Mediterranean Marine Science

Vol 16, No 3 (2015)

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doi: 10.12681/mms.1205

Short Communication

Mediterranean Marine Science
Indexed in WoS (Web of Science, ISI Thomson) and SCOPUS
The journal is available on line at http://www.medit-mar-sc.net
DOI: http://dx.doi.org/10.12681/mms.1205

Assessing species and stage-specific effects of preservation on fish oocytes over different temporal scales

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Handling Editor: Konstantinos Tsagarakis

Received: 13 January 2015; Accepted: 15 June 2015; Published on line: 7 September 2015

Abstract

This study assessed the effect of 10% neutral buffered formalin and three ethanol solutions of different concentration on Mediterranean sardine and European anchovy oocytes over several temporal scales (days, weeks, months). The two species exhibit differences both in the elemental composition and the shape of their oocytes, which allowed an appraisal of oocyte shrinkage dynamics in relation to oocyte shape, developmental stage and composition. We showed that the effect of the preservative on oocyte size is stage-specific while different preservation periods of ovarian material might lead to discrepancies among studies.

Keywords: sardine, anchovy, preservation protocols, spawning frequency, fecundity, reproductive biology.

Introduction

Oocyte size is a variable of increasing importance in fish reproduction studies. Given that the processing of ovarian material usually involves fixation and preservation, knowledge of the exact effect of the employed protocols is important in order to avoid bias in measurements of oocyte size and to make results between different studies and laboratories comparable (Heins & Baker, 1999; Klibansky & Juanes, 2007). Formalin-based fluids such as 10% neutral buffered formalin (BF-10) and Bouin's fluid are most commonly used for microanatomical work in fish reproduction studies since both are suitable for gonadal histology and fecundity measurements. However, formalin is known to cause a number of health issues to laboratory workers.

Alcohol solutions such as ethanol (EtOH; e.g. Klibansky & Juanes, 2007) or isopropanol (e.g. Heins & Baker, 1999) provide a comparatively good alternative to formalin since they are almost non-toxic. The main problem with alcohol solutions such as 70% EtOH (EtOH-70), which is one of the most common preservatives of animal tissue, is that they can cause severe tissue dehydration and could thus be inappropriate for methods that require accurate measurements of gonad and/or oocyte size. Moreover, other ethanol-based, formalin-free protocols such as the toxic Gilson's fluid or its modified non-toxic alternative proposed by Friedland *et al.* (2005), which are used to separate oocytes from ovarian tissue for fecundity measurements, were also shown to cause significant oocyte shrinkage (Friedland *et al*., 2005; Klibansky & Juanes, 2007).

The main objective of this study was to test the efficiency of EtOH-70 and two other EtOH solutions of lower concentration (25 and 50%) as preservatives of ovarian material for correct assessment of oocyte size in the Mediterranean sardine, *Sardina pilchardus*, and the European anchovy, *Engraulis encrasicolus*. The two species display similar reproductive biology but have oocytes of different shape and elemental composition, with anchovy oocytes being exceptionally oval and not-containing oil droplets (Ganias *et al*., 2014). As a consequence, this study further evaluated whether both short- and long-term preservation treatments affect oocytes of various shape, size, stage and elemental composition in different ways.

Material and Methods

Samples of reproductively active anchovy and sardine were collected in July-August 2010 and in October-November 2010, respectively, from a small purse seine vessel operating in Thermaikos Gulf, Northern Greece. Just after landing, samples were fixed in BF-10 and were transferred to the laboratory. After ca. 3 hours in the fixative, the BF-10 was carefully drained and the sample was subjected to moderate water flow for 2 extra hours. Individuals were then sexed and the maturity stage of females was scored macroscopically. Maturity observations continued until the hydration stage was represented by at least 3 individuals. We analyzed a large number of vitellogenic ovaries in order to cover a wide range of oocyte sizes. A total of 20 anchovies (3 hydrated) and 25

sardines (5 hydrated) were selected and used for subsequent analysis.

At the beginning of the trial period, four subsamples were dissected from each ovary, placed in small Petri dishes containing isotonic solution and subjected to moderate stirring for a few minutes for the oocytes to separate from each other. Then, subsamples were photographed using a PROgress C3 camera attached to a stereomicroscope. This procedure was repeated for all specimens in order to obtain oocyte measurements under the same conditions. Three of the subsamples were then removed and stored in small vials containing ethanol solutions of 25%, 50% and 70% each (EtOH-25, EtOH-50 and EtOH-70 respectively). The fourth subsample was kept in a vial containing BF-10 and was used as control. We adopted this scheme following the usual survey protocol, i.e. fixation in BF-10 and preservation either in BF-10 or EtOH.

The next day, oocytes from each vial were transferred to a Petri dish pre-filled with isotonic solution and photographed using the aforementioned procedure. Subsamples were placed in isotonic solution and not in the solution of origin due to concerns that lamp heating could cause uncontrolled alterations to the preservative and oocyte turbulence due to evaporation. Moreover, different solutions exhibit different optical density properties, and thus different absorbance spectra, which could affect oocyte measurements. For both species, this procedure was repeated almost daily for one week and then every 3-5 days until a trial period of 30 days for anchovy and 25 days for sardine was covered. The idea behind this temporal design was to identify the crucial changes in oocyte size, which usually occur during the first days of preservation, while oocyte size tends to stabilize thereafter (Witthames & Greer Walker, 1987). During the entire trial period, ovarian subsamples where kept in a dark room, under stable temperature conditions.

A replicate series of measurements was taken for the EtOH-25 subsamples in July 2011, i.e. almost 10 months after the trial period for anchovy and 7 months after the trial period for sardine. This extra series of measurements aimed at assessing the potential long-term effect of this preservation treatment on oocyte size.

A large archive of more than 1800 digital photomicrographs was created. To avoid measuring oocyte size either directly under the stereomicroscope or manually on each digital photomicrograph and thus reduce the workload, we employed an automated procedure, described in Ganias *et al*. (2010) using ImageJ (rsbweb.nih.gov/ij). A slightly different procedure was used for measuring anchovy oocytes, which were often "broken", using the aforementioned routine due to their extreme oval shape. Specifically, after conversion of the image type to 8-bit, the *Watershed Segmentation* plug-in was used (bigwww.epfl.ch/sage/soft/watershed/) through which both thresholding and segmentation of particles are performed after finding an optimum blurring algorithm (Gaussian blurring), which enhances the results of particle analysis.

Both species display multimodal oocyte size frequency distributions, each mode corresponding to succeeding batches of oocytes. To avoid differential contribution of different oocyte batches to the averaged oocyte size values, measurements were restricted to the spawning batch (see Ganias *et al*., 2010). This procedure led to one value of mean oocyte area per day *t*, species *i,* and preservation treatment *j* (OS_{tij}, in mm^2).

Mean oocyte size values on day-1 of the trial period (OS_{1ij}) did not differ significantly between the specimens of the two species (*t*-test: P>0.05). As a first step in our analysis we computed the 100% difference of each OS_{tij} from its respective value on day-1, OS_{1ij}, using the formula:

$$\%\Delta OS = \frac{OS_{tij} - OS_{1ij}}{OS_{1ij}} * 100 \quad (1)$$

Averaged $\%\Delta OS$ values (and 95% CIs) were then estimated for each 5days / species / preservation-treatment group. This analysis provided a first gross figure showing the effect of each preservation treatment on the size of the oocytes of the two species over time. However, the integrity of this analysis was rather poor since it did not allow to test the effect of each covariate (*day*, *species* and *maturity stage*) or their interactions on the time trends of oocyte size data.

Since measurements of oocyte size from each ovarian subsample were taken repeatedly, our study was based on a longitudinal experimental design. As a consequence, OS_{tij} data were modelled as a function of the covariates using linear mixed effects models (LMEMs). To analyze how OS_{tij} depends on the covariates over time, subsamples were treated as random effects whilst the day of measurements (*DAY*), species (*SP*), maturity stage (*MAT*: hydrated or not), and the interactions *DAY*SP* and *DAY*MAT* were treated as fixed effects. *DAY* was treated as a continuous variable whilst *SP* and *MAT* as discrete variables. A total of four LMEMs were constructed, one for each preservation treatment (BF-10, EtOH-25, EtOH-50 and EtOH-70). Quantile and residual inspection plots revealed that a Gaussian error structure model with an identity link was the most appropriate for the analysis of the OS_{tij} data. All data were analyzed using R v.3.1.0 (R Development Core Team, 2014) and the R package lme4 v. 0.999375-42. Upper-bound *p*-values for the analysis of variance (ANOVA) were computed using the pamer.fnc. function of the LMER Convenience Functions package. The bfFixefLMER_F.fnc function was used to back-fit the initial LMEMs on upper- bound p-values; the significance level for ANOVA was set at p=0.05.

Results

Figure 1 summarizes the effects of the four preservation treatments on the oocyte size of the two species. It is clearly evident that EtOH-50 and EtOH-70 caused sig-

nificant shrinkage both in anchovy and sardine oocytes. However, whilst in both solutions oocyte size in anchovy tended to stabilize after the first two weeks of treatment, shrinkage in sardine seemed to continue until the last days of the 30-day trial period. *%ΔOS* values for EtOH-50 and EtOH-70 at the end of the trial period were -15.4% (CI: ±3.9%) and -21.2% (CI: ±3.8%), respectively, for anchovy and -24.8% (CI: ±3.2%) and -34.8% (CI: ±3.4%), respectively, for sardine. For EtOH-25, the pattern between two species was even more differentiated; whilst in sardine there was a clear shrinkage of oocytes (*%ΔOS* at day 25=-12.4%), in ,anchovy the oocytes were shown to shrink during the first 5 days of the experiment mainly (Fig. 1) and stabilized thereafter. This pattern was also observed when testing the long term effect of EtOH-25 since *%ΔOS* for sardine dropped to -24.9% (CI: ±3.5%) 7 months after the trial period whilst for anchovy it remained almost stable at -7.2% (CI: ±2.9%) 10 months after the trial period. Statistical comparison of oocyte size between the last day of the trial period and the replicate measurements showed significant differences for sardine (paired *t*-test: P<0.001) but not for anchovy (paired *t*-test: P>0.1). Concerning the effect of BF-10, it appears to be consistent between the two species: there was a small shrinkage during the first three days of treatment (anchovy: -4.4% [CI: ±1.8%]; sardine: -1.9% [CI: 1.4%]) and this value remained almost constant at the end of the trial period for both species (anchovy: -4.8% [CI: ±3.4%]; sardine: -3.6% [CI: 2.1%]).

The data plots in LMEM analysis provided limited indications of a systematic relationship between a sub-sample's random effect for slope (i.e. the rate of oocyte shrinkage) and its random effect for the intercept (i.e. oocyte size at the beginning of the trial period). In addition, in all four models, the correlation between the intercept and the day of measurement was rather low (<0.2) thus confirming that the effect of the sub-samples should have been treated as random. We thus considered a model with two random-effects terms that had the same grouping factor (ovarian sub-sample) and different model matrices: the intercept for one term and the day of measurement for the other. Comparison of all models with the null model, i.e. the model that included the random effects only, showed that the addition of fixed effects performed significantly better compared to the null model (ANOVA: P<0.001).

As shown in Table 1, the absolute *t*-value for the effect of *DAY* in ethanol treatments increased from 1.7 in EtOH-25 to 4.4 in EtOH-50 and 7.4 in EtOH-70. This confirmed the increasing oocyte shrinkage that has already been observed in Figure 1 for ethanol solutions of increasing concentration. Concerning BF-10, back-fitting excluded *DAY* from the model, which confirmed that shrinkage only occurred during the first few days of treatment. The *DAY*MAT* interaction was negative and significant for the three ethanol solutions only, thus indicating that the hydrated oocytes exhibited higher shrinkage rates compared to the vitellogenic oocytes. The effect of the *DAY*SP* interaction was only significant for EtOH-25, which was related to the exceptional pattern that has been observed for this particular treatment between the two species, i.e. oocyte shrinkage in EtOH-25 being more prominent and persistent for sardine compared to anchovy. On the other hand, the effect of *SP* was negative and significant for the EtOH-50 and EtOH-70 treatments only despite the fact that mean oocyte size did not differ significantly between the two species at the onset of the trial period. We assume that this effect of *SP* is related to an abrupt shrinkage of sardine oocytes in both treatments during the first couple of days of the trial period (also observed in Fig. 1).

Table 1. Coefficients of the linear mixed effects models (LMEMs) used to analyze the effect of day of measurement (*DAY*), maturity stage (*MAT*; level 1=vitellogenic, level 2=hydrated) and species (*SP*; level 1=anchovy, level 2= sardine) on the size of oocytes for each of the four preservation treatments. The table includes only those coefficients that remained after back-fitting the initial models. *t*-values are given in parentheses.

Parameter	BF-10	EtOH-25	EtOH-50	EtOH-70
Null	0.21	0.21	0.21	0.23
	(8.37)	(8.59)	(9.02)	(7.87)
DAY	-	0.0001	-0.0007	-0.0011
		(1.72)	(-4.39)	(-7.44)
MAT	0.39	0.35	0.359	0.3695
	(19.86)	(18.41)	(18.65)	(15.44)
SP	-0.04	-0.04	-0.05	-0.06
	(-2.49)	(-2.65)	(-3.35)	(-3.48)
*DAY*MAT*	-	0.0001	-0.0004	-0.0319
		(-2.60)	(-11.42)	(-8.66)
*DAY*SP*	-	-0.0001	-	-
		(-3.26)		

Discussion

Oocyte shrinkage in BF-10 was similar for sardine and anchovy. This shrinkage occurred during the first days of treatment mainly and stabilized for the remaining trial period, with an overall value of 4.7% and 3.6% for anchovy and sardine, respectively, after ca. 1 month of treatment. These results are in agreement with previous studies that report either limited (*Thunnus alalunga:* Ramon & Bartoo, 1997; *Gadus morhua, Melanogrammus aeglefinus and, Hippoglossoides platessoides*: Klibansky & Juanes, 2007) or insignificant (*Cynoscion regallis*: Lowerre-Barbieri & Barbieri, 1993) size changes for oocytes kept in formalin. Given the limited shrinkage and non-significant time trends in oocyte size, BF-10% was used as the control during the trial period of both species.

For anchovy oocytes, preservation in EtOH-25 led to similar results with BF-10, causing an overall shrinkage of 6.5% at the end of the trial period. This value remained con-

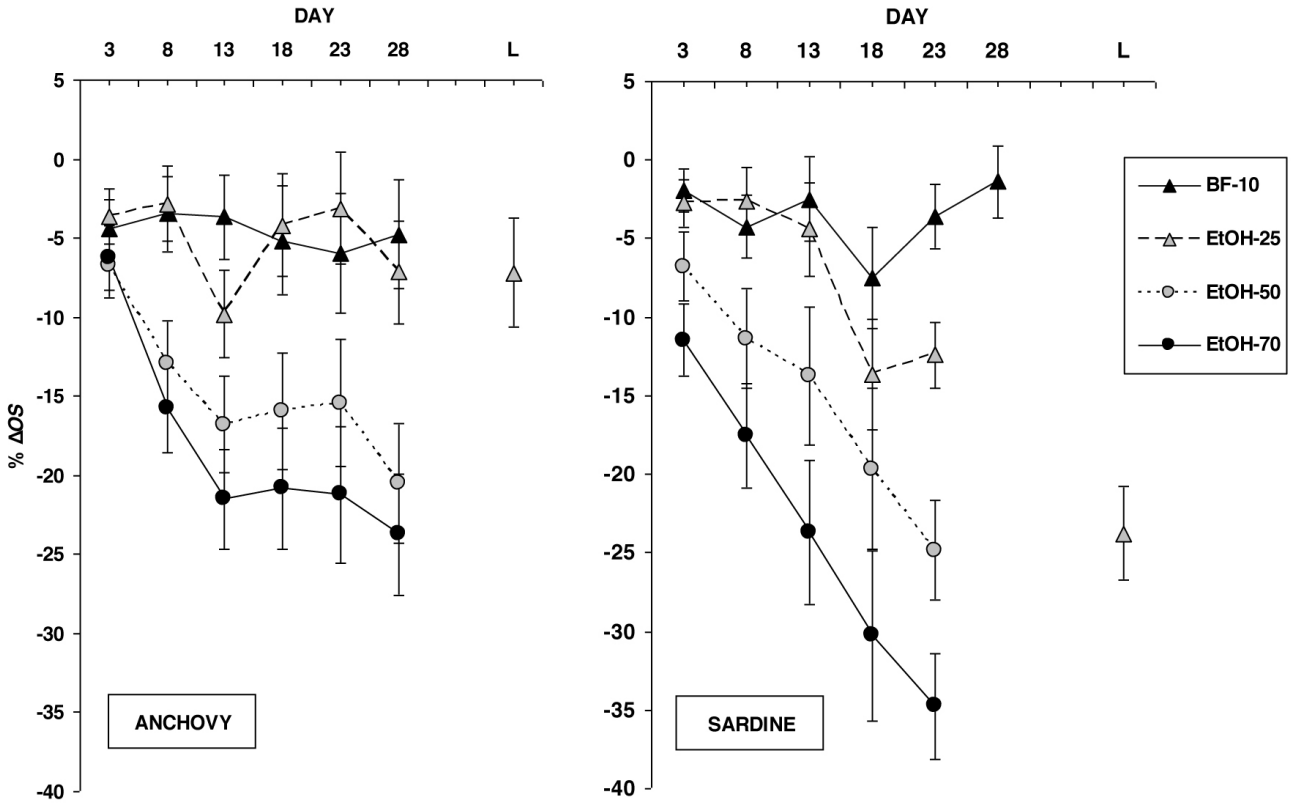

Fig. 1: Effect of four different preservation protocols (BF-10, EtOH-25, EtOH-50 and EtOH-70) on the size of the oocytes of the two species over time. Points represent the percent shrinkage (*%ΔOS*) values (and 95% CIs) for each 5days / species / preservation-treatment group.

stant for the long term treatment of ca. 10 months suggesting that EtOH-25 could be used as a good alternative to formalin for the preservation of anchovy ovarian material. Nonetheless, the effect of EtOH-25 in sardine was quite different since oocyte shrinkage at the end of the trial period was almost double compared to anchovy (12.4%) whilst shrinkage continued beyond this period reaching a value of ca. 24% after 8 months of treatment. The effect of EtOH-50 and EtOH-70 in sardine was even more pronounced (24.8% and 34.8% respectively at the end of the trial period) while for anchovy the effect was again significant though lower compared to sardine (20.5% and 23.7% respectively). In a parallel study, it was shown that preservation of hydrated Atlantic sardine, *S. pilchardus*, ovaries for ca. 1 year in BF-10 and EtOH-70 resulted in an average difference between the two treatments of ca. 37% (Ganias K., unpublished data). Therefore, shrinkage of sardine oocytes in EtOH-70 continues beyond the 1-month trial period, even if at slower rate.

In anchovy specimens, most shrinkage took place during the first two weeks of preservation whilst oocyte size tended to stabilize thereafter. Similar effects have been reported for plaice, *Solea solea*, oocytes by Witthames & Greer-Walker (1987) but also for cod, *Gadus morhua*, oocytes by Kjesbu *et al*. (1990) who both evaluated shrinkage rates using Gilson's solution. It is likely that shrinkage continues until substrates for ethanol get exhausted and the water content of the oocytes enters into osmotic equilibrium with the preservative. In addition, autoxidation of the alcohol preservatives makes them increasingly acidic with time, a fact that might change the levels of osmotic regulation and intensify the degradative process.

The observed differences in oocyte shrinkage rates between the two species are more complicated and difficult to explain. This comparison becomes even more complicated in view of the findings of Klibansky & Juanes (2007) who report that 3-4 months preservation in EtOH-70 caused marginal shrinkage in the oocytes of the Atlantic cod, *G. morhua*, and haddock, *Melanogrammus aeglefinus*, and no shrinkage at all in the oocytes of the American plaice, *H. platessoides*. Discrepancy between the two studies cannot be attributed to differences in the trial periods and the potential recovery of oocyte size during the longer EtOH-70 treatment reported by Klibansky & Juanes (2007). In addition, inter-specific differences in oocyte shape should be rejected as a cause for this discrepancy since anchovy, i.e. the species with the exceptionally oval oocytes, displays intermediate shrinkage levels between sardine and cod, haddock and plaice which all have typical, spherical oocytes. Surface area is similar for elliptical and spherical oocytes of the same volume, which suggests that different oocyte shapes offer the same relative surface areas for passive diffusion.

Contrary shape, differences in the elemental composition of the oocytes are predicted to cause important inter- and intra-specific differences in shrinkage rates. For pelagic spawners, such as anchovy and sardine, a major shift in oocyte composition occurs at hydration since free amino acids derived from yolk proteolysis cause an osmotic gradient that drives the influx of water. As a consequence, hydrated oocytes contain smaller protein and higher water fractions compared to vitellogenic oocytes. Indeed, in both species, LMEM analysis showed that hydrated oocytes exhibited higher shrinkage values compared to vitellogenic oocytes. Similarly, Witthames & Greer-Walker (1987) and Kjesbu *et al*. (1990) report, for plaice and cod respectively, that shrinkage was higher for hydrated oocytes compared to vitellogenic oocytes.

As already mentioned, differences in oocyte elemental composition are also predicted to cause inter-specific differences in shrinkage rates. Sardine oocytes contain numerous oil droplets throughout vitellogenesis, which fuse into a large oil drop at final oocyte maturation while anchovy oocytes lack such droplets (Ganias *et al.*, 2014). Given that exposure to ethanol solutions causes the lipid content of oocytes to dissolve, the observed differences between the shrinkage rates of the two species in EtOH solutions could be due to differences in their lipid content. Similarly, cod, plaice and haddock eggs lack oil droplets, which could explain the marginal effect of EtOH on the oocytes of these species reported by Klibansky & Juanes (2007). However, the big differences between the two studies as regards the shrinkage of oocytes preserved in EtOH-70 cannot only be explained by inter-specific differences in oocyte composition; differences in the experimental set-up between the two studies should also be considered. For example, in our study, we fixed ovaries in BF-10 [instead of EtOH-95 as in Klibansky & Juanes (2007)] and we restricted our measurements to the advanced batch of oocytes only in order to avoid biases caused by the differential contribution of the various batches to our measurements.

Acknowledgements

This study constitutes part of the dissertation thesis of MR submitted to the School of Biology, Aristotle University of Thessaloniki.

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