UNIVERSIDADE DE LISBOA
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MORPHOLOGY OF FISH EGGS: A COMPARATIVE METHOD FOR DIFFERENT SALINITIES AND PRESERVATIVES

Ana Filipa Miguel Carvalho

MESTRADO EM PESCAS E AQUACULTURA

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Dissertação orientada pelo:
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Resumo

A produção de ovos é um importante parâmetro na gestão das pescas e estudos de ecologia. As decisões relativas à gestão das pescarias são baseadas na abundância e composição do stock reprodutor; o nível de recrutamento poderá ser influenciado pela variação do tamanho e qualidade dos ovos, os quais são utilizados quer na identificação de espécies quer em modelos de processos ecológicos a operar durante os estágio iniciais de desenvolvimento dos peixes.

A salinidade é um dos factores mais importantes no estudo de organismos marinhos. A capacidade osmorregulatória de um organismo determina a sua tolerância à salinidade e, consequentemente, influencia a sua distribuição. Muitas espécies apresentam exportação larvar estuarina, o que faz com que estes estádios iniciais de desenvolvimento sofram grandes flutuações de salinidade, características destes locais.

A preservação de ovos é uma metodologia muito utilizada em trabalhos de campo de ecologia marinha, como estudos de dinâmica populacional e de fertilidade. A perda de tamanho e peso devido à preservação em soluções químicas é quase um processo inevitável para a maioria dos organismos, sendo os ovos extremamente vulneráveis a esta perda.

O presente trabalho teve como objectivo, analisar os efeitos de diferentes preservantes e de diferentes salinidades na morfologia de ovos de três espécies de peixes, dando-se especial ênfase à biometria dos ovos.

Para tal foram elaborados dois protocolos distintos, analisando-se as alterações na biometria dos ovos, no primeiro, a diferentes salinidades e, no segundo, em diferentes meios de preservação. Foram seleccionadas três espécies de peixes, o linguado-do-Senegal, *Solea senegalensis* Kaup, 1858, o sargo-legítimo, *Diplodus sargus* (Linnaeus, 1758) e a dourada, *Sparus aurata* Linnaeus, 1758. Os ovos provêm de peixes criados na Estação de Piscicultura de Olhão do INIAP/IPIMAR, em Olhão e foram recolhidos com colectores próprios instalados nos tanques durante a época

de reprodução. Os ovos viáveis foram separados dos inviáveis. Foram utilizados 5 replicados com aproximadamente 100 ovos para cada condição. Para a análise dos efeitos da salinidade foram utilizadas 5 salinidades diferentes (0, 5, 20, 35 e 50) e 5 tempos de medição (30 s, 3, 10, 30 e 60 m). Para estudar os efeitos dos vários meios de preservação, foram utilizados 3 concentrações diferentes de etanol (70, 90 e 99 % - etanol absoluto) e 4 de formol (4 e 10 % diluídos em água salgada (salinidade 35) e novamente as mesmas concentrações diluídas em água destilada). Os ovos foram medidos frescos (tempo 0) e novamente medidos após 1, 3, 5, 7, 14, 21, 30,60, 90 e 120 dias. Para a captação de imagens foi utilizada uma lupa estereoscópica equipada com uma câmara fotográfica e para a medição dos ovos o programa Image J®.

A troca de água entre ovos fertilizados e o meio externo há muito que foi proposta. Tal como esperado, nas duas espécies de Sparideos os ovos aumentaram significativamente de tamanho quando expostos a um meio hipotónico, ou seja com uma concentração de sais mais baixas que o meio interno. Uma vez que o fluido perivitelínico dos ovos dos teleósteos apresenta uma osmolaridade semelhante ao meio externo, é de prever que a salinidades a que o ovo e os progenitores se encontram normalmente, a variação em tamanho seja mínima ou inexistente. Já quando colocados em salinidades inferiores, a diferença entre a osmolaridade interna e externa aumenta, resultando na entrada de água para o ovo e consequentemente, o ovo aumenta de tamanho.

No que se refere ao linguado-do-Senegal, os resultados foram um pouco mais surpreendentes. Em salinidades inferiores à água do mar, nos primeiros 30 segundos há um aumento no tamanho do ovo, indicando que ocorreu entrada de água para o ovo. No entanto após este momento o ovo começou a decrescer, tendo mesmo acabado com um tamanho inferior ao inicial. Esta tendência decrescente após o choque inicial (pico máximo) foi também observada para as duas espécies de Sparideos, mas não tão marcada. Isto sugere que os ovos apresentam uma adaptação fisiológica que lhes permite regular o volume da célula, no entanto os resultados mostraram que não foram capazes de contrariar o choque inicial. Os

mecanismos osmorreguladores já foram extensivamente estudados em peixes, nomeadamente no linguado-do-Senegal, e mostraram que tanto pós-larvas, como juvenis e adultos têm capacidade de se adaptar a alterações de factores ambientais, como a salinidade e a temperatura. No entanto a formação destes órgãos osmorreguladores só ocorre em estados de desenvolvimento mais tardios. De acordo com registos fósseis, os ancestrais dos teleósteos viviam em água doce antes de voltarem ao mar. A osmolaridade do vitelo é semelhante aos fluidos corporais parentais e, portanto, hiposmótica em relação à água do mar. Daí que a existência de uma reserva de água no ovo antes da desova seja um pré-requisito para a sobrevivência deste. Este reserva resulta da presença de aminoácidos livres (FAA). A manutenção do volume da célula faz-se através da síntese/transporte transmembranar de FAA em ambientes hiperosmóticos e catabolização/excreção de FAA em ambientes hiposmóticos.

No que respeita aos efeitos da preservação nos ovos, foram observadas diferenças tanto na morfologia como na biometria. A preservação de ovos nas várias concentrações de formol resultou em ovos com tamanhos mais próximos dos frescos que a preservação em etanol. A maior retracção por parte dos ovos preservados em etanol deve-se às diferenças no modo de acção dos dois preservantes. O formol preserva a estrutura secundária das proteínas enquanto que no etanol, simultaneamente à fixação (coagulação das proteínas) ocorre um processo de desidratação. Em termos morfológicos, o formol diluído em água destilada apresentou-se como o melhor preservante, tendo os ovos preservados mantido uma aparência semelhante aos frescos. Os ovos preservados em formol diluído em água salgada, apresentaram uma elevada distorção resultante dos da elevada pressão osmótica criada pelos sais dissolvidos no meio. Os ovos em etanol ficaram rijos e brancos, resultando na indiferenciação das estruturas internas do ovo. A preservação é uma metodologia muito utilizada em estudos biológicos pelo que a escolha do preservante ideal é muito importante. Este deverá ser barato, não tóxico, não volátil, ter um elevado valor de preço/qualidade, capaz de inibir a autólise e ao mesmo tempo preservar a actividade enzimática e a reactividade

antigénica. Até ao momento não existe nenhum que cumpra todos os requisitos, até porque alguns destes critérios são mutuamente exclusivos, pelo que a escolha do preservante a utilizar deve ser feita tendo em conta as vantagens e desvantagens de cada um e consoante os objectivos do estudo. Sempre que possível, o tamanho dos ovos preservados deve ser ajustado tendo em conta as reduções de tamanho causadas pela preservação. Comparando o etanol com o formol, deverá ser utilizado o segundo se são necessárias análises morfológicas do ovo (e.g. análise de estruturas interiores) e quando são necessárias medições mais precisas. Se não for o caso, então o formol deverá ser substituído pelo etanol uma vez que é mais seguro, fácil de medir e permite posterior análises moleculares.

Palavras-chave: Tamanho dos ovos; Biometria; Morfologia; Preservação; Salinidade; Peixes

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Morphology of Fish Eggs: a Comparative Method for Different Salinities and Preservatives

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Abstract

Length measurements of fish eggs are necessary in many types of ecology studies and fisheries surveys. Those measurements are often made right after collecting or preservation may be required. If the preservative or the water used for measuring causes significant shrinkage/swelling, then those lengths cannot be used to indicate live lengths. The objectives of this study were to determine how preservation in different concentrations of formalin and ethanol and measurements made in different salinities affect the total lengths and morphology of eggs of three species of marine coastal fish (*Diplodus sargus*, *Solea senegalensis* and *Sparus aurata*). In the first experiment, eggs were measured in 5 different salinities (0, 5, 20, 35 and 50) and remeasured at 30s, 3, 10, 30 min and 1h after being placed in those salinities. For the second experiment, eggs were preserved in different concentrations of formalin and ethanol (4% Formalin, 10% Formalin, 4% Seawater-Formalin, 10% Seawater-Formalin, 70 % Ethanol, 90 % Ethanol, 99 % Ethanol). Eggs were measured at salinity in which they were collected (35) and remeasured at 1, 3, 5 days, 1, 2, 3 weeks, 1, 2, 3, 4 months after preservation. Most shrinkage occurred within the first day after preservation. In most cases, no significant differences in egg diameter variance (VP) were found between the four formalin-based preservatives although they were significantly different from the original length. Ethanol-based preservatives showed higher VP than formalin-based preservatives due to its dehydration process occur concurrently with the fixation process. Preservative choice should be made according to the study objective. In

lower salinities, after the initial shock (egg swell), egg size decreased, suggesting an adaptation mechanism to salinity variations; an extreme case was observed where, in lower salinities, *S. senegalensis* eggs ended smaller than at the beginning. Both seawater and salinity 50 didn't alter egg size significantly, suggesting that these are the appropriate salinities to measure these species eggs.

Keywords: Egg size; Biometry; Morphology; Preservation; Salinity; Fish.

Introduction

Study of Eggs

Egg production is an important parameter in fisheries management and ecological studies (Hempel, 1984). The decisions concerning fishing management are based, among others, in the dynamic of spawning biomass (Kjesbu, 1989), stock estimation and recruitment predictions, established through quantitative and qualitative studies of eggs and larvae (Hempel, 1984). Number, quality and egg size are important parameters to the basic population processes (Trippel, 1998). Considering the population level, the recruitment of an annual class can be influenced by the variation of egg quality and size along with fecundity (Kjesbu *et al.*, 1996), which makes egg biometry a basic and useful feature to an accurate assessment (Kjesbu, 1989).

Because larva size and its growth is directly correlated with egg size (Escaffre & Bergot, 1984; Hempel, 1984; Trippel, 1998), this is a parameter that can also be used to predict survival of the early stages of fish development, although this relation is not very clear in some species. Therefore, accurate measurements must me made in order to include this parameter in models of the early stages biological processes (Trippel, 1998).

Egg characters such as egg size, number and size of oil globules, chorion surface, yolk, pigmentation, and morphology of the developing embryo have little variation within the same species but vary between species (Ré & Meneses, 2009) (Figure 1).

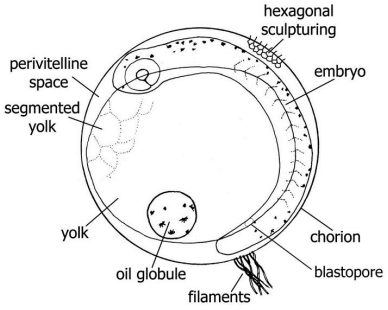


Figure 1: Anatomic features of early stages of fish eggs (Ré & Meneses, 2009)

However in many marine fish species, eggs (especially pelagic) are morphologically similar, spherical, with no special shapes or markings, egg size and pigmentation are among the parameters most frequently used for species identification. Nonetheless, many newly fertilized eggs are unpigmented which makes egg size the primary identification feature (Bagenal, 1971; Hempel, 1984).

Salinity

Salinity is one of the most important factors when studying a marine organism, and it is, therefore, a subject widely addressed in many studies (Alderdice *et al.*, 1979; Lee, 1981; Tucker Jr & Chester, 1984; Burke *et al.*, 1995; Bœuf & Payan, 2001; Gimenez & Anger, 2001; Sampaio & Bianchini, 2002; Arjona *et al.*, 2007; Shi *et al.*, 2008). The osmoregulatory capacity of an organism determines its salinity tolerance, and consequently, influences, in part, its distribution (Kinne, 1958; 1966; Holliday, 1969; Burke *et al.*, 1995; Swanson, 1998).

Many species migrate to inshore estuarine nursing grounds in early stages of development (Burke *et al.*, 1995; Cabral & Costa, 1999) because, generally, it is a place with great abundance of food resources and fewer predators. Estuaries are characterized by daily salinity fluctuations (tides); these changes, consequence of

water input from rivers (freshwater) and water from the ocean (seawater), present a challenge to the animal physiology (McLusky & Elliot, 1989). Because marine teleosts are hyposmotic to seawater and thus experience continuous water loss and salt gain over permeable surfaces, such as gills, some species developed adaptations to acclimate to salinity fluctuations (Griffith, 1974). Some of these adaptations include impermeabilization mechanisms to avoid too great a loss (Evans, 1975; 1984; 1993), using organs that are known to be functional in osmoregulation and seawater drinking (Alderdice *et al.*, 1979).

Mechanisms of ionic regulation of both adult and juvenile marine teleosts have been broadly reported (Evans, 1993; Bœuf & Payan, 2001; Sampaio & Bianchini, 2002; Arjona *et al.*, 2007) and recently, more studies in larvae have been conducted (Burke *et al.*, 1995; Schreiber, 2001; Shi *et al.*, 2008). However, little is known about their egg osmoregulatory physiology. Studies about the effects of salinity in fish eggs are mainly concern about the hatching success in different salinities (Alderdice *et al.*, 1979; Bœuf & Payan, 2001; Gimenez & Anger, 2001) rather than the effects on egg size.

It is widely known and proven that alterations in salinity causes changes in osmotic pressure that, without the ability of actively contradict or compensate this changes, results in cellular size/volume change (Kültz, 2001).

Adults of breams and soles, both euryhaline fishes, live in coastal marine waters and spawn offshore (Cabral & Costa, 1999; Bargelloni *et al.*, 2005), however, eggs and larvae are transported passively towards the shore and, in later stages, use estuaries as a nursing ground (Vasconcelos *et al.*, 2008).

Taking all this into consideration, the question about the salinity in which to measure this egg pops. In this spirit, one of this work's main goals is to study the effects of salinity in the egg biometry of three species of marine coastal fishes.

Preservation

Egg preservation is a method widely used in marine biology field studies. It's used in population dynamics studies, where egg counting and stage identification are required; also in fertility studies where preservation is also used as way of increasing the resistance of eggs for easier handling (Steedman, 1976), when long time storage is required (Black & Dodson, 2003) or to prevent/minimize the degradation of the sampled material (Ré *et al.*, 2005).

Shrinkage and weight loss are consequences widely reported for larva, juvenile and adult fishes due to chemical preservation, such as alcohol and formalin based preservatives. It is an inevitable consequence for most organisms (Krishna, 1948; Steedman, 1976; Markle, 1984; Tucker Jr & Chester, 1984; Thibault-Botha & Bowen, 2004) but the exact effects on eggs remain unspecified. Marine organisms preserved in a 4 % formalin solution suffer twice the osmotic pressure caused by the surrounding seawater (Steedman, 1976), which, in turn, causes water to leave, resulting in a size reduction. Variation in retraction/swelling vary according to the species and its physiology; an organism with a soft covering is more likely to suffer higher variations due to osmotic pressures than a hard covered organism (Thibault-Botha & Bowen, 2004).

Formalin is known as a universal preservative due to its rapid action, availability, cost, and ability for long-term storage. Its action includes three processes happening simultaneously, although at different rates: a very rapid penetration that stops autolysis, covalent bonding and cross-linking. It's the preservative of choice in most laboratories (Buesa, 2008). Despite these clear advantages of formalin preservation, there is significant increasing health concerns for formaldehyde exposure (Black & Dodson, 2003), so other preservatives started to be more used in replacement of formalin, which is the case of ethanol.

Although the use of alcohol and formalin based solutions are widely used methodologies for egg preservation (Steedman, 1976), but because there isn't a standard protocol in egg preservation, different types of preservative and duration

are used, which generate different data that is not comparable between them. The second main objective of this study is to verify the effects of formalin and ethanol preservation in egg morphology of three species of fish, with special emphasis to egg biometry.

Materials and Methods

Two experiments were outlined with the purpose of studying the effects of different salinities and preservatives in the morphology of fish eggs.

Species

Three marine costal species were selected for this study, being one more estuarine, the senegalense sole (*Solea senegalensis* Kaup, 1858), one more coastal, the white seabream (*Diplodus sargus* (Linnaeus, 1758)) and one marine, the gilthead seabream (*Sparus aurata* Linnaeus, 1758), although all use estuaries as nursing grounds (Burke *et al.*, 1995; Cabral & Costa, 1999).

Brood stock maintenance

S. senegalensis brood stock was kept under artificial temperature (18,2 ºC) and natural photoperiod. Water salinity was 35. Tanks had 28 females (out of 56 animals) and a density 5.9 $kg.m^{-3}$. All females had approximately the same size (average of 54.0 ± 2.93 cm).

Both *S. aurata* and *D. sargus* were reared under artificial temperature (19 ºC), salinity 35 and photoperiod with day light rhythms. *S. aurata* tanks had 24 females (fish density: 6.5 $kg.m^{-3}$) and *D. sargus* tanks had 12 females (fish density: 2,7 $kg.m^{-3}$), both tanks with a sex ratio of 1:2 (males:females). *S. aurata* females

with approximately 49.5 ± 4.95 cm in length and *D. sargus* with an average of 41.0 ± 3.13 cm).

Collection of Eggs

Eggs were collected from brood stocks that naturally spawned in the tanks in the facilities of INIAP/CripSul in November, for *S. senegalensis* (in pre-hatch stage) and June for *D. sargus* and *S. aurata* (both in neurula stage). The eggs, due to their hyponeustonic characteristics, were collected with 500 µm nets installed as egg collectors in the tanks during the spawning season. The water flux of the surface layer was directed through a system of water jets that led the eggs to the collectors. The eggs were collected in the morning before feeding in order to prevent contamination with food particles. Viable eggs were separated from the nonviable through floating criteria. Floating eggs were considered viable and the non-floating eggs, which sank to the bottom of the incubator, were considered nonviable (Lahnsteiner & Patarnello, 2003). The eggs were then placed in a goblet with water from the tanks. The replicates (n=5) consisted in a sample of approximately 100 fertilized eggs collected from a tank.

Salinity Experiment

Five different salinities were selected (0, 5, 20, 35 and 50); two near the limits existent in an estuary, plus freshwater, seawater and hypersaline water.

Eggs were placed in Petri dishes and water with the different salinities was added to each trial. After 30 seconds the eggs were measured and again at 3, 10, 30 and 60 minutes. An exponential time scale was used, mainly because most of the alterations are expected to occur in the beginning of the experiment.

Samples of *Sparus aurata* eggs had a great amount of nonviable eggs, even after the separation through the floating criteria (Lahnsteiner & Patarnello, 2003). However,

only viable eggs were measured. Due to laboratory problems, it wasn't possible to collect data for this species for the 60-minute period.

Preservation Experiment

Two different preservatives were selected among the most used in egg preservation and egg biometry studies, ethanol and formalin (Steedman, 1976; Markle, 1984; Goswami, 2004; Shi *et al.*, 2008). Several concentrations were considered: ethanol at 70%, 90% and 99%, and seawater-formalin at 4% and 10%, and formalin (with distilled water) at 4% and 10%. Formalin based preservatives were obtain through dilution of 37% formaldehyde aqueous solution.

Each egg sample was placed in 25 ml of one of the preservatives mentioned above and the measurements were made every two days for the first week, then once a week for the first month and every month during 4 months (days 1, 3, 5, 7, 14, 21, 30, 60, 90 and 120). Again, an exponential time scale was also considered for this experiment for the same reasons, but with longer periods.

Data acquisition and processing

A stereomicroscope (Zeiss Stemi 2000C) equipped with a camera (Canon 350D) was used for the image caption, with the highest resolution possible. The images were treated in Photoshop® and the diameter measurements made with Image J® software. From the 100 eggs in each replicate, 20 were randomly measured with a scale of 262.01 pixels/mm.

For all the species, the initial diameter value considered (time = 0) for every condition was the average diameter (IAD) obtained for the 30 seconds time with the water in which they were collected, meaning salinity 35, and the variation percentage of the diameter (VP) was calculated (VP = (∅-IAD)/IAD, and expressed in percentage).

Factorial analysis of variance (ANOVA) with subsequent Tukey's test was used to compare the means of the variation percentage of egg diameter between the different conditions (salinity or preservation) and times, using Statistica® (version 8.3). A level of significance of 5 % was used.

Results

Salinity Experiment

The two Sparidae species spawned transparent eggs with one oil droplet and the senegalese sole spawned transparent eggs with small scattered oil globules. Egg size (diameter ± standard error) was 1.0018 mm (± 0.0016) for the senegalese sole, 1.0060 mm (± 0.0021) for the gilthead seabream and 0.9358 mm (± 0.0026) for the white seabream (Table 1 – in annex).

The white seabream (Figure 2A) showed the highest variation value observed for the three species (4.72 %). In salinity 35, as well as in salinity 50, the variation was small ($P > 0.05$), however, when in salinities lower than seawater (< 35), egg size increased significantly ($P<0.0001$).

In salinities lower than 35, in spite of the sharp increase in the diameter in the first 30 seconds, the senegalese sole eggs (Figure 2B) ended up significantly smaller than at the beginning ($P<0.01$). The lower the salinities, the bigger the variation observed for this species; both highest (3.67 %) and lowest (-1.71 %) variation values observed for this species were recorded in freshwater. In hypersaline water, egg size followed the inverse tendency showed for the lower salinities, however the differences observed were not significant ($P>0.05$). Like *D. sargus*, egg size in salinity 35 showed a constant tendency.

The gilthead seabream (Figure 2C) showed a similar trend as the other Sparidae species when placed in salinities lower than the seawater. Significant differences in egg size were only verified in salinities 0 and 5 ($P<0.0001$).

In all species, the variation on the diameter was always inferior to 5 %. In general, the majority of the variations occurred during the first 10 minutes, which is confirmed by the absence of significant differences between the 10 and 60 minute-time ($P>0.05$).

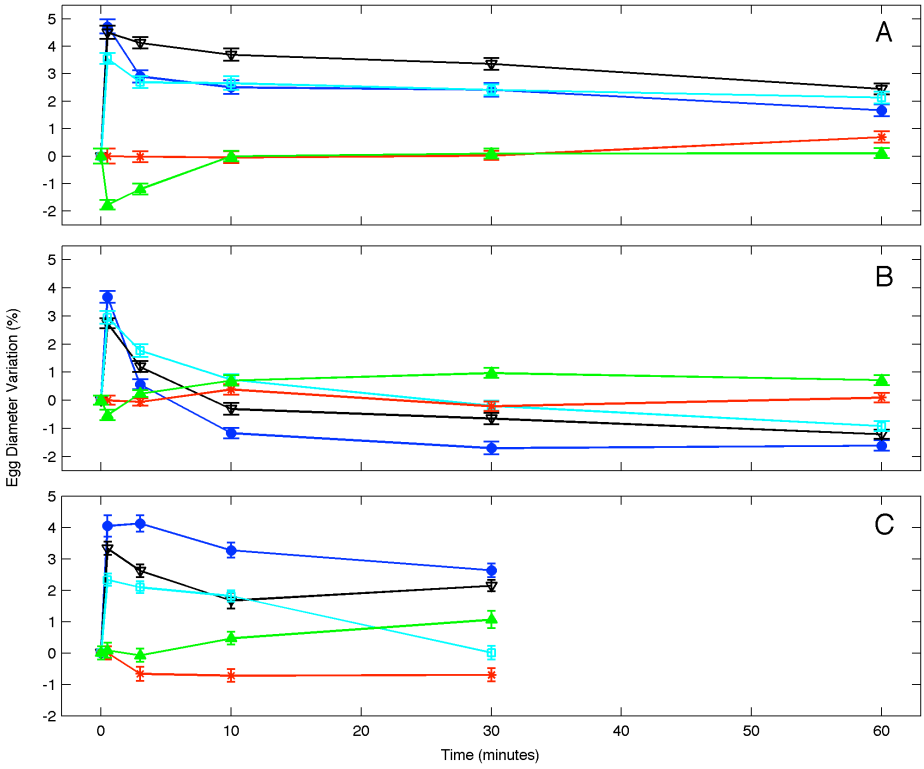


Figure 2: Variation percentage of the diameter of eggs (mean ± S.E.) in time in different salinities (Salinity 0; Salinity 5; Salinity 20; Salinity 35; Salinity 50). Species: A - *Diplodus sargus*; B - *Solea senegalensis*; C - *Sparus aurata*.

Preservation Experiment

For all three species, ethanol preservation exhibited higher percentage variations in diameter than formalin preservation (Figure 3). Shrinkage tendency was similar in all species; both 90 and 99 % ethanol preservation resulted in smaller eggs than 70 % ethanol preservation, except for *S. senegalensis* (Figure 3B), in which this last preservative caused 12 % shrinkage. Both *D. sargus* and *S. senegalensis* (Figure 3A;

3B) displayed similar shrinkage values (approximately 9 %) for 90 and 99 % ethanol preservation (P>0.95).

For both *D. sargus* and *S. aurata* (Figure 3A; 3C), all formalin based preservatives had similar effects in terms of egg size, resulting in a shrinkage of approximately 2 % (P<0.05). As for *S. senegalensis*, formalin preservatives showed significant differences (P<0.001) between them, except for formalin 4 and 10 %, which were quite similar (P>0.5). The 10 % seawater-formalin resulted in a constant egg size opposed to the 2-3 % shrinkage of the other formalin-based preservatives.

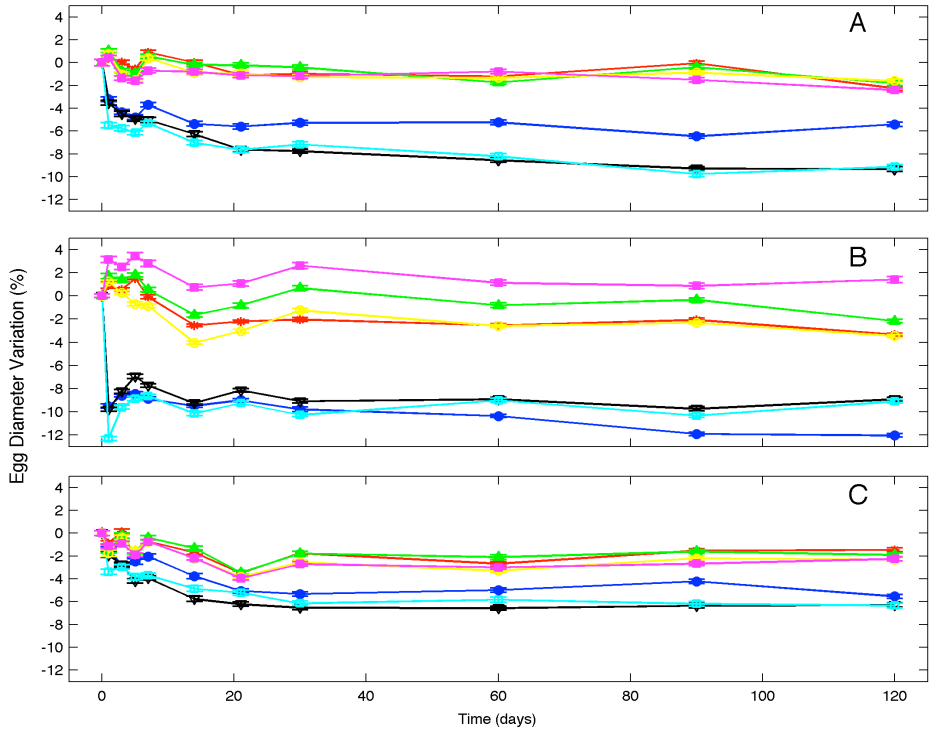


Figure 3: Variation percentage of the diameter of eggs (± S.E.) in time and in different preservatives (Ethanol 70 %; Ethanol 90 %; Ethanol 99 %; Formalin 4 %; Seawater-Formalin 4%; Formalin 10 %; Seawater-Formalin 10%). Species: A - *Diplodus sargus*; B - *Solea senegalensis*; C - *Sparus aurata*.

In all species standard errors ranged from 0.1230 to 0.3384 % (Figure 4; 5). In general, freshwater showed higher values comparing to other salinities. Considering the scale in question, standard error didn't fluctuate much, remaining close to the initial standard error (time 0, salinity 35).

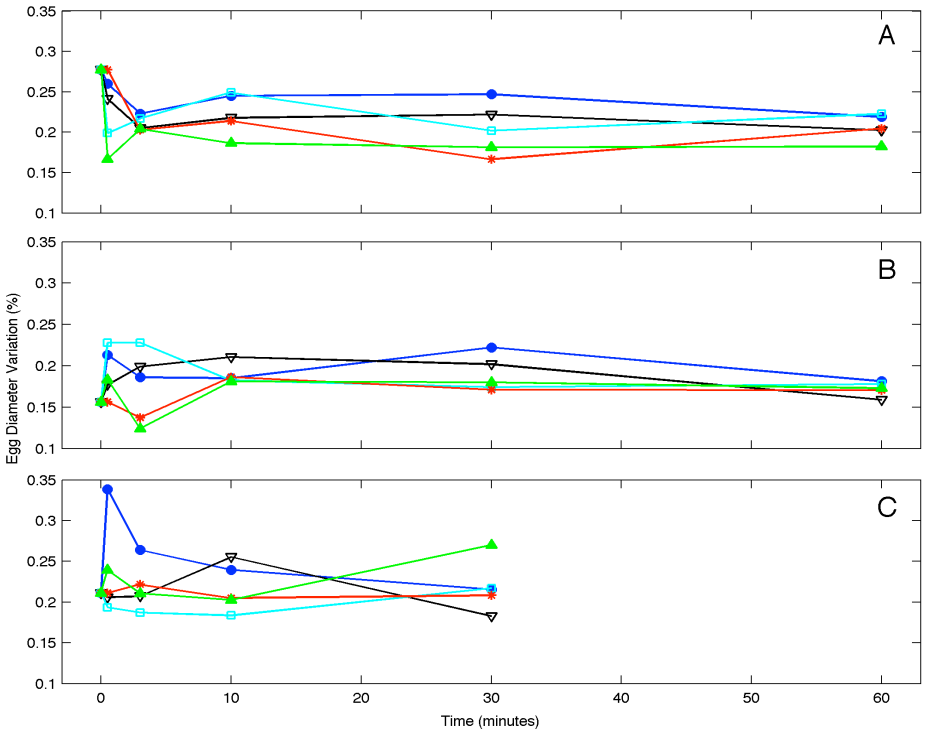


Figure 4: Standard error of the variation percentage of the egg diameter in time and in different salinities (Salinity 0; Salinity 5; Salinity 20; Salinity 35; Salinity 50). Species: A - *Diplodus sargus*; B - *Solea senegalensis*; C - *Sparus aurata*.

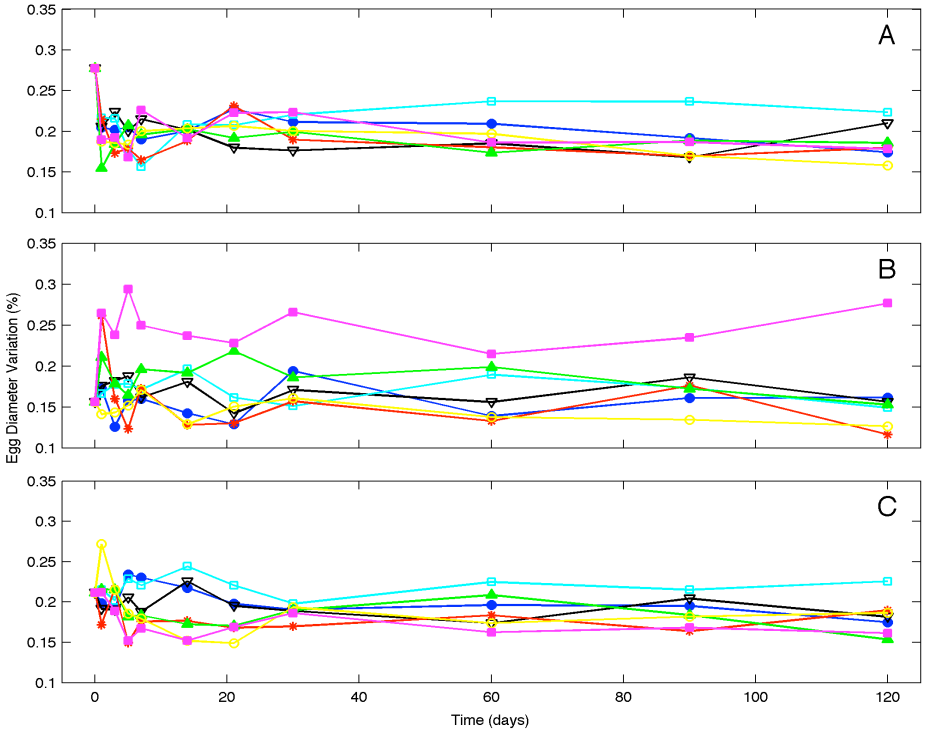
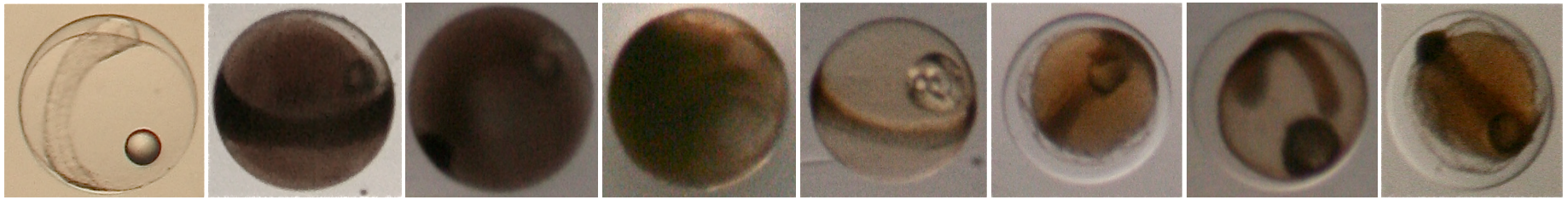
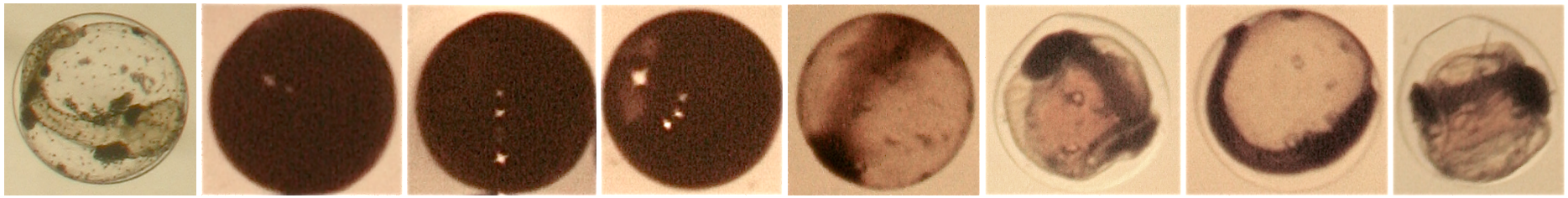


Figure 5: Standard error of the variation percentage of the egg diameter in time and in different preservatives (Ethanol 70 %; Ethanol 90 %; Ethanol 99 %; Formalin 4 %; Seawater-Formalin 4%; Formalin 10 %; Seawater-Formalin 10%). Species: A - *Diplodus sargus*; B - *Solea senegalensis*; C - *Sparus aurata*.

Diplodus sargus



Solea senegalensis



Sparus aurata

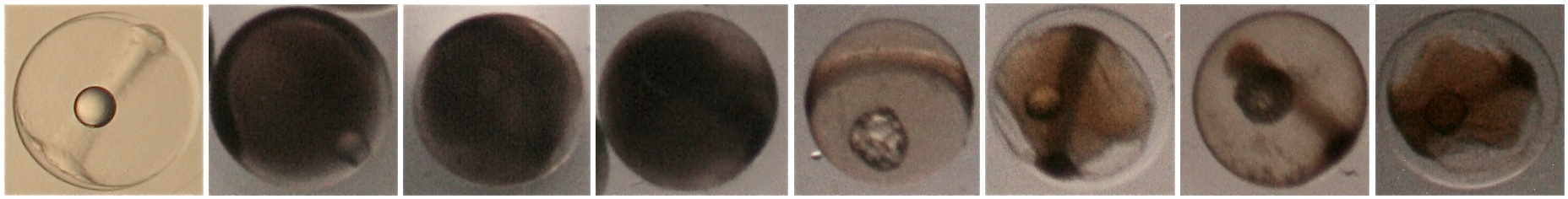


Figure 6: Egg morphology before (A - fresh) and after preservation (B – Ethanol 70 %; C - Ethanol 90 %; D– Ethanol 99 %; E– Formalin 4 %; F – Seawater-Formalin 4%; G– Formalin 10 %; H - Seawater-Formalin 10%). Note: Eggs not to scale

Egg morphology changed according to the preservative used (Figure 6). In ethanol-based preservatives (Figure 6B-D), eggs became white and in the higher concentrations (90 and 99 % ethanol), almost opaque. All egg structures, easily identified in the without preservative, became unidentifiable. In seawater-formalin (Figure 6F; 6H), egg became distorted and the perivitelline space increased. In freshwater-formalin (Figure 6E; 6G), eggs didn't show many morphological changes, although the oil globule suffered some alterations.

Discussion

The egg diameter determined for the gilthead seabream and the Senegalese sole was approximately 1 mm, the same as reported by Lahnsteiner & Patarnello (2003) for the first species and Dinis (1992) and Yúfera *et al*. (1999) for the second. The diameter in the white sea bream was 0.94 mm, the same as reported by Brownell (1979).

Salinity

Water exchange between fertilized eggs and external medium has long been proposed (Loeffler & Lovtrup, 1970; Loeffler, 1971; May, 1974). As expected, the size of the fertilized eggs exposed to salinities lower than the seawater (salinity 35) changed significantly in both breams. These results are partly in accordance with Shi *et al.* (2008) that observed egg size of the pomfret *Pampus puncatissimus* decreasing in salinities above 35 and increasing in lower salinities. Holliday (1969) suggested that the osmolarity of the perivitelline fluid of teleost eggs tends to be similar to that of the outside medium (Davenport *et al.*, 1981), so the higher diameters must be a consequence of the greater difference between internal and external osmolarity, resulting in water uptake (Thibault-Botha & Bowen, 2004).

However, our results showed no significant differences between egg size in salinity 35 and 50, which would be expected to decrease.

For salinities lower than seawater, although there was a significant increase the senegalese sole egg size in the first 30 seconds (that correspond to the expected effects of hyposaline water on marine fish), in time, egg size decreased. Although the egg size in the end in the other two species was bigger than at the beginning, the same pattern of diminishing size after the initial shock occurred in all species, suggesting a common property of egg adaptation to lower salinities. *S. senegalensis* is a marine teleost that inhabits coastal waters and riverine estuaries, and post-larvae, juveniles and adults are know to be capable of adapting to substantial changes in environmental salinity and temperature (Imsland *et al.*, 2003; Arjona *et al.*, 2007), however no data is available for this species eggs.

According to fossil records, teleost fish ancestors lived in freshwater before entering the sea during the Triassic period, which implies that their eggs, broadcasted freely into the environment by their oviparous parents, had become adapted to the freshwater condition. However, when teleosts eventually started spawning in the sea, eggs encountered osmotic problems (Finn & Kristoffersen, 2007). Yolk osmolarity is similar to the parental body fluids, and therefore, hyposmotic to seawater (Solemdal, 1967; Lønning & Davenport, 1980). So, instead of an osmotic influx, the problem in seawater is a continuous water efflux. The transition from freshwater to seawater demands osmotic adaptations, especially for eggs since osmoregulatory organs do not develop until later development stages. According to Holliday and Jones (1965), full osmotic regulation during the early embryonic stage is not completed until blastopore closure. So, marine fish eggs must contain a water reservoir before spawning in the hyperosmotic seawater (Finn & Kristoffersen, 2007). Some examples of adaptations strategies include cell volume regulation, adaptive regulation of ion transport, adaptive regulation of water and ion permeabilities, protection of macromolecular structures and function and controlled accumulation of organic osmolytes in order to close the osmotic gap

between conserved intracellular electrolyte concentration and extracellular osmolarity (Kültz, 2001).

Newly spawned marine fish eggs have a total amino acid content of 40-60 % of their dry mass and in the case of marine pelagic fish eggs, free amino acid pool (FAA) constitute up to 50 of the total amino acid pool. This FAA pool is dominated, in case of the pelagic eggs, by neutral amino acids such as leucine, isoleucine, valine, alanine and serine and represents about 50 % of the yolk osmolarity. Studies have shown that the FAA pool is a consequence of a yolk protein hydrolysis during oocyte maturation. Concurrently with the establishment of the FAA pool there is an osmotic influx of water that results in a rapid increase in oocyte volume, which is responsible for the increased buoyancy. This higher water content (normally > 90 %) also prepares the embryo for development in a hyperosmotic environment so it may survive until osmoregulatory organs are formed. Also, the FAA pool of a given species may vary with life stage and rearing salinity. It has been shown that marine invertebrates are able to adapt to a changing salinity by adjusting their osmolyte concentration, almost exclusively through regulation organic osmoytes, mostly FAA. In hyperosmotic environments, the increase of FAA is done via synthesis or transmembrane transport and in hyposmotic environments, FAA are catabolised or excreted (Rønnestad *et al.*, 1999).

Egg buoyancy is often regarded as a limiting factor, which determines where embryonic development may be successful (Nissling *et al.*, 1994). It has been shown by Holiday & Jones (1967) that the concentration of the yolk in plaice *Pleuronectes platessa* eggs can be regulated from the time of fertilization and in this way the buoyancy is maintained. The size of the eggs is, therefore, related to the amount of yolk present even in these pelagic eggs.

Several mechanisms of egg osmolarity fixation have been proposed. Two hypothesis arise trying to explain the production of ovoplasm concentration stability; either the ovoplasm membrane is impermeable to salts and water, which seems very unlikely, or it must possess efficient ion pumps to extrude salts. However dye tests showed

that chorion is very permeable to relatively large molecules (Davenport *et al.*, 1981). Only Alderdice *et al*. (1979) reported significant differences between osmolarities of perivitelline fluid of the Pacific herring eggs (*Culpea pallasi*) and the external medium, suggesting some kind of adaptation preventing water uptake/loss. Also Lønning & Solemdal (1972) stated that variations in chorion thickness allowed flatfish eggs to float in relatively low salinities in the Baltic Sea, which means that no or little water entered these eggs.

However more studies in this species eggs must be made in order to verify the physiological reasons and mechanisms that are preventing egg swelling in hypotonic waters and shrinkage in hypertonic water.

Preservation

Due to its wide use in many biological study methodologies, the effects of long term preservation on fish larvae, juveniles and adults are well documented; nevertheless studies about effects on eggs aren't abundant. It has been reported significant modifications in body morphometry, weight and pigmentation, always depending on the species, size of specimens, preservative used and duration of preservation (Markle, 1984; Tucker Jr & Chester, 1984; Cunningham & Granberry Jr, 2000; Koumoundouros *et al.*, 2000; Black & Dodson, 2003).

When preserved in formalin-based solutions, egg size decreased about 2%, which is in accordance with Steedman (1976) that reported slight variations in formalin preserved eggs, comparing to high shrinkage from ethanol preservation. Formalin preserves the secondary structure of proteins (Mason & O'Leary, 1991), insolubilizing them in more than 90% (Buesa, 2008), which confers the hard but flexible appearance (Steedman, 1976).

According to Goswami (2004), the concentrated formalin should be diluted preferably with water from the sampling area in order to avoid undesirable osmotic effects. However our results showed that seawater-formalin preserved eggs

suffered high distortion, making measuring much harder and less accurate. The perivitelline space increased in volume and the yolk and embryo volume decreased. Egg distortion was higher in 10 % seawater-formalin than in 4 % seawater-formalin. This distortion was caused by the increase in osmotic pressure due to the salinity present in the solution (Thibault-Botha & Bowen, 2004).

Moreover, if the sampling takes place near shore where water salinity fluctuates a lot, the decision on which water salinity to measure the eggs isn't straightforward and could lead to the undesirable osmotic driven shrinkage.

In all species ethanol preservation caused higher egg shrinkage (between 6 and 12 %), comparing to the 2 % caused by formalin preservation. This higher shrinkage is due to the dehydration that, in alcohol-based preservatives, occurs simultaneously with the fixation process (Buesa, 2008). This shrinkage is higher with higher concentrations of ethanol (Steedman, 1976), although this study showed that 90 and 99 % ethanol preservation cause similar shrinkage. This was also observed by Cunningham & Granberry Jr (2000) where 80 and 100 % ethanol preservation showed no significant differences in size variation between them although they caused the most shrinkage among the other ethanol-based preservatives in the study.

Contrary to what has been reported for adult fishes and larvae (Kabbarah *et al.*, 2003; Cox *et al.*, 2006), ethanol preservation caused morphological differences in the eggs. All egg characters, present and easily identifiable before preservation, became unrecognizable after 3 days of preservation. All eggs became almost opaque white.

Brood stocks of Sparidae species are able to produce large quantities of eggs, however egg quality varies greatly (Lahnsteiner & Patarnello, 2004). Replicates of *Sparus aurata* had a great amount of nonviable eggs, although they were floating and therefore considered viable for the sampling through the floating criteria (Lahnsteiner & Patarnello, 2003). Because ethanol preservation causes

indifferentiation in the egg structures, it is hard to distinguish viable from nonviable eggs. This task becomes practically impossible in 90 and 99% ethanol.

Within the same spawning event there is a high variance in egg size (Hempel, 1984). No major alterations nor variation tendencies in standard errors were observed comparing the initial standard error (resulting from the inherent variability of the egg samples) with all the other. This could mean that variations in egg size are due to the preservative/salinity and might be dependent on the initial egg size value.

As mentioned above, formalin is known as a universal preservative that preserves tissues by cross-linking proteins. Its use in preservation methodologies for more than 100 years and all the accumulated scientific knowledge along with its cost, convenient storage, good lipid preservation, makes it very suitable for most studies. However, there are considerable disadvantages on the use of formalin. Besides being a carcinogen, formalin is also a very hazardous substance to the environment and its disposal is expensive because it requires specialized contractors or has to be "neutralized" in the laboratory with some very expensive neutralizing agents (Buesa, 2008). Some studies showed that many antibodies that work on fresh or alcohol preserved tissue don't work on formalin-preserved tissues, which suggest that proteins epitopes are modified or removed by formalin. Also, according to (Gugic *et al.*, 2007), formalin may be too successful in preventing autolysis and in coagulating cell content into insoluble substances, which prevents posterior molecular analysis.

Ethanol is a fast fixative and preservative, acting by coagulation of proteins (Buesa, 2008). It is volatile (Goswami, 2004) which makes long-term storage, without supervision, a problem. Shrinking and hardening are also expected consequences when using ethanol as a preservative, but samples can be used for genetic analysis (Black & Dodson, 2003).

When comparing formalin and ethanol in terms of measurement easiness, ethanol preservation transforms eggs into perfect marbles, which makes measurements much easier. Also, when using automatic egg measurements, the high contrast from

the eggs and the background resulting from ethanol preservation makes sizing up more accurate (personal observation).

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Final Considerations

Salinity is an environmental factor that influences fish distribution. Because salinity tolerance is species specific, extrapolations to other species aren't accurate. Therefore, more studies should be made, using more species from different habitats, such as freshwater, brackish water, marine (entire life cycle in seawater), hypersaline and try to see any pattern concerning egg volume regulation or any other adaptation to salinity variations; also biochemical and physiological analysis to eggs during this salinity stress to understand the mechanisms involved in the volume regulation.

An optimal preservative should be able to inhibit autolysis while preserving enzyme activity and antigen reactivity. However, some of these requirements are mutually exclusive. At present, no preservative is able to fulfil all of these criteria, so the choice of the preservative must be made according to the objective of the study.

Because shrinkage due to preservation is species specific, comparison between preserved species isn't accurate and for that reason it shouldn't be made, unless a correction factor (species and preservative specific) is applied to the measurement. For that, more studies like this should be made in order to calculate correction factors for more species and more preservatives. So, whenever possible, egg size of preserved samples should be adjusted to account for the size reductions caused by preservation. Formalin should be used in place of ethanol when morphological analysis (such as egg structures) and minimal shrinkage are important. If that is not the case, then ethanol should be used because it's safer, easier to measure and allows posterior molecular analysis.

Annex

Table 1: Time-series of egg diameters (mean ± standard error) (in mm) exposed to the different salinities (Summary Table for the Salinity Experiment)

Species	Salinity	Time (minutes)					
		0	½	3	10	30	60
Solea senegalensis	0	1.0018 ± 0.0016	1.0386 ± 0.0021	1.0075 ± 0.0019	0.9901 ± 0.0019	0.9847 ± 0.0022	0.9856 ± 0.0018
	5	1.0018 ± 0.0016	1.0292 ± 0.0018	1.0138 ± 0.0020	0.9987 ± 0.0021	0.9953 ± 0.0020	0.9896 ± 0.0016
	20	1.0018 ± 0.0016	1.0313 ± 0.0023	1.0195 ± 0.0023	1.0092 ± 0.0018	0.9998 ± 0.0017	0.9926 ± 0.0018
	35	1.0018 ± 0.0016	1.0018 ± 0.0016	1.0011 ± 0.0014	1.0056 ± 0.0019	0.9997 ± 0.0017	1.0027 ± 0.0017
	50	1.0018 ± 0.0016	0.9965 ± 0.0018	1.0042 ± 0.0012	1.0088 ± 0.0018	1.0115 ± 0.0018	1.0089 ± 0.0017
Diplodus sargus	0	0.9358 ± 0.0026	0.9800 ± 0.0024	0.9630 ± 0.0021	0.9593 ± 0.0023	0.9584 ± 0.0023	0.9514 ± 0.0020
	5	0.9358 ± 0.0026	0.9779 ± 0.0023	0.9744 ± 0.0019	0.9704 ± 0.0020	0.9672 ± 0.0021	0.9587 ± 0.0019
	20	0.9358 ± 0.0026	0.9690 ± 0.0019	0.9609 ± 0.0020	0.9606 ± 0.0023	0.9584 ± 0.0019	0.9557 ± 0.0021
	35	0.9358 ± 0.0026	0.9358 ± 0.0026	0.9356 ± 0.0019	0.9354 ± 0.0020	0.9360 ± 0.0016	0.9423 ± 0.0019
	50	0.9358 ± 0.0026	0.9192 ± 0.0016	0.9246 ± 0.0019	0.9358 ± 0.0017	0.9367 ± 0.0017	0.9368 ± 0.0017
Sparus aurata	0	1.0060 ± 0.0021	1.0467 ± 0.0034	1.0475 ± 0.0027	1.0389 ± 0.0024	1.0325 ± 0.0022	
	5	1.0060 ± 0.0021	1.0395 ± 0.0021	1.0323 ± 0.0021	1.0228 ± 0.0026	1.0276 ± 0.0018	
	20	1.0060 ± 0.0021	1.0295 ± 0.0019	1.0271 ± 0.0019	1.0243 ± 0.0018	1.0061 ± 0.0022	
	35	1.0060 ± 0.0021	1.0060 ± 0.0021	0.9994 ± 0.0022	0.9988 ± 0.0021	0.9990 ± 0.0021	
	50	1.0060 ± 0.0021	1.0069 ± 0.0024	1.0053 ± 0.0021	1.0107 ± 0.0020	1.0167 ± 0.0027	

Table 2: Time-series of egg diameters (mean ± standard error) (in mm) exposed to the preservatives salinities (Summary Table for the Salinity Experiment. E – Ethanol; F - Freshwater-formalin; Fs – Seawater-formalin).

Species	Preservative	Time (days)									
		1	3	5	7	14	21	30	60	90	120
Solea senegalensis	E 70 %	0.9064 ± 0.0017	0.9155 ± 0.0013	0.9171 ± 0.0016	0.9128 ± 0.0016	0.9068 ± 0.0014	0.9116 ± 0.0013	0.9037 ± 0.0019	0.8979 ± 0.0014	0.8823 ± 0.0016	0.8811 ± 0.0016
	E 90 %	0.9035 ± 0.0018	0.9191 ± 0.0018	0.9320 ± 0.0019	0.9241 ± 0.0016	0.9089 ± 0.0018	0.9201 ± 0.0014	0.9107 ± 0.0017	0.9123 ± 0.0016	0.9040 ± 0.0019	0.9124 ± 0.0016
	E 99 %	0.8784 ± 0.0017	0.9054 ± 0.0018	0.9128 ± 0.0018	0.9150 ± 0.0017	0.9001 ± 0.0020	0.9091 ± 0.0016	0.8988 ± 0.0015	0.9114 ± 0.0019	0.8983 ± 0.0017	0.9106 ± 0.0015
	F4 %	1.0109 ± 0.0026	1.0072 ± 0.0016	1.0169 ± 0.0012	1.0008 ± 0.0017	0.9763 ± 0.0013	0.9796 ± 0.0013	0.9813 ± 0.0016	0.9764 ± 0.0013	0.9810 ± 0.0018	0.9683 ± 0.0012
	Fs4 %	1.0188 ± 0.0021	1.0158 ± 0.0018	1.0198 ± 0.0016	1.0071 ± 0.0020	0.9854 ± 0.0019	0.9934 ± 0.0022	1.0086 ± 0.0019	0.9937 ± 0.0020	0.9983 ± 0.0017	0.9801 ± 0.0015
	F10 %	1.0142 ± 0.0014	1.0044 ± 0.0014	0.9949 ± 0.0015	0.9930 ± 0.0017	0.9613 ± 0.0013	0.9714 ± 0.0015	0.9892 ± 0.0016	0.9757 ± 0.0014	0.9786 ± 0.0013	0.9670 ± 0.0013
	Fs10 %	1.0332 ± 0.0027	1.0267 ± 0.0024	1.0361 ± 0.0029	1.0298 ± 0.0025	1.0092 ± 0.0024	1.0126 ± 0.0023	1.0280 ± 0.0027	1.0132 ± 0.0022	1.0104 ± 0.0024	1.0158 ± 0.0028
Diplodus sargus	E70 %	0.9058 ± 0.0019	0.8953 ± 0.0019	0.8910 ± 0.0019	0.9012 ± 0.0018	0.8857 ± 0.0019	0.8835 ± 0.0021	0.8864 ± 0.0020	0.8868 ± 0.0020	0.8754 ± 0.0018	0.8851 ± 0.0016
	E90 %	0.9027 ± 0.0019	0.8936 ± 0.0021	0.8897 ± 0.0019	0.8887 ± 0.0020	0.8771 ± 0.0019	0.8644 ± 0.0017	0.8632 ± 0.0017	0.8557 ± 0.0017	0.8489 ± 0.0016	0.8483 ± 0.0020
	E99 %	0.8845 ± 0.0020	0.8820 ± 0.0020	0.8783 ± 0.0017	0.8861 ± 0.0015	0.8701 ± 0.0020	0.8643 ± 0.0019	0.8687 ± 0.0021	0.8590 ± 0.0022	0.8444 ± 0.0022	0.8503 ± 0.0021
	F4 %	0.9395 ± 0.0020	0.9361 ± 0.0016	0.9306 ± 0.0017	0.9443 ± 0.0015	0.9359 ± 0.0018	0.9254 ± 0.0022	0.9267 ± 0.0018	0.9245 ± 0.0017	0.9353 ± 0.0016	0.9147 ± 0.0017
	Fs4 %	0.9456 ± 0.0014	0.9293 ± 0.0017	0.9270 ± 0.0019	0.9410 ± 0.0018	0.9341 ± 0.0019	0.9337 ± 0.0018	0.9320 ± 0.0019	0.9196 ± 0.0016	0.9321 ± 0.0018	0.9188 ± 0.0017
	F10 %	0.9432 ± 0.0018	0.9262 ± 0.0017	0.9218 ± 0.0017	0.9395 ± 0.0019	0.9274 ± 0.0019	0.9269 ± 0.0019	0.9239 ± 0.0019	0.9234 ± 0.0018	0.9276 ± 0.0016	0.9208 ± 0.0015
	Fs10 %	0.9399 ± 0.0018	0.9229 ± 0.0018	0.9207 ± 0.0016	0.9293 ± 0.0021	0.9283 ± 0.0018	0.9254 ± 0.0021	0.9249 ± 0.0021	0.9285 ± 0.0017	0.9217 ± 0.0017	0.9133 ± 0.0017
Sparus aurata	E70 %	0.9919 ± 0.0020	0.9779 ± 0.0019	0.9810 ± 0.0024	0.9854 ± 0.0023	0.9681 ± 0.0022	0.9551 ± 0.0020	0.9525 ± 0.0019	0.9558 ± 0.0020	0.9636 ± 0.0020	0.9504 ± 0.0018
	E90 %	0.9867 ± 0.0019	0.9786 ± 0.0019	0.9638 ± 0.0021	0.9665 ± 0.0019	0.9481 ± 0.0023	0.9434 ± 0.0020	0.9404 ± 0.0019	0.9399 ± 0.0017	0.9420 ± 0.0021	0.9427 ± 0.0018
	E99 %	0.9719 ± 0.0022	0.9762 ± 0.0020	0.9672 ± 0.0023	0.9687 ± 0.0022	0.9571 ± 0.0025	0.9536 ± 0.0022	0.9442 ± 0.0020	0.9474 ± 0.0023	0.9438 ± 0.0022	0.9422 ± 0.0023
	F4 %	0.9973 ± 0.0017	1.0073 ± 0.0022	0.9864 ± 0.0015	0.9987 ± 0.0018	0.9890 ± 0.0018	0.9710 ± 0.0017	0.9884 ± 0.0017	0.9791 ± 0.0018	0.9906 ± 0.0016	0.9913 ± 0.0019
	Fs4 %	0.9905 ± 0.0022	1.0039 ± 0.0022	0.9896 ± 0.0018	1.0020 ± 0.0018	0.9927 ± 0.0017	0.9712 ± 0.0017	0.9882 ± 0.0019	0.9849 ± 0.0021	0.9898 ± 0.0018	0.9869 ± 0.0015
	F10 %	0.9883 ± 0.0017	1.0021 ± 0.0022	0.9903 ± 0.0019	0.9984 ± 0.0018	0.9839 ± 0.0015	0.9675 ± 0.0015	0.9804 ± 0.0019	0.9729 ± 0.0017	0.9839 ± 0.0018	0.9829 ± 0.0019
	Fs10 %	0.9945 ± 0.0021	0.9968 ± 0.0019	0.9870 ± 0.0015	0.9985 ± 0.0017	0.9839 ± 0.0015	0.9662 ± 0.0017	0.9787 ± 0.0019	0.9759 ± 0.0016	0.9791 ± 0.0017	0.9833 ± 0.0016