**Abstract**—The lack of information concerning the preservation of ovarian material of fish species inhibits standardization of methods for determining fecundity and measuring oocytes. The effects of four preservatives (10% phosphate-buffered formalin, modified Gilson's solution, 70% ethanol, and freezing) on ovarian material weight and oocyte size were quantified for prespawning Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*), and American plaice (*Hippoglossoides platessoides*). Effects of preservation were similar between Atlantic cod and haddock but different between Atlantic cod and American plaice for nearly all comparisons. Although all treatments affected the weight of ovarian material, freezing caused the most change and formalin caused the least. Such significant species-specific effects should be quantified in the calculation of life history characteristics, such as fecundity, to minimize error. This is one of few studies dedicated to evaluating the effects of preservation on oocytes and ovarian material and is the first to evaluate multiple preservatives on species.

# Species-specific effects of four preservative treatments on oocytes and ovarian material of Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*), and American plaice (*Hippoglossoides platessoides*)

**Nikolai Klibansky**

**Francis Juanes (contact author)**

Email address for F. Juanes: juanes@forwild.umass.edu

Department of Natural Resources Conservation
University of Massachusetts
160 Holdsworth Way
Amherst, Massachusetts 01003

Because of the weak relationship between spawning stock biomass and stock reproductive potential (Marshall et al., 1998, 2003), stock assessment scientists recommend incorporating basic reproductive biology (such as fecundity) into estimates of stock reproductive potential. Estimating fecundity and other reproductive biological parameters often requires oocytes or ovarian material, preserved chemically or by freezing. Ovaries can sometimes be weighed fresh, but often, as when they are collected aboard ships where special marine scales are not available, they must be preserved until they are weighed in the laboratory.

Common preservatives can affect the size and weight of oocytes differently among species. Formalin can increase the mean diameter of catfish eggs by 4–11% (Tan-Fermin, 1991), and the mean diameter of cod eggs by 3.5% (Svaasand et al., 1996), but does not affect the weight of salmon eggs (Fleming and Ng, 1987). Common preservatives have also been shown to affect oocyte size of the same species differently in different studies. Schaefer and Orange (1956) found that standard Gilson's solution and formalin had the same effect on oocyte diameter of yellowfin (*Neothunnus macropterus*) and skipjack (*Katsuwonus pelamis*). In contrast, Joseph (1963) found the mean diameter of oocytes of these same tuna

species preserved in standard Gilson's solution to be 24% smaller than the mean diameter of oocytes preserved in formalin.

To enable accurate comparisons of fecundity information among populations and years, data should be collected and analyzed by a standard method and a standard preservative should be used for storing oocytes and ovarian material. But because little research has been conducted on the effects of preservatives on oocyte size, the selection of a preservative is usually based more on popular use than empirical knowledge—a process that may perpetuate the use of inferior preservatives and unnecessary toxins. Preservatives that have been used in past fecundity research may be adequate for determining fecundity but may not preserve ovarian material well enough for related analyses, particularly histology.

The three most commonly employed chemical treatments for ovarian material are 10% buffered formalin (3.7% formaldehyde), standard Gilson's solution, and freezing. Formalin and freezing are best used for short-term preservation (up to two years) of ovarian material; however, formalin is ideally employed as a fixative. Standard Gilson's solution also preserves ovarian material but was developed to dissolve the interstitial material that holds oocytes together

**Table 1**

Summary information for all Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*), and American plaice (*Hippoglossoides platessoides*) sampled on Georges Bank (GB) and in the Gulf of Maine (GOM). Maturity stage is based on Tomkiewicz et al. (2003a). Month = month of sampling; Jan = January, Feb = February, Mar = March, etc. $n$ = number of individuals sampled.

| Species | Month | Region | Spawning season (peak) | Maturity stage | $n$ |
|---------|-------|--------|------------------------|----------------|-----|
| Atlantic cod | Feb 04 | GB | Nov–May (Feb–Mar) | Ripening, stage IV | 19 |
| Haddock | Feb 04 | GB | Jan–May (Mar–Apr) | Ripening, stage IV | 16 |
| Atlantic cod | May 04 | GOM | Nov–May (Mar–May) | Ripening, stage IV | 23 |
| American plaice | May 04 | GOM | Feb–Jun (Apr–May) | Ripening, stage IV | 16 |

so they could then be counted and measured more easily (Simpson, 1951).

In this analysis, the effects of four preservatives on ovarian material weight and oocyte diameter of three species of commercially important Northwest Atlantic groundfish are evaluated. The preservatives are formalin (0.037 formaldehyde, 0.015 methyl alcohol, <0.01 sodium phosphate dibasic, <0.01 sodium phosphate monobasic, 0.93 deionized water), modified Gilson's solution (0.10 60% ethanol, 0.015 nitric acid, 0.008 glacial acetic acid, 0.88 distilled water), 70% ethanol, and freezing and are hereafter referred to simply as formalin, Gilson's, ethanol, and freezing. The three species used in this project included two gadids, Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*), and one pleuronectid, American plaice (*Hippoglossoides platessoides*). These species are hereafter referred to as cod, haddock, and plaice. All are historically important groundfish species in the Northwest Atlantic that have been reduced to low numbers over the past century (Boreman et al., 1997) and are now managed together in the Northeast multispecies fishery. Reproductive biology and ecology are similar among these species: they are all iteroparous, determinate, batch spawners exhibiting group-synchronous ovarian organization (Murua and Saborido-Rey, 2003). Although data are abundant for these species on many characteristics used to predict stock reproductive potential, such as maturity and sex-ratio, data on fecundity and oocyte size remain very limited (Tomkiewicz et al., 2003b).

## Materials and methods

### Ovary collection

All ovarian specimens were taken from fish caught in bottom trawls in the Northwest Atlantic during peak spawning (Table 1). In February 2004 the ovaries of 19 ripening (classified as stage IV by the maturity key developed by Tomkiewicz et al. [2003a]) cod and 16 haddock were collected by bottom trawling in the western portion of Georges Bank (GB). In May 2004 the ovaries of 23 ripening cod and 16 ripening plaice were collected by bottom trawling in the inshore waters of the Gulf of Maine (GOM).

### Preservation and measurement

For all specimens collected on GB (cod and haddock), fresh weight of the left ovary was measured at sea, to the nearest 1.0 g, with a Marel marine balance (Marel Food Systems, Gardabaer, Iceland). These ovaries were then preserved in formalin. Specimens collected in the GOM (cod and plaice) could not be weighed at sea and therefore were packed in ice until they could be weighed to the nearest 0.001 g in the laboratory 24 hours later. The left ovarian lobes of 17 cod caught in the GOM were reweighed after 48 hours on ice to determine if time on ice affected ovary weight.

Within minutes of weighing, the entire left lobe of most specimens was placed in a 1-L jar containing a volume of formalin approximately equal to four times the volume of the ovary. When the left lobe was too large (>250 g) to fit through the opening of the jar, a large portion weighing 250 g or less was cut off and weighed and preserved as above. These whole lobes and lobe portions of ovarian material remained in formalin for 158–175 (mean=169) days and were then reweighed to the nearest 0.001 g. These are hereafter referred to as lobe-formalin samples.

From the center of the right ovary of each fish, four 1.5-mL subsamples of ovarian material (i.e., one subsample per fish for each of four treatments: formalin, Gilson's, ethanol, and freezing) almost entirely comprising vitellogenic oocytes, were removed with a 3-mL plastic syringe tube, the end of which was cut off at the zero mark. The collection of subsamples from fresh ovaries in this way was very easy, gave no evidence of damaged oocytes, and repeatedly produced subsamples with a mean of 1.54 g ($n$=155, coefficient of variation [CV]=3.70). Across fish species, differences in oocyte size and density within and between left and right ovarian lobes are uncommon (West, 1990), and oocyte size tends not to vary among different locations in cod ovaries (Kjesbu and Holm, 1994). Still, in this study ovarian material was always taken from the same part of each ovary in order to ensure that subsamples of

oocytes removed from the same localized population of oocytes all had the same mean size and density. Each of these subsamples was then preserved in one of four ways, such that one subsample from each fish was placed in each treatment (i.e., one replicate per fish within a treatment). Only subsamples from specimens collected in May in the GOM (i.e., 23 cod and all 16 plaice) were weighed to the nearest 0.001 g before preservation.

In the formalin treatment (termed the sub-formalin treatment in weight comparisons to distinguish it from the lobe-formalin treatment), a subsample of ovarian material was placed in a vial (10-mL plastic vial with screw cap) containing 5 mL of formalin. Similarly in the Gilson's treatment, a subsample of ovarian material was placed in a vial containing 5 mL of modified Gilson's solution. This modified form of Gilson's solution was employed because it does not contain mercuric chloride, and is thus not toxic, but still effectively preserves and separates oocytes (Friedland et al., 2005). These samples were capped and shaken to ensure oocytes were all thoroughly immersed in the preservative, and were then stored upright in a rack at room temperature. In the ethanol treatment, a subsample of ovarian material was placed in a vial and immersed in 4 mL of 95% ethanol for approximately 15 seconds, before 1.5 mL of distilled water was added to the sample through a graduated pipette, before the solution was diluted to 70% ethanol. Samples in this treatment were also shaken and stored at room temperature. Briefly submerging the material in 95% ethanol is meant to act as a fixative, whereas 70% ethanol is better for long-term preservation (Black and Dodson, 2003). In the freezing treatment a subsample of ovarian material was immersed in a vial of distilled water. Because of our concern that the expansion with freezing too much water might crack the vials, 3 mL of water was used rather than 5 mL as in the other treatments. These samples were also shaken, but then placed (upright) in the freezer so that the ovarian material would be frozen solid in ice. The samples were frozen in this way to preserve the shape of the oocytes and to stop them from drying out or becoming freezer damaged.

Subsamples that were weighed fresh (i.e., those collected in May) were reweighed after 97–111 (mean=102) days of preservation and then returned to their preservative vials. To weigh a preserved subsample, the entire content of a vial was poured into a 40-$\mu$ mesh, nylon cell-strainer fitted atop a hand operated vacuum pump. Excess fluid was then removed from each sample by repeatedly squeezing the handle of the hand pump until preserved ovarian material was all that remained in the strainer. The strainer and its contents were then weighed to the nearest 0.001 g, and the known weight of the strainer was subtracted to find the preserved subsample weight.

In addition to the four treatments mentioned above, a fifth split-formalin treatment (so termed because preservation was split into two phases described below) was used for the left ovarian lobes. After 91–131 (mean=111) days of preservation in formalin, each left lobe was removed from its jar and a 1.5-mL subsample was removed from its center and placed in a vial containing 5 mL of formalin. Because they were not preweighed, these subsamples were used only for examining preservative effects on oocyte size. These subsamples remained in vials for another 33–92 (mean=63) days before they were analyzed. Including the total time that split-formalin samples were preserved, the time that samples from all treatments were preserved was 117–164 (mean=145) days before the mean oocyte diameter of each sample was estimated.

The method used here for measuring oocytes is largely based on the work of Thorsen and Kjesbu (2001) but is described here because of differences in details. To start, a vial was shaken vigorously for 30–60 seconds to break apart any oocytes still connected. Samples in modified Gilson's solution did not require shaking because the oocytes were already chemically separated. Most other samples broke apart very well from this shaking, especially those with larger oocytes. Samples in ethanol and samples containing the smallest oocytes were more difficult to break apart and required more shaking. After the vial was shaken, a transfer pipette was used to agitate the oocytes by the action of drawing in and expelling the solution rapidly, so that all oocytes were effectively mixed randomly in suspension and would not settle out by size. While the suspension was being agitated, a portion of the vial's contents was drawn and quickly emptied into a Petri dish containing ≈2 mm of the respective preservative and a drop of 5% Palmolive soap solution which helped the oocytes spread out in the dish and kept them from floating. Oocytes were then added to the Petri dish until the bottom of the dish was filled but so that the oocytes could still be spread out without clumping. A black lid was then placed on the dish to serve as a contrasting background, and an image of the sample was captured with a flatbed scanner. Each sample was scanned at 1200 dpi in 16-bit gray scale and a contrast setting of 18 with the use of an Epson Perfection 1670 scanner (Epson, Long Beach, CA). The same selection marquis was used for each sample so that all images were exactly the same size: 3688×3671 pixels, within a 12.9 MB uncompressed TIFF file.

Because samples in most treatments were stored at room temperature, no temperature adjustment was needed before they were scanned. For samples in the freezing treatment, however, care was taken to be sure that vials were thawed one hour before they were scanned out of concern that oocyte diameter may be affected by how much time thawed samples were left in water.

Once scanned, each image was analyzed in the freeware program Scion Image (Scion Image, version beta 4.0.2, Scion Corporation, Frederick, MD) by first setting the scale of each image to 1200 pixels per inch (472 pixels per cm), and the measurements to micrometers. The lower limit of the density slice tool was then set to 40 and the upper limit ranged from 180 to 205, and was typically 195. The upper limit varied because the grey value of the oocyte margins varied between samples depending on how dark the oocytes were. The Scion

Image software used those grey values to determine the perimeter of the particles; therefore standardizing the upper limit of the density slice among samples would result in less accurate measurements. Then the "analyze particles" command was run on particles from 150 to 1500 pixels in area. The process was also set to include interior holes and to ignore particles touching the perimeter as the oocytes were measured.

The output of this process produced four columns of data: area, perimeter, major axis length, and minor axis length for each particle. These data were then transferred to a Microsoft Excel spread sheet (Microsoft® Office Excel 2003, Microsoft Corporation, Redmond, WA), where a macro was run to filter out measurements of non-oocytes by accepting only particles within a narrow range of roundess values. The macro then calculated the mean oocyte diameter of each sample, as well as other descriptive statistics, and produced a percent frequency histogram for the sample of roundness-filtered particles.

Ideally all samples would have been analyzed after the same amount of time that they had been preserved, although it was not practical to do so. Thus time (days) preserved was recorded for each sample and the average time that the sample was preserved was compared between groups where appropriate.

## Statistical analyses

The preserved weight of a lobe or subsample of ovarian material was compared to its fresh weight. Percent change in weight was calculated with Equation 1:

$$\%\Delta_{Wt} = \left( (Wt_{Preserved} - Wt_{Fresh}) / Wt_{Fresh} \right) \times 100, \quad (1)$$

where $\%\Delta_{Wt}$ = the percent change between $Wt_{Preserved}$ and $Wt_{Fresh}$;

$Wt_{Preserved}$ = preserved weight of a sample of ovarian material; and

$Wt_{Fresh}$ = fresh weight of that sample.

A positive $\%\Delta_{Wt}$ indicated an increase in weight due to preservation.

It was not logistically possible to measure the diameters of fresh oocytes; therefore it was necessary to use one of the preservative treatments as a control treatment. The formalin treatment was chosen for this purpose, because it is a standard preservation method and thus was expected to have the most consistent effect on oocyte size. In this experiment, the other four treatments (Gilson's, ethanol, freezing, and split-formalin) were considered experimental treatments. Change in mean oocyte diameter due to preservation in the experimental treatments was quantified by using Equation 2:

$$\%\Delta_{OD} = \left( (OD_{Experimental} - OD_{Control}) / OD_{Control} \right) \times 100, \quad (2)$$

where $\%\Delta_{OD}$ = the percent difference in mean oocyte diameter between $OD_{Experimental}$ and $OD_{Control}$;

$OD_{Experimental}$ = the mean oocyte diameter of a subsample of an ovary preserved in one of the four experimental treatments; and

$OD_{Control}$ = the mean oocyte diameter of a subsample of the same ovary preserved in the formalin treatment.

A positive $\%\Delta_{OD}$ indicates that the mean oocyte diameter of a subsample in the experimental treatment is larger than in the formalin treatment.

For examining $\%\Delta_{Wt}$ and $\%\Delta_{OD}$, samples were grouped by experimental treatment within species, and then in the case of cod where samples were collected from two regions, the samples were further grouped by region. $T$-tests were conducted within these groups to test the null hypothesis ($H_0$) that $\%\Delta = 0$ for each experimental treatment. Assessment of normality of each group by examining boxplots and histograms indicated that data did not require transformation. Statistical analyses were performed with SAS (version 9.1, SAS Institute Inc., Cary, NC). Each test had $n_1 + n_2 - 2$ degrees of freedom (Tables 2 and 3) where $n_1$ and $n_2$ were the total numbers of observations from each group. Where t-tests were conducted in groups, significance ($\alpha$) levels were adjusted by the sequential Bonferroni procedure (Quinn and Keough, 2002), to minimize family-wise type-I error rate.

When a significant difference in $\%\Delta_{OD}$ was found between two groups, time preserved was investigated as a confounding factor, although any major change in oocyte size due to preservation typically happens within one day (Kjesbu et al., 1990). A $t$-test of time preserved between the groups was conducted in which the ratio of time preserved in experimental treatment to time preserved in control treatment was used as a metric of time preserved, because the calculation for $\%\Delta_{OD}$ includes $OD_{Control}$ in the denominator (see Eq. 2). If no significant difference was found, we concluded that the difference in $\%\Delta_{OD}$ was not due to time preserved. If a significant difference was found, a one-tailed $t$-test of $\%\Delta_{OD}$ between these groups was conducted. Assuming that the sign of the slope of the relationship between time preserved and oocyte diameter is constant after the initial changes that occur within days of preservation, the group with a higher time preserved value should have exhibited a greater $|\%\Delta_{OD}|$. Thus the $H_0$ for each one-tailed $t$-test was: |mean $\%\Delta_{OD}$| of group preserved for more time ≤ |mean $\%\Delta_{OD}$| of group preserved for less time. If this analysis implicated time preserved as a likely cause of a significant difference in $\%\Delta_{OD}$, then the effect of the experimental treatment would be considered confounded.

In situations where there was a significant difference in $\%\Delta_{OD}$ and time preserved, but the sign of $\%\Delta_{OD}$ was different between the groups, no such test was performed. Instead it was concluded that the difference in $\%\Delta_{OD}$ was not due to time preserved, based on the assumption that the sign of the slope of the relationship between time preserved and oocyte diameter is constant.

**Table 2**

The effects of method and treatment on ovary weight among the species Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*), and American plaice (*Hippoglossoides platessoides*), within the region Georges Bank (GB), the Gulf of Maine (GOM) or within both regions combined (Combined), and between regions for Atlantic cod. *T*-tests compare percent change in fresh weight among species within each treatment group. The methods column designates whether ovaries were used as 1.5-mL subsamples (Sub) or as entire lobes (Lobe). df = degrees of freedom, *t* = *t*-statistic, * = statistically significant at the appropriate Bonferroni adjusted $\alpha$-value.

| Species | Method | Region | Treatment | $t$ | df | $P$-value | $\alpha$-value[1] |
|---|---|---|---|---|---|---|---|
| Atlantic cod | Sub | GOM | Formalin | −0.24 | 38 | 0.81 | 0.05 |
| American plaice | Sub | GOM | Gilson's | 3.05 | 37 | 0.0042* | 0.025 |
| | | | Ethanol | −9.61 | 37 | <0.001* | 0.013 |
| | | | Freezing | 7.47 | 32 | <0.001* | 0.017 |
| Atlantic cod | Lobe | GB | Formalin | −3.73 | 40 | <0.001* | 0.05 |
| Atlantic cod | Lobe | GOM | | | | | |
| Atlantic cod | Sub | GOM | Formalin | 6.06 | 44 | <0.001* | 0.05 |
| Atlantic cod | Lobe | GOM | | | | | |
| Atlantic cod | Lobe | GB | Formalin | −1.19 | 36 | 0.24 | 0.05 |
| Haddock | Lobe | GB | | | | | |
| Atlantic cod | Lobe | Combined | Formalin | 1.33 | 59 | 0.19 | 0.05 |
| Haddock | Lobe | GB | | | | | |

[1] $\alpha$-values lower than 0.05 were adjusted by the sequential Bonferroni procedure for multiple comparisons (Quinn and Keough, 2002).

Similar analyses were conducted when a significant difference in $\%\Delta_{Wt}$ was identified between two groups. For these comparisons, however, days preserved was used as the metric of time preserved, since fresh weights were taken for all these samples.

## Results

### Effect of preservation on weight of ovarian material

All four experimental treatments had significant effects on subsample weight for cod and plaice (Fig. 1), except for plaice samples in the freezing treatment ($P=0.12$), which also had the highest standard error (SE) of $\%\Delta_{Wt}$ (SE=4.93). For cod, standard error of $\%\Delta_{Wt}$ was also highest in the freezing treatment (SE=3.37) along with mean $\%\Delta_{Wt}$ (mean=51.32). Mean $\%\Delta_{Wt}$ was lowest for cod in the ethanol treatment (mean=−4.21) but highest for plaice (mean=25.3). In the lobe-formalin treatment there was a significant difference in $\%\Delta_{Wt}$ for GB cod ($P=0.036$), GOM cod ($P<0.001$), all cod combined ($P<0.001$), and haddock ($P=0.002$). These data were not available for plaice.

Mean $\%\Delta_{Wt}$ was not significantly different between cod and haddock in the lobe-formalin treatment regardless of whether haddock was compared with cod samples restricted to GB ($P=0.24$, $\alpha=0.03$) or when all cod were combined ($P=0.28$, $\alpha=0.05$). Mean $\%\Delta_{Wt}$ of subsamples was significantly different between cod and plaice for the ethanol ($P<0.001$), freezing ($P<0.001$), and Gilson's ($P=0.0042$) treatments (Table 2). There was no difference in $\%\Delta_{Wt}$ between cod and plaice in the subformalin treatment ($P=0.81$). This test marks the only comparison between cod and plaice where no difference was found. There was a significant difference in mean $\%\Delta_{Wt}$ ($P<0.001$) for cod between GB (mean=1.41) and the GOM (mean=6.79) in the lobe-formalin treatment. For cod from the GOM, there was a significant ($P<0.001$) and nearly threefold difference in mean $\%\Delta_{Wt}$ between lobe-formalin (mean=6.79) and sub-formalin (mean=19.32) treatments.

For the 17 ovaries collected from cod caught in the GOM, all of which were weighed after 24 and then 48 hours on ice (fresh weights were not available), mean percent change in weight during this interval was significantly different from zero ($P=0.02$). However this change was very slight (mean=−0.32, SE=0.11).

### Effect of preservation on oocyte diameter

Oocyte diameter in the experimental treatment was significantly different from that of the control treatment (Fig. 2), in all but three comparisons. Significant differences were not detected for plaice ($P=0.22$) in the ethanol treatment or for GB cod ($P=0.59$) or haddock ($P=0.37$) in the split-formalin treatment. Of the four experimental treatments, the freezing treatment produced the highest mean $\%\Delta_{OD}$ for GB cod (mean=18.81), GOM cod (mean=26.99), GB and GOM cod combined (mean=23.29), and haddock (mean=28.88). Plaice samples in the freezing treatment also exhibited a significant positive change ($P<0.001$, mean=9.90), but a larger change was exhibited in the Gilson's treatment

**Table 3**

The effects of experimental treatment (Gilson's, ethanol, freezing, and split-formalin) on oocyte diameter among the species Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*), and American plaice (*Hippoglossoides platessoides*) within the regions of Georges Bank (GB), the Gulf of Maine (GOM), or within both regions combined (Combined), and between regions for Atlantic cod. *T*-tests were used to compare mean percent difference[1] in oocyte diameter between experimental and control (formalin) treatments among species. *t* = *t*-statistic, df = degrees of freedom, * = statistically significant at the appropriate Bonferroni adjusted $\alpha$-value.

| Species | Region | Treatment | *t* | df | *P*-value | $\alpha$-value[2] |
|---|---|---|---|---|---|---|
| Atlantic cod | GB | Gilson's | 5.19 | 40 | <0.001* | 0.013 |
| Atlantic cod | GOM | Ethanol | −1.79 | 40 | 0.082 | 0.05 |
| | | Freezing | −3.91 | 37 | <0.001* | 0.025 |
| | | Split-Formalin | −4.21 | 39 | 0.001* | 0.017 |
| Atlantic cod | GB | Gilson's | 2.49 | 33 | 0.018 | 0.017 |
| Haddock | GB | Ethanol | −2.09 | 33 | 0.045 | 0.025 |
| | | Freezing | −2.62 | 33 | 0.013 | 0.013 |
| | | Split-Formalin | −1.11 | 32 | 0.28 | 0.05 |
| Atlantic cod | Combined | Gilson's | 0.36 | 56 | 0.72 | 0.05 |
| Haddock | GB | Ethanol | −1.75 | 56 | 0.085 | 0.017 |
| | | Freezing | −1.92 | 53 | 0.06 | 0.013 |
| | | Split-Formalin | 0.72 | 54 | 0.48 | 0.025 |
| Atlantic cod | GOM | Gilson's | 2.84 | 37 | 0.0073* | 0.05 |
| American plaice | GOM | Ethanol | −5.39 | 37 | <0.001* | 0.017 |
| | | Freezing | 11.40 | 32 | <0.001* | 0.013 |
| | | Split-Formalin | 3.41 | 36 | 0.002* | 0.025 |
| Atlantic cod | Combined | Gilson's | 4.33 | 56 | <0.001* | 0.013 |
| American plaice | GOM | Ethanol | −5.74 | 56 | <0.001* | 0.025 |
| | | Freezing | 6.18 | 51 | <0.001* | 0.017 |
| | | Split-Formalin | 2.98 | 55 | 0.0043* | 0.05 |

[1] Defined by the following: (*TreatmentDiameter*−*FormalinDiameter*) × 100), where *TreatmentDiameter* = the mean oocyte diameter of a subsample of an ovary preserved in one of the experimental treatments and *FormalinDiameter* = the mean oocyte diameter of a subsample of the same ovary preserved in the formalin treatment.

[2] $\alpha$-values lower than 0.05 were adjusted by the sequential Bonferroni procedure for multiple comparisons (Quinn and Keough, 2002).

(mean=−18.43). Of the four experimental treatments, the split-formalin treatment produced the smallest mean $\%\Delta_{OD}$ for GB cod (mean=−0.31), GOM cod (mean=2.90), GB and GOM cod combined (mean=1.41), and haddock (mean=0.77). Plaice samples in the split-formalin treatment also exhibited only a small change (mean=−2.59), but the smallest change (mean=0.94) was in the ethanol treatment.

Mean $\%\Delta_{OD}$ was not significantly different between cod and haddock for any treatment (Table 3), regardless of whether haddock samples were compared with cod samples restricted to GB or when cod from GB and GOM were combined. Mean $\%\Delta_{OD}$ was significantly different between cod and plaice for all treatments, also regardless of whether cod samples were restricted to GB or when all cod were combined. There was a significant difference in mean $\%\Delta_{OD}$ for cod from GB and cod from the GOM in the Gilson's (*P*<0.001, $\alpha$=0.013), freezing (*P*<0.001, $\alpha$=0.025), and split-formalin (*P*=0.001,

$\alpha$=0.017) treatments, but not the ethanol treatment (*P*=0.08, $\alpha$=0.05).

In the comparison between lobe-formalin and subformalin samples, analyses showed time preserved could not have caused the difference in preserved weight between these groups. Although significant differences in $\%\Delta_{Wt}$ of subsamples were detected between cod and plaice in the Gilson's, ethanol, and freezing treatments, time preserved between species was consistent for all treatments.

There were only five comparisons where it was appropriate to investigate time preserved as a possible cause for differences in $\%\Delta_{OD}$ between two groups. These comparisons were the following: cod from GB and GOM in the Gilson's, freezing, and split-formalin treatments, and cod (GB and GOM combined) and plaice, in the Gilson's and freezing treatments. The $H_0$ that mean $\%\Delta_{OD}$ of group preserved longer ≤ mean $\%\Delta_{OD}$ of group preserved shorter was only rejected in the comparison

between cod (GM and GOM combined) and plaice in the freezing treatment ($P<0.001$).

For four haddock samples in the freezing treatment, mean oocyte diameter was measured immediately after the sample was freshly thawed, and then again after 48 hours of refrigeration. Mean oocyte diameter of the samples decreased by 3–10% after refrigeration, although the average decrease of 4.4% was not significantly different from zero ($P=0.11$), perhaps because of the small sample size.

## Discussion

Of the preservatives tested in this study, the results with formalin were most similar among individuals and species, and the results from freezing were the least



**Figure 1**

Percent difference in ovarian material weight ($\%\Delta_{Wt}$) for each species (Atlantic cod [*Gadus morhua*]), haddock [*Melanogrammus aeglefinus*], and American plaice [*Hippoglossoides platessoides*]) and region, grouped by treatment. For Atlantic cod, observations are displayed together in the combined group and are then displayed separately by region (i.e., Georges Bank or the Gulf of Maine). The horizontal line at zero on the vertical axis indicates values where the percent difference in weight between preserved and fresh ovarian material was equal to zero. Circles indicate outliers. Asterisks indicate that mean $\%\Delta_{Wt}$ was significantly different from zero at the appropriate Bonferroni adjusted $\alpha$-value. Box length is equal to the interquartile range which contains the central 50% of the observations (25% above and below the median). Whiskers extend 1.5 box-lengths above and below the limits of the box.
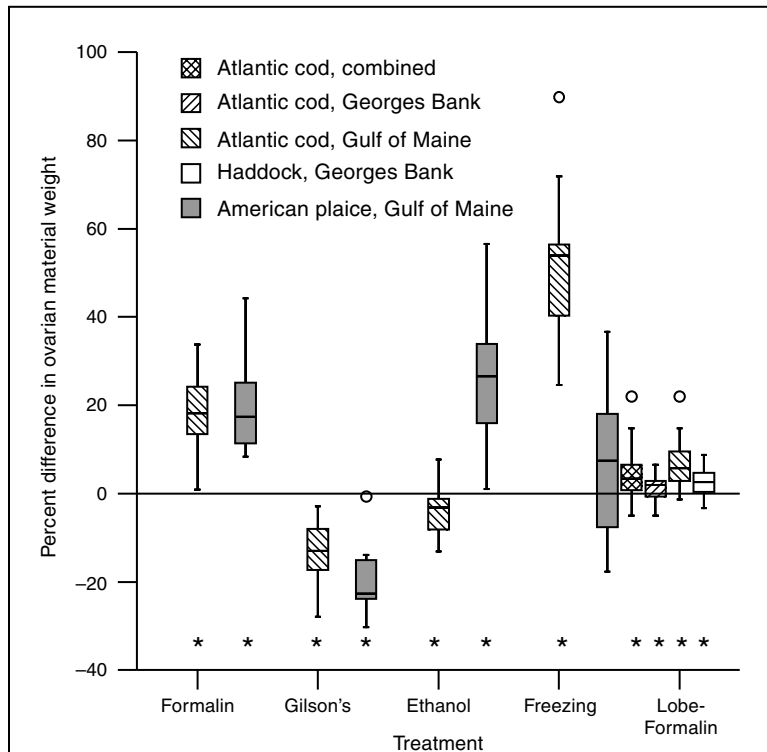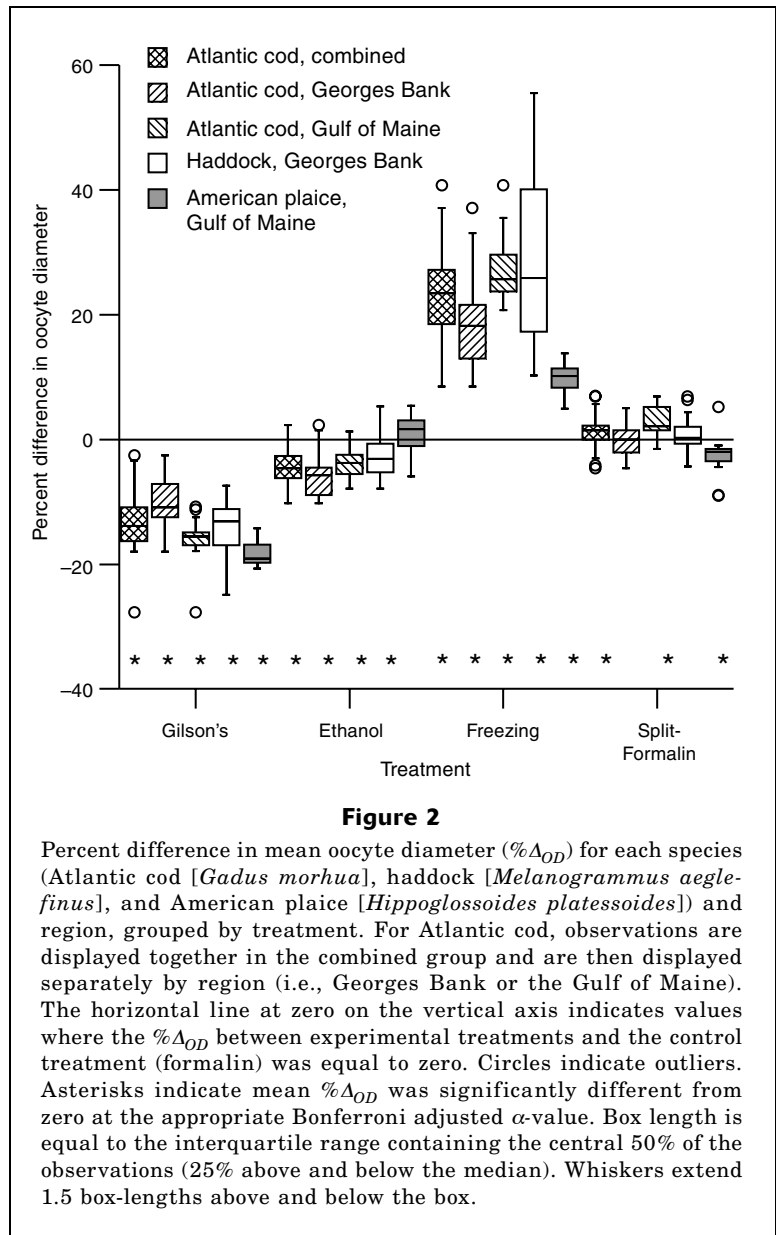
consistent among individuals within and among species. In terms of image quality, samples preserved in formalin were clearest and contained the least debris. In weight comparisons between cod and plaice, the subformalin treatment was the only one where no significant difference between the two species was found. Of the tested preservatives, the best option for a standard preservative was formalin, especially when samples might be used for histology, where postovulatory follicles and fine cellular structures must be preserved.

When realized fecundity is estimated from total potential fecundity, estimates may be biased if samples are collected too late in the season and spawning has already begun, or if atresia is likely to occur between sampling and the time of spawning. Both of these biases are quantified through histological analysis. In species or stocks where the developing stage is difficult to identify or where high rates of atresia are expected, histological analysis is necessary to assure that estimates of realized fecundity are accurate. Although Gilson's solution and ethanol may be used for fecundity research and may be superior in certain situations for particular species, they do not preserve tissue well enough for histological analysis, and thus they are undesirable in many situations.

Formalin is a common preservative for ovarian material and is widely used to fix and preserve animal tissue. Although our results show differences between formalin samples and Gilson's and frozen samples that contrast with results of studies on other species, the effects of formalin preservation were very consistent among the species we studied. Formalin preservation resulted in an increase in sample weight in all species, and when compared between species these changes were similar.

The differences between the formalin and split-formalin treatments for all species (Fig. 2), although significant for GB and GOM cod combined, GOM cod, and plaice, were quite small and therefore indicated that formalin preserves oocyte size similarly whether ovarian material is in large membrane-bound lobes or in 1.5-mL subsamples. However, the large and significant difference in $\%\Delta_{Wt}$ of samples of GOM cod ovarian material between the lobe-formalin and subformalin treatments indicates a conflict because oocyte diameter and ovarian material weight are inherently related. If ovarian material weight increases much more in one treatment than another, oocyte size should as well. The reason for this disagreement may lie in the fact that after subsamples in the split-formalin treatment were removed from whole preserved lobes, they were preserved as subsamples for several weeks before the

oocytes were measured. During that time, swelling that was inhibited by the dense packing of oocytes in the ovarian lobe may have occurred; therefore by the time they were measured, oocytes were very similar in size to those preserved in the sub-formalin treatment.

Standard Gilson's solution has been a common oocyte preservative since it was developed. Joseph (1963) found that tuna oocytes preserved in standard Gilson's solution were 24% smaller than those preserved in 4% formalin. In contrast Schaefer and Orange (1956) concluded that mean diameters of oocytes from the same tuna species, preserved in standard Gilson's solution and formalin (concentration not specified) were similar, although they did not present statistical evidence to support this conclusion. Cod, haddock, and plaice oocytes preserved in modified Gilson's solution were 13.28%, 13.77%, and 18.43% smaller, respectively, than those preserved in 10% formalin. These results were all significant, and are similar to Joseph's (1963) results. The discrepancy between our results and those of Joseph (1963) could be attributed to interspecies variation. Although differences were identified, we consider the effects of modified Gilson's solution to be fairly consistent among species.

Ethanol is rarely used to preserve ovarian material used in fecundity studies, but because Black and Dodson (2003) used ethanol to successfully fix and preserve water fleas (*Daphnia pulex*) and their eggs, and ethanol caused less distortion and change in body size than a solution of sucrose and 4% formalin, it was evaluated in this study. Oocytes preserved in this treatment were of sufficient quality for use in digital image analysis. Ethanol also had a bleaching effect on oocytes, which resulted in greater contrast between oocytes and the black background. The ethanol treatment tended to cause smaller changes to oocyte size and ovarian material weight than other treatments, except for the large increase in subsample weight in plaice. In ethanol, $\%\Delta_{Wt}$ was negative for cod, $\%\Delta_{OD}$ was negative for cod and haddock, and both were positive for plaice. Such results make it difficult to generalize about the effects of ethanol on ovarian material across taxa and may make this treatment less desirable than others.

Freezing is one of the most common methods of ovary preservation, but this treatment has not been used to preserve oocytes for digital image analysis. Although freezing was expected to result in many ruptured oocytes, oocytes in the frozen treatment maintained a very round shape and rarely broke even after vigorous shaking and agitation. Freezing the oocytes solid in distilled water may have improved the ultimate quality



**Figure 2**

Percent difference in mean oocyte diameter ($\%\Delta_{OD}$) for each species (Atlantic cod [*Gadus morhua*], haddock [*Melanogrammus aeglefinus*], and American plaice [*Hippoglossoides platessoides*]) and region, grouped by treatment. For Atlantic cod, observations are displayed together in the combined group and are then displayed separately by region (i.e., Georges Bank or the Gulf of Maine). The horizontal line at zero on the vertical axis indicates values where the $\%\Delta_{OD}$ between experimental treatments and the control treatment (formalin) was equal to zero. Circles indicate outliers. Asterisks indicate mean $\%\Delta_{OD}$ was significantly different from zero at the appropriate Bonferroni adjusted $\alpha$-value. Box length is equal to the interquartile range containing the central 50% of the observations (25% above and below the median). Whiskers extend 1.5 box-lengths above and below the box.

of the samples by limiting the exposure of oocytes to the oxidizing and desiccating effects of air. Although the quality of the images obtained from these samples was high enough for measurement and counting, percent change in ovarian material weight and oocyte diameter of frozen specimens proved to be very variable and often very high (Figs. 1 and 2).

Although the mean diameter of cod, haddock, and plaice oocytes preserved in the freezing treatment was 23.29%, 28.88%, and 9.90% larger, respectively, than the mean diameter of oocytes in the formalin treatment, Ramon and Bartoo (1997) found that the mean diameter of tuna eggs in 10% buffered formalin was generally 5–10% larger than the mean diameter of oocytes from frozen ovaries. It may not be surprising to find a difference between the species we studied and the tunas, but

such a substantial difference showing an opposite trend is somewhat surprising and is probably largely due to the difference in freezing methods. Although freezing ovarian material in water may improve the quality of the preserved oocytes, it is probably responsible for the large and variable changes that we observed. Because ovarian material is placed in hypotonic distilled water, it will absorb the water, thus increasing the size and weight of the oocytes. If it takes a long time for ovarian material in water to become isotonic, the material may not be osmotically stable by the time it is frozen and even by the time the oocytes are measured, which could cause the observed changes and variation in size and weight. In future research ovarian material should be frozen in an isotonic solution to prevent oocytes from swelling.

The initial reason ovary samples were preserved in a variety of treatments was to determine if oocytes preserved by these methods could be used in digital image analysis. The only studies we are aware of that employ digital image analysis to count and measure fish oocytes preserve them in either formalin (Thorsen and Kjesbu, 2001; Yoneda and Wright, 2004) or modified Gilson's solution (Friedland et al., 2005). All treatments in our study preserved ovarian material of cod, haddock, and plaice well enough to permit easy identifcation and measurements of oocytes. Clumping was a factor for many samples in all but the Gilson's treatment, but most clumps could be broken up well by shaking the vial for 30–60 seconds. Very tight clumps could be broken apart by rapidly drawing in and expelling oocytes and solution for 30–120 seconds with a 10-mL glass pipette with an ≈1 mm diameter and a plastic, thumb-wheel pipette pump. A glass pipette worked better than a plastic transfer pipette, probably because its rigidity allows more suction to be created as fluid is quickly drawn into it. With simple, albeit occasionally time-consuming (up to five minutes) mechanical separation, all clumps could be sufficiently broken up so that the oocytes could be measured and counted.

Considering all comparisons for all three species, cod and haddock oocytes and ovarian material are affected similarly by a given type of preservative, whereas effect on plaice tends to differ with different preservatives. This is not unexpected because cod and haddock are in the same family, Gadidae, whereas the narrowest taxonomic group common to cod and plaice is the subdivision Euteleostei. One would expect tissues of closely related species would have similar chemical properties, and should thus be affected similarly by preservatives. Although it could be inferred from results among studies that differences in preservation exist among fish species, specific differences have not been reported before the present study.

A difference in the effect of preservation between cod samples from GB and those from GOM is also reported. This difference is unexpected but is supported by the results of comparisons of oocyte size in the Gilson's, freezing, and split-formalin treatments, and by the comparison of ovarian material weight in the lobe-formalin treatment. Samples from GB were collected in February, whereas samples from the GOM were collected in May, which may somehow contribute to this difference. Still, it is unclear why time of year or region of sample collection would affect preservation and we suggest this as an area worthy of future research.

We successfully evaluated and quantified the effects of several preservatives on the ovarian material of several fish species, but perhaps more importantly we demonstrated how preservation can add variation to seemingly simple measurements like ovary weight and oocyte size, and can have different effects between species. Thus we stress the importance of consistent experimental methods and suggest that in studies of preserved ovarian material it should not be assumed that the effects of different preservatives and preservation treatments are consistent.

## Acknowledgments

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