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Studies on the reproduction of multiple-spawning fishes often involve estimates of batch fecundity and oocyte size (Hunter & Goldberg 1980, DeMartini & Fountain 1981, Hunter et al. 1985, Brown-Peterson et al. 1988). Because data collection and laboratory analysis are rarely concurrent, oocytes which are preserved and hardened are generally used for these analyses. It is critical, therefore, to have a method of oocyte preservation which does not damage or destroy oocytes and has a determinate effect on oocyte size.

The preferred oocyte preservative (Bagenal & Braum 1978, Snyder 1983, Cailliet et al. 1986) has been a modified Gilson's solution: 100 mL 60% ethanol or methanol, 880 mL water, 15 mL 80% nitric acid, 18 mL glacial acetic acid, and 20 g mercuric chloride (Snyder 1983). The benefit of using Gilson's is its ability to harden oocytes while chemically separating them from ovarian tissue. However, a number of problems are associated with this procedure, including degeneration of hydrated oocytes (Hunter et al. 1985, Schaefer 1987, Brown-Peterson et al. 1988); substantial and continuous oocyte shrinkage, reported to range from 15% to 24% (DeMartini & Fountain 1981, Schaefer 1987, Witthames & Greer Walker 1987); a relatively long fixation period of several days to a few weeks (Cailliet et al. 1986); and the extreme toxicity of mercuric chloride (West 1990).

Formalin solution (4–10%) has also been used to preserve whole fish ovaries (Bagenal & Braum 1978, Hunter 1985, Cailliet et al. 1986). It is recommended by Hunter et al. (1985) as the only preservative appropriate for use with the hydrated oocyte method. This is because the hydrated oocyte method estimates batch fecundity by calculating the number of hydrated, unovulated oocytes in gravid ovaries, and Gilson's destroys hydrated oocytes (Hunter et al. 1985).

Formalin preservation has the advantages over Gilson's of (1) preserving hydrated as well as other oocytes over long periods of time, (2) having a short fixation period and (3) low shrinkage rates, varying from 0 to 7% (Hiemstra 1962, Fleming & Ng 1987, Hislop & Bell 1987), and (4) relative ease of handling (Hunter et al. 1985, Cailliet et al. 1986, Schaefer 1987, West 1990). Its greatest disadvantage is that oocytes and ovarian tissue may become fixed into a hard mass, making it extremely difficult and tedious to separate oocytes without damage (Schaefer & Orange 1956, Bagenal & Braum 1978, Cailliet et al. 1986).

In this paper we propose a new, two-step method to obtain hardened, separated oocytes for fish reproduction studies. Oocytes are physically separated before being preserved in formalin, thus maintaining the advantages of formalin fixation and preservation while also providing well-separated oocyte samples. The objectives of this paper are to (1) describe this new method and evaluate its effectiveness, (2) determine the shrinkage rates of weakfish *Cynoscion regalis* oocytes separated and preserved by this method, after 3–4 and 6–7 mo preservation, and (3) assess the appropriateness of this method for use with the hydrated oocyte method of estimating batch fecundity (Hunter et al. 1985).

Methods

Twenty-eight weakfish *Cynoscion regalis*, with ovaries in the hydrated but unovulated developmental stage, were collected in the summer of 1991. Fresh (unpreserved) oocytes were removed from the right ovary of each fish and spread onto a microscope slide. Twenty hydrated oocyte diameters were then measured, after a minimum sample size of 15 oocytes was determined using the iterative method described in Sokal & Rohlf (1981) (S=0.05, α=0.05; P=0.90, δ=0.06 mm). An ocular micrometer in a dissecting microscope was used to measure oocyte diameters to the nearest 0.038 mm (1 micrometer unit at a total magnification of 24×). Measurements were taken along the median axis of the oocyte, parallel to the horizontal micrometer gradations (Macer 1974, DeMartini & Fountain 1981).

Ten of these fish were also used to estimate batch fecundities gravi-

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metrically (Bagenal & Braum 1978), using the hydrated oocyte method (Hunter et al. 1985). A 0.2 g subsample of fresh oocytes was taken from the middle of the right ovary, and all hydrated oocytes in each subsample were counted under a dissecting microscope at a magnification of 24×.

Oocytes were separated from one another and the ovarian membrane through a washing process. Each ovary was slit longitudinally, turned inside out, and held under vigorously flowing tapwater. This flushed the oocytes out of the ovarian membrane and into a 0.01 mm mesh sieve, which was held beneath the ovary. Oocytes collected in the sieve were again rinsed with fully-flowing tapwater to help separate them from one another. The whole procedure took 5–10 min per ovary.

After draining the water, oocytes were transferred to containers where they were preserved in 2% neutrally-buffered formalin. This formalin concentration was chosen because it was the lowest possible concentration that would ensure proper oocyte preservation while minimizing changes in oocyte size and appearance.

The equipment necessary for the washing process is very basic. We used two standard faucets (2 cm diameter), with flow rates of 133 and 286 mL/s, respectively. Both faucets had sufficient hydraulic pressure to dislodge oocytes of all stages from ovarian tissue. However, the faucet with the higher flow rate, and thus greater water pressure, worked best. Any sieve with mesh small enough to retain less-developed oocytes, and deep enough to keep them from being flushed over the edge during washing, can be used as a collecting sieve. We used a sieve made from a piece of nylon plankton net (0.01 mm mesh) inserted between two sections of 10 cm diameter PVC pipe, with a depth (from lip to the mesh layer) also of 10 cm (Fig. 1).

Preserved oocytes were measured 3–4 mo after collection and, again, 6–7 mo after collection. Samples were stirred before oocytes were removed to reduce bias due to settling differences caused by oocyte size or density. Oocytes were then dipped out of the formalin with a spoon and placed in a gridded petri dish. The first 20 undamaged hydrated oocytes were measured along the median axis as described for fresh oocytes. Oocyte damage, due to the washing process, was evaluated by assessing the percentage of damaged oocytes in subsamples of 50 hydrated oocytes from each of 10 preserved samples. We considered as damaged those oocytes which were partially collapsed and thus not appropriate for diameter measurements.

Batch fecundities were also estimated gravimetrically from preserved samples (after 3–4 and 6–7 mo preservation) using oocyte samples from the same 10 fish originally used to estimate batch fecundities from fresh samples. Oocytes were stirred, decanted into a sieve, drained of formalin, and washed with tapwater. Oocytes were removed from the sieve, spread on the bottom of a petri dish, and blotted dry with tissue paper. A 0.2 g subsample was then transferred to a gridded petri dish. A small amount of tapwater was added to keep the oocytes moist and to help distribute them evenly over the bottom of the dish.

One-way analysis of variance (ANOVA) was used to evaluate differences between fresh and preserved oocyte diameters and batch fecundity estimates. Individual females were used as blocks to remove the effect of variation among females. To compare batch fecundities based on fresh samples with those based on preserved samples, it was important to evaluate the within-ovary positional effect. This was necessary because fresh oocyte samples were taken from the middle of the ovary, whereas preserved samples came from mixed areas (due to the washing process). Hydrated oocytes were counted in 0.2 g oocyte samples taken from the anterior, middle, and posterior areas of 28 fresh ovaries.

Mean oocyte shrinkage was calculated for each of the 28 ovaries after 3–4 and 6–7 mo preservation. Mean oocyte shrinkage was then plotted against mean fresh hydrated oocyte diameter to evaluate whether oocyte shrinkage was consistent over the size-range of hydrated oocytes.

All data were analyzed using statistical methods available through the Statistical Analysis System (SAS 1988). Model assumptions were evaluated by examination of residuals (Draper & Smith 1981). Batch fecundity data was $\log_{10}$-transformed to meet the assumption of homogeneity of variances.

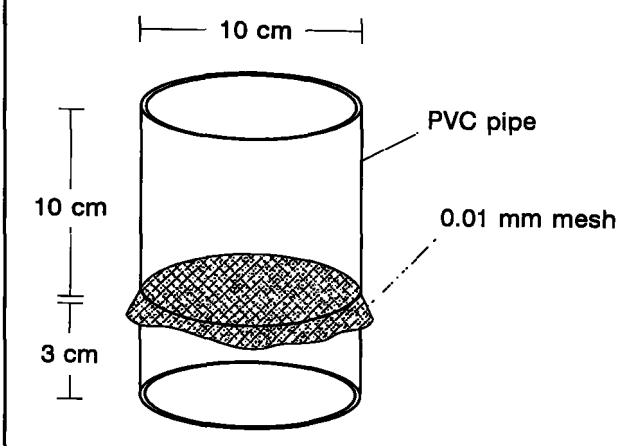

Figure 1
Schematic representation of the sieve used to collect weakfish *Cynoscion regalis* oocytes dislodged during the washing process.

Results

We successfully used this separation technique on ovaries in all stages of development and observed little or no damage to the oocytes (Figs. 2, 3). The percentage of damaged oocytes ranged from 0 to 6%, with an average of 2%. None of these, however, were structurally damaged, i.e., no empty chorions were found. Because such low percentages of slightly-degenerated hydrated oocytes can also be found in fresh oocyte samples—i.e., some hydrated oocytes are never ovulated and will be resorbed, and some ovulated oocytes are never spawned (e.g., Clark 1934, DeMartini & Fountain 1981)—we considered oocyte damage due to the washing process to be negligible.

Oocytes in all stages (primary growth to hydrated) were obtained in sufficiently large numbers and correct proportions to develop oocyte size-frequency distributions (Fig. 2). For most ovaries it was possible to flush virtually all oocytes out of the ovarian membrane. However, we found it was easier to dislodge oocytes in well-developed ovaries than in early-developing or resorbing-phase ovaries.

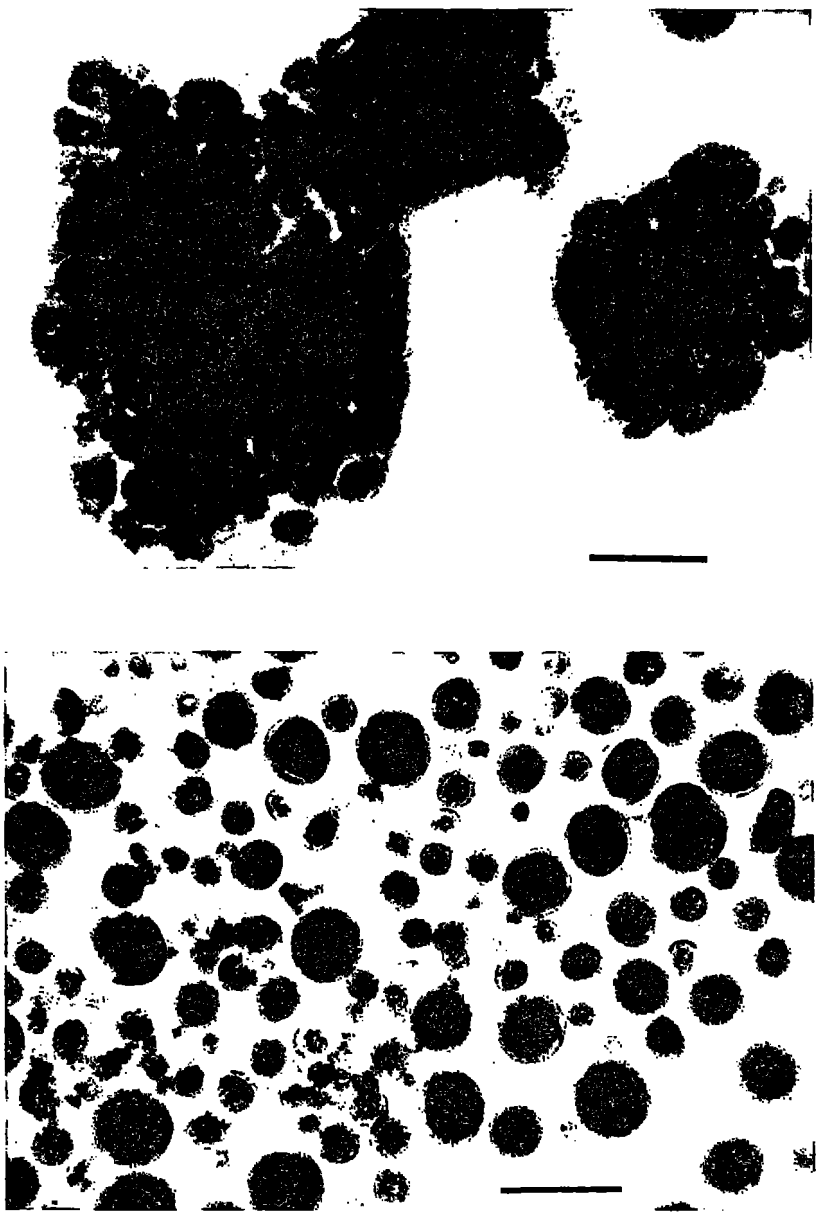

Figure 2
Appearance of weakfish *Cynoscion regalis* oocytes in various developmental stages: **(top)** fresh and **(bottom)** after hydraulic separation and fixation in 2% formalin. Bars=1 mm.

Formalin (2%) successfully fixed and then preserved weakfish oocytes for over 6 months with minimal effect on their appearance and size. There was no need for a separate, higher-concentration fixative. Atretic and hydrated oocytes were more opaque after preservation, but much less so than when kept at higher formalin concentrations. After 6 months, hydrated oocytes were still easily recognized by their larger size and greater translucence than were less-developed oocytes (Fig. 3). Most oocytes, in all stages, retained their spherical shape.

Hydrated oocytes had a highly significant decrease in diameter after preservation, with a range of 0–11% shrinkage after 3–4 mo in preservative (F=223.25, N=560, P<0.01). The average oocyte shrinkage, however, was only 5% and after more than 6 months, oocyte shrinkage had not significantly increased (F=1.91, N=560, P=0.17). Mean shrinkage of hydrated oocytes preserved for 3–4 and 6–7 mo showed no relationship with their original mean fresh diameters (Fig. 4), indicating that an oocyte's stage in the hydration process did not affect its rate of shrinkage.

Batch fecundities estimated from fresh oocyte samples were

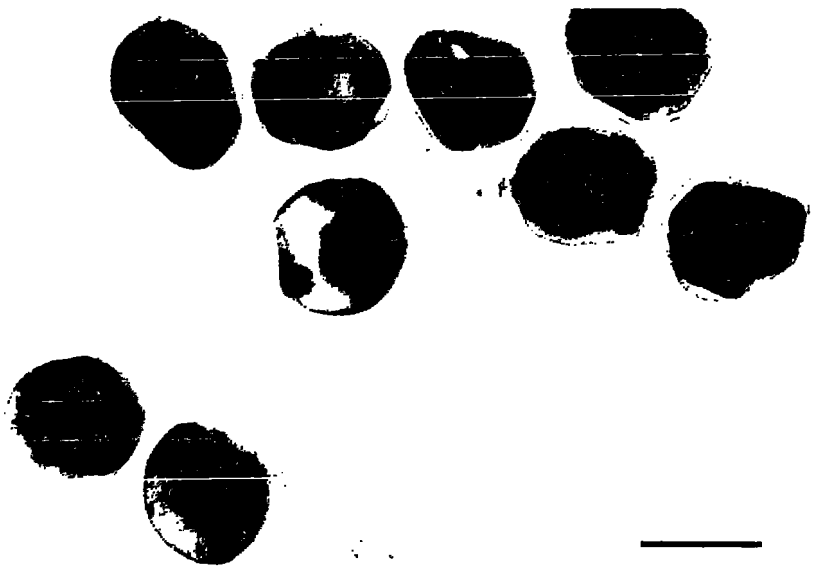

Figure 3
Appearance of weakfish *Cynoscion regalis* hydrated oocytes after 6–7 mo preservation in 2% formalin. Bar=1 mm.

not significantly different (F=0.0027, N=10, P=0.14) from those estimated from samples preserved for both 3–4 and 6–7 mo (Table 1). Batch fecundities estimated from fresh and preserved samples could be compared because no positional effects were found between counts from different areas (ANOVA, F=0.91, N=28, P=0.41).

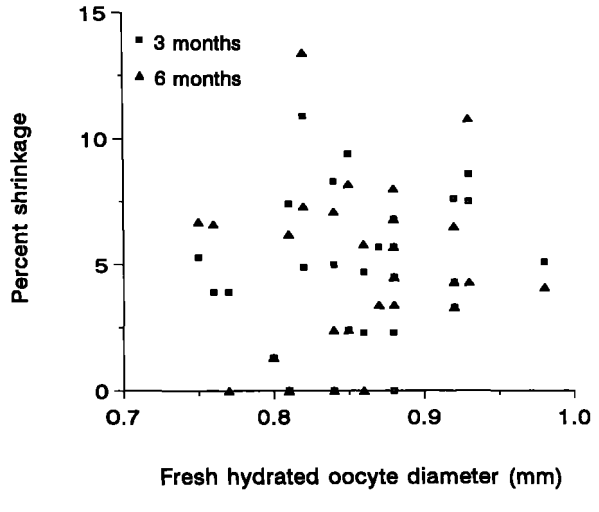

Figure 4
Mean shrinkage of hydrated oocytes of weakfish *Cynoscion regalis* after 3–4 and 6–7 mo preservation in 2% formalin.

Discussion

There is need for a reliable method of separating and preserving fish oocytes. Generally, separated and preserved oocytes are used to estimate oocyte size and develop oocyte size-frequency distributions, as well as in the hydrated oocyte method of determining batch fecundity. Researchers often want to evaluate changes in egg size over the spawning season, or between spawning seasons (e.g., DeMartini 1990), and oocyte size-frequencies are used to assess whether fish have determinate or indeterminate fecundity (Hunter & Macewicz 1985). The hydrated oocyte method appears to be the easiest and most accurate way to determine batch size in serial spawners (Hunter et al. 1985). All of these types of analyses are integral to reproductive studies of multiple-spawning fishes, and yet their accuracy depends on using either fresh oocyte samples or oocyte samples separated and preserved in a reliable fashion.

Although still widely used, researchers are beginning to recognize a number of problems with the use of Gilson's solution as a preservative. It degenerates hydrated oocytes (Hunter et al. 1985, Schaefer 1987, Brown-Peterson et al. 1988), making it impossible to use the hydrated oocyte method to estimate batch fecundity (Hunter et al. 1985). It causes a high rate of

Table 1
Batch fecundities of weakfish *Cynoscion regalis* estimated from fresh oocyte samples and from oocyte samples preserved for 3–4 and 6–7 mo in 2% formalin.

Fish#	Fresh count	After 3 mo preservation	After 6 mo preservation
1	159,400	140,400	154,700
2	101,700	104,800	95,700
3	190,000	212,700	208,300
4	158,900	176,300	156,800
5	171,100	174,000	181,400
6	155,100	149,000	143,300
7	208,400	167,800	223,100
8	161,000	154,200	191,800
9	144,700	127,200	148,300
10	209,000	172,200	216,100

oocyte shrinkage (DeMartini Fountain 1981, Schaefer 1987, Witthames & Greer Walker 1987), which could mask gaps found naturally in oocyte size-frequency distributions. Gilson's solution also causes continuous shrinkage over time (Witthames & Greer Walker 1987), which could make any comparisons of egg diameter during the spawning season, or between consecutive years, meaningless unless all samples were preserved for the same amount of time.

Formalin at low concentrations (3–5%) meets the requirements of both an oocyte fixative and preservative (Markle 1984). It prevents microbial activity, with minimal effect on shape, cell contents, and osmolarity. As a preservative, it maintains this state, is relatively mild, stable, and long-lasting (Snyder 1983, Markle 1984). Although formalin is commonly used to preserve ichthyoplankton samples (Snyder 1983), it has not been commonly used for adult fish-reproduction studies. This is due to the tendency for formalin to fix the whole ovary into a hard mass, from which it is difficult to separate individual oocytes (Schaefer & Orange 1956, Bagenal & Braum 1978).

By physically separating the oocytes before preservation in formalin, our method overcomes the problem of oocyte separation while maintaining the advantages of using formalin as a preservative. This method is inexpensive, quick, and much less toxic than Gilson's, providing researchers with undamaged oocytes of all stages, with little effect on appearance or size. This new method has been successfully used on weakfish and two other sciaenids (Atlantic croaker *Micropogonias undulatus*, and black drum *Pogonias cromis*; unpubl. data), and, given the similarity of teleost ovaries, should be applicable to a wide range of species. Additionally, because oocytes are preserved in a low concentration of formalin, similar to the preservation of most plankton samples, hydrated oocytes processed in this fashion would be comparable to those collected and preserved during plankton studies. This would make it possible to better link adult fish-reproduction studies with those from egg surveys.

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