dilutions of dry sea urchin sperm were prepared. An aliquot of each dilution was transferred into each of 3 containers (i.e., 3 container types per dilution), and sperm density determined after overnight storage of the formalin fixed sample.

3. Long-term storage effect. Samples that have been fixed at remote study sites may require storage and transport back to a laboratory prior to filtration. The delay between preservation and filtration may affect sperm density. To determine the effects of long-term storage on sperm concentration, a second sample from each of the containers generated in the sperm concentration effect experiment were stained and filtered 28 d after the initial filters were prepared.

4. Time before preservation effect. Collections of water samples by SCUBA divers during *in situ* spawning events often require 30 min or more to complete. The time interval between collection and fixation may introduce bias if sperm cells degrade while in the collection containers. To quantify short-term degradation of sperm, sea urchin sperm was diluted in glass containers and then samples preserved 0.5, 1, 2 and 24 h after dilution. These samples were compared to a replicate set that was preserved immediately after the dry sperm was diluted.

5. Pre-filter effect. The presence of debris in natural waters can lead to clogging of filters. This is especially problematic because field samples may have low sperm concentrations and thus require large sample volumes. In such instances, the use of a pre-filter that removes larger debris may be necessary. To examine the effects of using pre-filters, 4 replicate samples were prepared using a 40 μm plankton mesh pre-filter placed directly above the membrane filter in the filtering apparatus and compared to 4 samples prepared without pre-filtering.

6. Reduced volume staining effect: Instead of staining our 20 ml sample, we first reduced the sample volume by filtration to 1 ml before staining with AO. The advantage of this method is that the volume of AO contaminated filtrate is substantially reduced. To examine the effects of the procedure on our measurements of sperm density, we compared sperm densities of 4 samples that were reduced to 1 ml to the sperm densities of four replicate samples in which the entire volume was stained prior to filtration.

Application of technique

The Caribbean gorgonian coral *Plexaura kuna* is a gonochoric, broadcast spawner. Females release large eggs (600-800 μm) during synchronous spawning events (Brazeau and Lasker 1989; Lasker et al. 1996). Gamete release by males is not directly visible, but fertilization potential of water samples indicates that it follows a similar temporal pattern of release as the females (Lasker et al. 1996). Lasker and Stewart (1993) estimated sperm release based on extrapolations from histologic sections, but no direct measures are available. We used the AODC technique to estimate sperm densities within aquaria in which male branch explants were maintained and to estimate *in situ* concentrations at different distances from a spawning male colony. Field measurements were made during July 1995 at Tiantupo Reef, a shallow patch reef located near the Smithsonian Tropical Research Institute field station in the San Blas Is., Panama.

Water samples were collected during spawning events, soon after eggs were sighted in the water column. Collections were made at distances of 2 m upstream and 0, 1, 2, and 4 m downstream of the study colony. Four replicate samples were collected at each distance in 125 ml glass jars with Teflon-lined polypropylene caps. Each sampling series began at the furthest downstream distance (4 m) and proceeded upstream to minimize the effects of turbulence created by the divers. Each sample required 10-20 s to collect with an entire series being completed in under 5 minutes. Samples were transported back to the research station where 20 ml of each sample were preserved in a glass scintillation vial with 5% formalin (final concentration). Total time between collection and fixation was approximately 0.5-3 h. Samples were filtered and stained the following morning. Water samples were also collected from spawning males in 18 l aquaria and preserved as noted above.

RESULTS

The distinct morphology and green-yellow fluorescence of the acridine orange stained DNA make the spermatozoa easily discernible from debris on the membrane. The triangular heads and thread-like flagella were readily visible at 160 x. At 400 x the red-orange acrosome and midpiece of the sperm could be discerned.

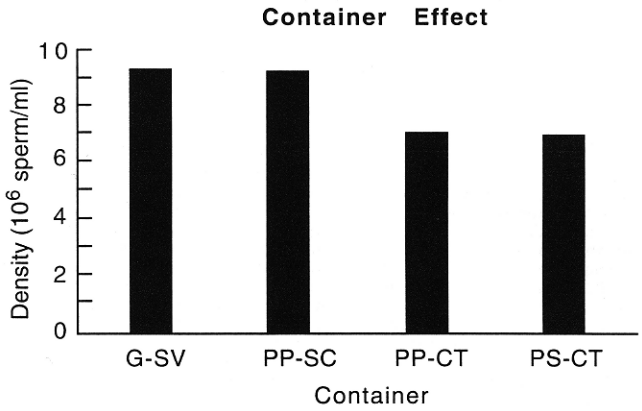

Fig. 1: Comparison of four collection containers on sea urchin sperm densities: glass scintillation vial (G-SV), 120 ml polypropylene sample cup (PP-SC), 50 ml polypropylene centrifuge tube (PP-CT), and 50 ml polystyrene centrifuge tube (PS-CT).

Storage of preserved sea urchin sperm in the glass scintillation vials for 24 h lead to a significant 17% decrease in measured sperm density (paired t-test comparing density on filters prepared immediately after preservation against filters prepared 24 h after preservation, $t=16.82$, $df=3$, $p<0.05$). Container effects were identified when the sea urchin sperm densities observed in glass scintillation vials were compared to specimen cups and centrifuge tubes. Densities measured in the four container types differed significantly between the containers (Fig 1, $F=9.983$, $df=3,8$, $p<0.05$). The reduction in sperm density was greatest in the containers with the greatest surface to volume ratios, as can be seen in the comparison of the polypropylene specimen cup and the polypropylene centrifuge tube. The polypropylene centrifuge tube, which has a higher surface to volume ratio, had a greater effect on measured sperm density than the polypropylene specimen cup.

Both sperm density and container effects were evident in the sperm concentration effect experiment in which sperm densities in containers were compared with each other at sperm concentrations of approximately 10^4, 10^5 and 10^6 sperm/ml. Aliquots of sea urchin sperm that were transferred into the polypropylene specimen cup and polypropylene centrifuge tube had lower measured sperm densities than the glass scintillation vial (Friedman two-way analysis of variance, $\chi^2= 29.1$, $df=2$, $p,0.001$). There was a greater percent decrease of sperm density in containers in which sperm were initially more dilute.

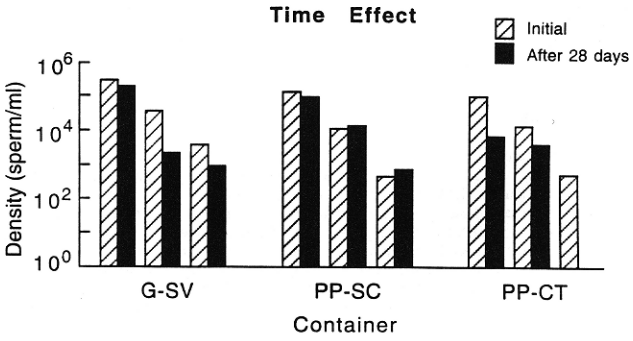

Fig. 2: The effect of long-term storage on aqueous sea urchin sperm samples stored in various containers: glass scintillation vial (G-SV), 120 ml polypropylene sample cup (PP-SC), and 50 ml polypropylene centrifuge tube (PP-CT). Comparisons of the observed effect at initial concentrations of approximately 10^4, 10^5, and 10^6 were made for each container.

Fig 2 illustrates the decrease in sperm density in three test containers after 28 d of additional storage. The figure also demonstrates this effect at three different initial dilutions of sperm. Extended storage of the preserved sample reduced apparent sperm density (Kruskal-Wallis test, H=7.87, df=3, p=0.049). Sperm counts in the glass scintillation vial and the polypropylene centrifuge tube both decreased after 28 days, with more severe decreases observed in the initially lower density samples. Although an initial drop in sperm density was observed in the polypropylene specimen cup, density was stable over the 28 d time period.

Leaving sea urchin sperm samples in the glass scintillation vial for short periods of time (0.5, 1, and 2 h after spawning) prior to preservation had no effect on observed sperm density, and there was no significant difference when these samples were compared with a fixed but otherwise identical sample (t=2.657, df=2, p>0.05). Although there was no significant difference in density, signs of cell degradation were evident in samples that were allowed to age to 2 h without fixation. When left unfixed for 24 h, a sample that initially contained 1.9 x 10^6 sperm/ml had no discernable sperm. *Plexaura kuna* sperm from *in situ* samples collected early in the spawning event and kept in collection vessels (125 ml glass jars) for up to three hours did not exhibit the structural deterioration observed in sea urchin sperm at 2 h. This may indicate a longer half-life for the gorgonian sperm.

The stability of fixed sea urchin sperm samples was greatly increased after filtration and mounting on slides. Prepared filters were recounted at 5 and 13 mo after the initial counts were performed, and no significant difference was found between the observed densities (t=-0.338, df=9, p>0.37). There was no discernable decrease in the fluorescence of these samples, even after 24 months. Samples of both sea urchin and *P. kuna* sperm continue to fluoresce 3 or more years after preparation.

In situ P. kuna sperm samples (20 ml) were processed without the use of a pre-filter, and no clogging of the filter was observed. Comparison between sea urchin sperm samples processed with a pre-filter and those without showed no significant difference in sperm density (t=0.901, df=3, p> 0.2). The comparison between sea urchin sperm stained in solution and samples stained on the filter demonstrated no significant difference between the two sample sets (t=0.169, df=3, p> 0.4).

The *in situ* collections performed on spawning *P. kuna* in Panama yielded density estimations that ranged from 0 to 10^3 sperm/ml. Results from sampling on July 17 and 18, 1995 are presented in Fig 3. The highest densities measured were collected directly at the spawning male colony (0 m) and decreased rapidly as distance from the colony increased in either the upstream or down stream directions. Collections from aquaria (18 l) yielded density estimates that ranged from 0 to 10^5 sperm/ml, indicating that 15-25 cm long branches can release up to 10^9 sperm on a single night.

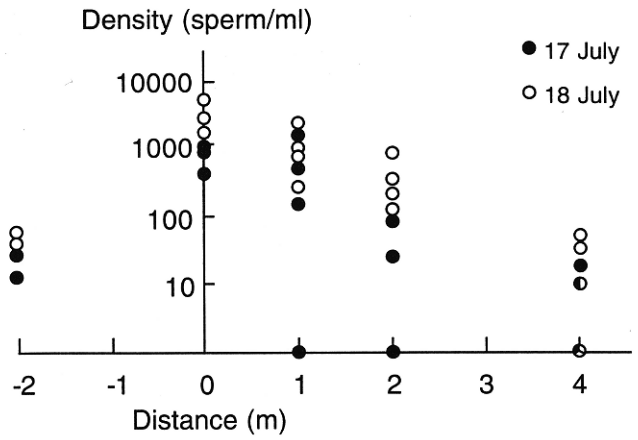

Fig 3: *In situ* effects of distance on the sperm densities for *Plexaura kuna* male.

DISCUSSION

AODC measurements of sperm density can provide a simple and reproducible methodology capable of measuring density as low as 10 sperm/ml. Although the technique requires laboratory analysis of samples, the initial steps can be carried out in any field setting in which a filtration apparatus is available within 24 h of collecting the samples.

A number of techniques that do not require fluorescence microscopy may also have potential as alternatives to the AODC procedure. However, these alternatives are subject to many of the same limitations and biases as the AODC technique. Shapiro et al. (1994) present a direct count technique similar to ours. In their technique the water samples are stained with rose bengal and filtered through cellulose acetate filters. Gorgonian sperm were not visible when samples were prepared following their technique (Lasker unpubl. data).

Use of a hemocytometer for counting cells in a liquid medium is more cost effective and is less time consuming then the AODC method. However, it requires high densities (>10^3 sperm/ml) for accurate estimates of sperm density. The AODC method can provide reproducible analysis of samples that contain sperm at densities as low as a few sperm/ml. Use of a hemocytometer must also be completed on site during spawning events or shortly thereafter in order to insure that the cells contained in the sample have not degraded. This provides immediate data, but takes up time in the field and produces results that cannot be verified later. Attempts at bypassing the time constraints by preservation of sperm samples with an aldehyde for later enumeration will be subject to the same preservation and storage effects that we have discussed. Sperm in samples that are kept for over 24 h also clump, which will further reduce the accuracy and reproducibility of hemocytometer counts. If they are undertaken, hemocytometer counts should be made within 24 hours or less of collection and samples analyzed toward the end of that time period may yield less accurate counts than the first sample counted.

Techniques using flow cytometry have proven to be reliable as well as expedient for enumerating cells (Monfort and Baleux 1992). However, the equipment involved is expensive and certainly not intended for use in the field. Additionally, the stability and time constraints seen in the use of a hemocytometer are not eliminated by the use of flow cytometry.

Although the AODC technique is both sensitive and readily conducted in field settings, it does have limitations. First, sample preparation requires either multiple preparations using different sample volumes or some initial estimate of density, and second, the technique underestimates density by 17% or more depending on the procedures followed.

The AODC technique is most reproducible when the density of sperm on the membrane filter is in the range of 20-60 sperm per field. In order to insure that sperm are present on filters in sufficient density for counting, an initial estimate of sperm density is helpful. If sperm are more dense than expected then the density of sperm on the filter can make enumeration difficult. This can be avoided by preparing multiple filters with different volumes or dilutions. In high density samples an eyepiece retical can be used to analyze smaller areas (3.24 X 10^{-4} μm^2) of the membrane rather than the entire field (1.62 X 10^{-3} μm^2).

A more difficult density problem occurs when samples are not concentrated enough for accurate estimates of density. This can be averted by collecting larger sample volumes. A 20 ml sample has a minimal detectable density of 10.8 sperm/ml (one sperm found on one of ten fields). The use of larger volumes of water will increase the density of sperm on the membrane but may lead to difficulties with debris on the membrane. AO non-specifically stains debris in the samples and creates areas on the membrane that cannot be counted. This problem affected only a small percentage of our sample preparations and seldom affected an entire slide, but could become problematic if larger volumes are filtered.

A factor that had a much greater affect on our ability to score filters occurred when filters were not allowed to dry long enough before the addition of immersion oil. When excess water was present, the filters did not clear properly and the sperm were impossible to count.

Difficulties with fluorescence quenching also arose in field samples. Reductions in incubation times or stain concentration created poorly stained samples that

quenched quickly. Quenching also occurred when filtered seawater was substituted for distilled water in acridine orange solution preparation. Acridine orange did not dissolve as well in filtered seawater as it did in distilled water and some samples prepared in this manner quenched before counts on a single field could be completed.

Field samples that have been stained poorly may be salvageable by further staining at a later time. By removing the cover slip and adding 3 μl of 10 μg/ml 4'6-diamidino-2-phenylindole (DAPI), we have been able to stain sperm cells on the membrane and view them under ultraviolet (365 nm) illumination. DAPI as well as AO has been used for enumeration of bacteria (Porter and Feig 1980). There was an overall decrease in nonspecific binding and background fluorescence when DAPI is used, making sperm cells more discernable and easy to enumerate. This suggests that DAPI may prove to be a superior stain for field samples. The occasional failure of staining in the field also suggests the advisability of incorporating positive controls in the analyses.

All of the containers tested in our experiments reduced sperm density below the respective control, and the severity of the effect correlated with the container's surface to volume ratio. Accuracy of the procedure can be increased in the laboratory by filtering samples immediately upon fixation. Elimination of the error among field samples may be more difficult. Possible procedures that should be tested include immediate fixation by collecting samples into containers that already contain formalin or immediate freezing of samples.

Glass containers yielded the most accurate sperm density measurements, and the 17% reduction in sperm density we observed in the glass containers was relatively small compared to the several order of magnitude range in sperm density observed in the field samples (Fig 3). The relationship between sperm density and fertilization rates for *Plexaura kuna* and among many marine broadcast spawning species follows a sigmoidal function of the logarithm of sperm density (Vogel et al. 1982; Levitan et al. 1991; Benzie and Dixon 1994; Lasker, unpubl. data). Thus on most occasions the AODC technique has the resolution to distinguish conditions that will yield high fertilization from those that will yield low fertilization rates. The lack of resolution of the AODC estimates may become problematic for predicting fertilization success when *in situ* concentrations occur near the inflection point of the sperm density-fertilization success curve and when absolute estimates of fertilization kinetic constants are desirable. None-the-less the AODC technique provides a reproducible technique for estimating sperm densities across a range of field conditions. Incorporation of sperm density data into field studies will enable us to compare male reproductive strategies across a variety of environments and taxa.

ACKNOWLEDGMENTS

Our thanks to Elizabeth Beiring, Mary Alice Coffroth, Rafel Coma, William Hamilton, Edith Hare, Thomas Insalaco Walter Kapela, Kristin Kittle, Allen Siegal and Sara Weatherup for their efforts on this project. Our work in San Blas was made easier and more pleasant by the efforts of the staff of the Smithsonian Tropical Research Institute and our special thanks to Reynaldo Tapia, the San Blas Station Manager. We thank the Kuna Indians and the Republic of Panama for permission to work in San Blas and are grateful for the support of the National Science Foundation (OCE 9217014), the National Geographic Society, the Howard Hughes Medical Institute Undergraduate Biology Initiative, and the Smithsonian Tropical Research Institute.

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