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Validación y estandarización de una técnica para biopsia ovárica en el pargo flamenco, *Lutjanus guttatus* (Steindachner, 1869)

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ABSTRACT

The standardization and validity of a biopsy technique for the assessment of the ovarian development of the spotted rose snapper, *Lutjanus guttatus* (Steindachner, 1869), was studied. Ovarian oocytes at different stages were present along the length of the ovaries. Late vitellogenic oocytes are distinguished by their opacity under transmitted light. Diameters should be measured within 36 hours after fixation in 1 % formalin in 0.9 % ClNa solution, before their cytoplasm is cleared with Serra solution, to avoid distortions. Vitellogenic oocytes showed unimodal diameter frequency distributions, their means were not significantly different between the right and left ovaries ($P > 0.05$), and increased significantly from the anterior to posterior regions ($P < 0.05$). A sample size of 50 late vitellogenic oocytes was calculated for diameter mean estimates. Statistical analyses of *in vivo* cannulated oocytes were not significantly different ($P > 0.05$) from duplicate *in vitro* samples from the same ovarian region, as well as from paired *in vivo* samples. Estimates of the bias corrected coefficient of variation ($P < 0.05$) from five females were 1.10 ± 0.27 to 1.20 ± 0.30. The results of the present study led to accurate and precise information on ovarian development for selection of breeders in induced spawning experiments.

Key words: adjustment and justification tests, *Lutjanus guttatus,* ovarian *in vivo* sampling.

RESUMEN

Se estudió la estandarización y validación de una técnica de biopsia para la evaluación del desarrollo ovárico del pargo flamenco, *Lutjanus guttatus* (Steindachner, 1869). Ovocitos en diferentes estadios estaban presentes a lo largo de los ovarios. Los ovocitos vitelogénicos se distinguen por su opacidad bajo luz trasmitida. Los diámetros deben ser medidos dentro de las primeras 36 h después de su fijación en una solución de formalina al 1 % en 0,9 % de NaCl, antes de que su citoplasma sea clareado con solución Serra, para evitar distorsiones. Los ovocitos vitelogénicos mostraron una distribución de frecuencias de sus diámetros con una sola moda y sus medias no fueron significativamente diferentes entre los ovarios derecho e izquierdo ($P > 0,05$), y se incrementó significativamente de las regiones anterior a posterior ($P > 0,05$). Se calculó un tamaño de muestra de 50 ovocitos vitelogénicos tardíos para los estimados de los diámetros medios. Los análisis estadísticos de los ovocitos obtenidos con cánula *in vivo* no fueron diferentes significativamente de muestras duplicadas *in vitro*, así como de muestras pareadas *in vivo*, de la misma región ovárica ($P > 0,05$). Los estimados del coeficiente de variación corregidos para sesgos ($P <$ 0,05) de cinco hembras fueron 1,10 ± 0,27 a 1,20 ± 0,30. Los resultados del presente estudio aportaron información exacta y precisa sobre el desarrollo ovárico para la selección de reproductores en experimentos de inducción del desove.

Palabras clave: pruebas de ajuste y justificación, *Lutjanus guttatus*, muestreo ovárico *in vivo*.

INTRODUCTION

Methods for hormone-induced spawning of fish are becoming more refined and improved. Two critical areas include the stage of sexual development at which the individual females will be most receptive to a particular procedure and the appropriate hormone and dosage administered (Tamaru *et al.*, 1988). Particular attention must be paid to broodstock selection and maturation criteria (Shelton 1989). Shehadeh (1975) and Sundararaj (1981) recommended high priority to the development of simple, reliable, quick, and inexpensive methods for determining the broodstock stage of ovarian development. *In vivo* intra-ovarian oocytes sampled by standardized and validated biopsy procedures for each species make it possible for oocyte development stages to be objectively and accurately expressed (Shehadeh *et al.*, 1973; Kuo *et al.*, 1974; Garcia, 1989). These procedures can enable the identification of suitable broodstock for induced spawning, increasing the efficiency of inducement therapies (Crim and Glebe 1990).

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The majority of culture marine fishes can be hormone-treated when the final oocyte maturation (FOM) has not started and the germinal vesicle can be seen in a central position after the oocyte cytoplasm is cleared (Alvarez-Lajonchère *et al.*, 1991; Berlinsky *et al.*, 1996). This is the case of the spotted rose snapper, *Lutjanus guttatus* (Steindachner, 1869) (Ibarra-Castro and Alvarez-Lajonchère, 2009). However, for some species, hormone-induced ovulation is recommended to be applied after FOM has started (Rottmann *et al.* 1991; Mylonas *et al.* 1997), and most freshwater fishes, such as common carp and Chinese carps (W.L. Shelton, Oklahoma State University, 2011, personal communication to L.A.-L.).

The use of biopsy techniques has been extended to different species. For some of them the procedures have been adapted to the characteristics of their sexual development, and tests have been made to standardize and validate the procedures with group synchronous oocyte development species (Markmann and Doroshov 1983; Alvarez-Lajonchère *et al.*, 1983; Rodriguez and Garza, 1986; Tamaru *et al.*, 1988), and asynchronous oocyte development species (Garcia 1989; Franscescon *et al.* 1992; Alvarez-Lajonchère *et al.*, 2001; Coward and Bromage, 2001; Ferraz *et al.*, 2004). Harvey and Carolsfeld (1993) recommended these studies when new species are to be bred, pointing out that success would depend on the ability to judge the correct oocyte maturity required to achieve a successful induced breeding.

Snappers (Family Lutjanidae) are important food and recreational species throughout the tropical and subtropical regions, with high price and strong demand. Spotted rose snapper is a popular Pacific snapper in several American countries. It is a batch spawner with asynchronous ovarian development and a long reproductive season with several spawning periods (Cruz-Romero *et al.*, 1996). Ibarra-Castro and Duncan (2007) applied a biopsy technique similar to one described for common carp (Rodriguez and Garza 1986) to develop an induced spawning protocol with GnRHa implants for wild spotted rose snapper breeders, although they reported important variations on spawning success, egg fecundity, and egg quality.

The objectives of the present study were to develop a biopsy technique to determine the characteristics of ovarian development of the spotted rose snapper, as well as standardize and statistically validate the procedure as a reliable reference to improve the female selection for induce spawning trials.

MATERIALS AND METHODS

Experimental Fish

The study was carried out during the main spawning season of spotted rose snapper from spring to autumn (26-28 ºC) at Sayulita Beach, Nayarit (México). Mature females were caught from coastal reef areas with a beach seine operated by scuba fishermen, and immediately transported to the laboratory, in 500-L high density polyethylene tanks filled with seawater (35 g/l and 28 ºC) continuously aerated by a battery operated blower during the 2-h trip.

At the laboratory each female was anesthetized with 300 ppm of 2-phenoxyethanol (Sigma Chemical Co., Toluca, Mexico) to reach the stage of deep anesthesia (total loss of equilibrium). Body weight (BW) and total length (TL) of the experimental fish were 290-450 g and 260 – 291 mm, respectively.

Validation and Standardization tests

I. Comparisons between the right and left ovarian lobes. Three females were sacrificed by immersion in 300 ppm of 2-phenoxyethanol (Sigma Chemical Co., Toluca, Mexico) to reach the stage of surgical anesthesia, followed by spinal dislocation. Pair samples of the anterior, central and posterior regions were taken from the left and the right ovaries of each female, and 50 oocytes from each sample were observed and measured to the nearest 10-µm under transmitted light using a compound microscope with a calibrated ocular micrometer, 1-1.5 h after their fixation in the preservation solution of 1 % formalin in 0.9 % NaCl. Afterwards, measurements of oocyte diameters at the late vitellogenic stage were carried out to complete 50 per sample.

II. Establishing the observation and fixation techniques. Fresh samples of intra-ovarian oocytes from the posterior ovarian region of four females were removed with a flexible polyethylene catheter (PE No. 160, Clay Adams with an inner diameter of about 1.1 mm and an outer diameter of 1.6 mm), blown into glass vials with a solution of 1 % formalin in 0.9 % NaCl, vigorously shaken, and afterwards introduced in a Sedgwick Rafter counting chamber for observation under transmitted light using a compound microscope with a calibrated ocular micrometer. About 200-300 oocytes were collected in each biopsy sample, and 120 vitellogenic oocytes were observed and their diameter measured to the nearest 10-µm while fresh and after fixation in a preservation solution of 1 % formalin in 0.9 % NaCl, at 1, 2, 4, 8, 24, 36 h intervals. Another oocyte sample was introduced in a Sedgwick Rafter counting chamber and 120 oocytes were measured within the preservation solution for 1-1.5 h, and again 3 min after the addition of a few drops of Serra solution(6:3:1 v/v ethanol: pure formaldehyde (37 %): pure glacial acetic acid), which is usually used to clear the cytoplasm for the identification of any evidence of

oocyte maturation (Makeeva *et al.* 1988; Shelton 1989; Stoeckel and Neves 1992). Late vitellogenic oocytes were identified by the complete opacity of their cytoplasm under transmitted light, resulting from packed yolk globule accumulation (Shehadeh *et al.* 1973) before cytoplasm clearance. The Wallace and Selman (1981) classification system for oocyte development stages was followed, together with the corresponding stages described by Kuo *et al.* (1974), as well as those described by Yaron (1995) for final maturation stages.

III. Identifying differences in oocytes development stages and diameters along the length of the ovaries, to determine sampling region. Six females were sacrificed with the procedure described in section (I) above. Samples of the anterior, central and posterior regions were taken from the left ovary of each female, and 120 oocytes were observed and measured as described in section (II). Afterwards, measurements of oocytes at the late vitellogenic stage were carried out to complete 120 per sample. Each sample of 120 late vitellogenic oocytes was observed again 2-3 min after the addition of a few drops of Serra solution, searching for evidence of oocyte maturation (e.g., lipid-droplet coalescence or germinal vesicle migration, yolk-globule coalescence, and hydration).

IV. Calculating sample size to estimate mean diameters and power for ANOVA. The iterative procedure described by Sokal and Rohlf (1995) was applied with the diameters measured as described in section (II) of 120 cannulated late vitellogenic oocyte samples from the posterior region of five females, and their means and standard errors of the means were calculated.

The formula and assumptions applied through iteration were:

$N \geq 2\,(\sigma/\,\delta)^2\,\{t_{\delta\,[\mathrm{Cv}]} + t_{2(1-P)[\mathrm{V}]}\}^2$

Where N = sample size.

σ = true standard deviation ≈ s (= CV * $\bar{Y}$/100).

$\bar{Y}$ = mean.

δ = smallest true difference between two means of three ovary sites, where a 5% difference between two means of the three ovary sites is desired.

υ = degrees of freedom if the standard deviation of the samples with three groups and "n" replications (= possible sample size to be applied through iteration in the formula) per group.

α = significance level = 0.01.

P = desired probability that a difference will be significant (the intended power of the test) = 95 % certainty.

CV = coefficient of variation previously estimated from 120 oocytes samples from five females = 6 %.

$t_{\delta\,[V]}$ and $t_{2(1-P)\,[V]}$ = values from a two tailed t-table with v degrees of freedom and corresponding to probabilities of α and 2 (1 – *P*), respectively.

V. Determining if biopsy samples are representative of the ovarian region from which they were taken. Statistical analyses of paired *in vivo* and *in vitro* samples from six females were carried out. *In vivo* intra-ovarian samples were taken by inserting the polyethylene catheter into the gonad pore up through the oviduct approximately 2-3 cm from the point of entry, and 120 oocytes were observed and measured as described in section (II). The *in vitro* samples were taken from the posterior region of the left ovary after the females were sacrificed following the above described procedure. In each sample, 120 late vitellogenic oocyte diameters were measured as describe in section (II).

VI. Calculating the precision of estimates of mean oocyte diameter obtained by the biopsy procedure. Statistical analyses of 10 *in vivo* samples obtained with a catheter from the posterior region of five females, and 50 late vitellogenic oocyte diameters measured as described in section (II) from each sample were carried out, and the bias corrected coefficient of variation with its confidence limits was calculated following the formula recommended by Sokal and Rohlf (1995):

$CV^* \pm t_{0.05\,[N]}\, Sv^*$

Where CV* = the bias corrected coefficient of variation = CV* = (1 + 1/4n) CV

CV = the coefficient of variation without the bias correction = 100 SD / mean

SD = standard deviation

Scv* = unbiased estimate of the standard error of the coefficient of variation = (1 + 1/4n) Scv

Where: n = number of females

Scv = biased estimate of the standard error of the coefficient of variation = CV/√2n

Statistical Analyses

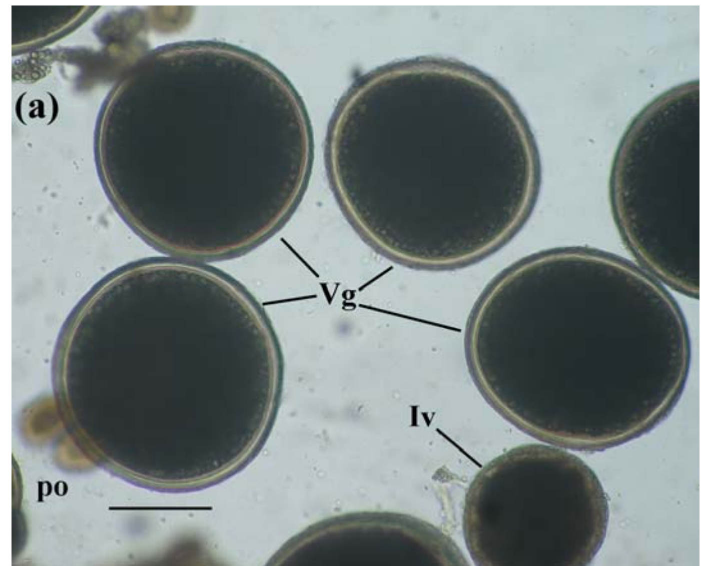
All data were subject to normality and homogeneity of variance analyses. Statistical analyses were performed using GraphPad Prism® versión 5.0 for Windows (GraphPad Software Inc 1992–2007, La Jolla, USA). Statistical differences between oocyte mean diameters were calculated by one-way analysis of variance (ANOVA), followed by a Tukey's multiple comparison test when significant differences were detected. Differences between frequency distributions of the diameters were calculated by Chi-square determinations, and Student's t-tests were used to compare means of paired data sets. In the case of percentages values of the development stages, their mean ± SE were calculated from six females for each of the ovary regions and compared at each stage using Log transformation by a one-way ANOVA test. Differences were accepted as significant when $P \leq 0.05$ (Sokal and Rohlf 1995) or otherwise specified. When analyses were carried out for several females, the corresponding statistic range was specified.

RESULTS

I. Comparisons between the right and left ovarian lobes. Both ovary lobes (right and left) of the three females had oocytes in different development stages showing no significant difference between stages of development of the three ovarian regions from the two ovarian lobes in each female (ANOVA, $F_{0.05\ (1)\ 2,\ 11}$ = 0.007 – 0.033, $P \geq 0.99$). The diameters of late vitellogenic oocytes from the three regions of the two ovarian lobes of the three females showed no significant differences between their means ($t_{0.05\ (1),\ 98}$ = 0.149–0.318) or their frequencies ($X^2_{0.05,\ 7}$ = 0.33-6.27).

II. Establishing the observation and fixation techniques. The ovaries of all females examined had oocytes at different development stages. The vitellogenic oocytes were characterized by opaqueness when observed under transmitted light without the clearing solution. Intensity varied from partial opaqueness, almost always darkened at the center and decreasing towards the edges in early vitellogenic oocytes, to completely opaque in late vitellogenic oocytes (Figure 1a). There were no significant distortions of preserved oocytes, in mean diameter ($t_{0.05\ (1),\ 238}$ = 0.244-1.429) or diameter frequency distributions ($X^2_{0.05,\ 7}$ =2.042-13.336), within the first 36 h after their immersion in the fixation solution, compared to fresh oocytes. After no more than 3 min, the Serra solution had cleared the cytoplasm, making possible to observe the packed yolk globules, while the germinal vesicle was visible as a dark spherical structure in a central position, and lipid droplets were not visible (Figure 1b), which corresponds to stage I of Yaron (1995) scale, and to the end of the tertiary yolk globule stage of Kuo *et al.*, (1974). However, after more than 3 min in the Serra solution there was a significant oocyte shrinkage of about 4-6 % (20 – 26 µm) in mean diameter ($t_{0.05,\ 238}$ = 0.330-4.351).

III. Identifying differences in oocyte development stages and diameters along the length of the ovaries, to determine the sampling region. There were no significant differences in development stages of the three ovarian regions from the six females (ANOVA, $F_{0.05\ (1)\ 2,\ 11}$ = 0.003–0.034, $P \geq 0.99$). Yolk vesicle stage oocytes or pre-vitellogenic oocytes (115-230 µm) ranged from 52.3 ± 11.5 % to 61.9 ± 13.0 %; early vitellogenic oocytes (primary yolk globule stage of Kuo *et al.* 1974) (220-325 µm) ranged from 15.7 ± 1.9 % to 19.2 ± 2.9 %; late vitellogenic and post vitellogenic oocytes (tertiary yolk globule stage of Kuo *et al.* 1974) (325-550 µm) ranged from 22.2 ± 11.5 % to 24.7 ± 11.7 %, and oocytes showing atresia were very few (1.7 ± 0.3 % to 2.3 ± 0.4 %). No mature oocytes were found or was there any indication that the final maturation stage had started. Late vitellogenic oocytes had a unimodal diameter frequency distribution, with varying frequencies by individual females.



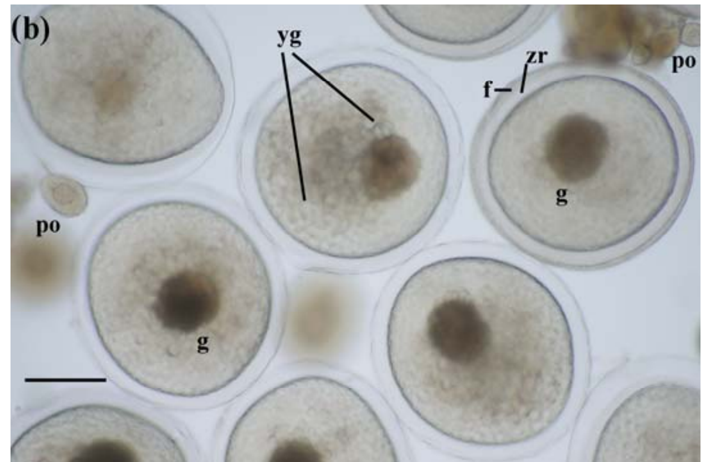


Figure 1. Photomicrographs of spotted rose snapper, *Lutjanus guttatus*, intra-ovarian follicles obtained by biopsy and observed under transmitted light (horizontal bars = 200 µm), showing: a) several completely opaque late vitellogenic oocytes (Vg) and one in early vitellogenesis (Iv); b) late vitellogenic oocyte cleared by Serra solution, with the germinal vesicles (g) centrally located, follicular layer (f), zona radiata (zr), packed yolk globules (Yg), and primary oocytes (po) can be seen.

The comparison of paired *in vitro* samples of each of three ovary portions (anterior, central, and posterior) from the left ovary lobes of the six females were not significantly different in mean oocyte diameters ($t_{0.05(1),\ 238}$ = 0.15-0.32) or diameter frequency distribution ($X^2_{0.05,\ 7}$ = 0.33-6.27). A significant difference ($P < 0.05$) between diameters of oocytes from the anterior to the posterior regions taken from the left ovary lobe of six females was observed (ANOVA, $F_{0.05\ (1)\ 2,\ 147}$ = 4.899-8.48, P<0.0001), with the oocytes of the posterior region being significantly larger than the oocytes from the anterior region, in all cases (Tukey, $Q_{0,05,\ 1,\ 98}$ = 4.390–9.826, $P < 0.05$) (Figure 2). The mean diameter

increased from the anterior to the posterior regions in 31.8 ± 7.1 µm (24-48 µm), being 5-12 % larger than that of the anterior region. The middle portion of the posterior ovary region could be attained at about 2-3 cm from the genital pore.

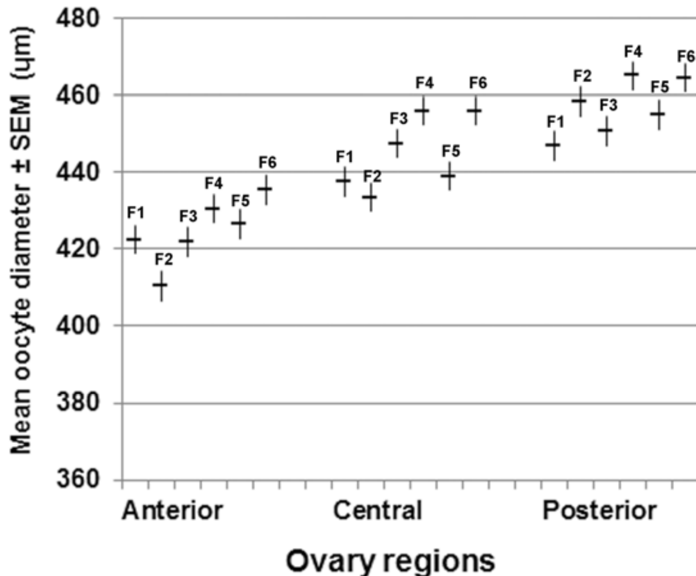


Figure 2. Oocyte measurements (mean oocyte diameter and 95% confidence intervals, represented by the horizontal and vertical bars, respectively), of 120 late vitellogenic oocyte from the ovarian samples obtained *in vitro* from the anterior, central, and posterior ovary regions of six individual females of spotted rose snappers, *Lutjanus guttatus*.

IV. Calculating sample size to estimate mean diameters and power for ANOVA. According to our calculations, a sample size of 50 oocytes was required for an ANOVA, with a 95 % certainty of detecting a 5 % difference between diameter means at the 1 % level of significance.

V. Determining if biopsy samples are representative of the ovarian region from which they were taken. No statistical analyses of paired *in vivo* and *in vitro* samples taken from the posterior region of ovaries of six females showed no significant differences in mean diameters (P = 0.2658-0.3872) ($t_{0.05}$ 242 = 0.2875-0.5056) or diameter frequency distributions (P > 0.05) (X^2 7 = 3.067-11.341) (Figure 3). Duplicate *in vivo* samples taken from the posterior region of the ovaries of four females did not show significant differrences in late vitellogenic oocyte frequency distribution (P > 0.05) (X^2 7 = 6.243-9.888) or mean diameters (P = 0.177-0.471) ($t_{0.05}$ 242 = 0.074-0.927).

VI. Calculating the precision of estimates of mean oocyte diameter obtained by the biopsy procedure. The bias corrected coefficients of variation with confidence limits for 10 means of repeated *in vivo* samples of 50 oocytes each from five females were: CV* ± t $_{0.05\ [10]}$ Scv* = 1.10 ± 0.27 to 1.2 ± 0.30. The bias corrected coefficient of variation calculated from 10 means of repeated *in vivo* samples of the first 20 oocytes measured in each *in vivo* sample was CV* ± t $_{0.05\ [10]}$ Scv* = 3.90 ± 0.60.

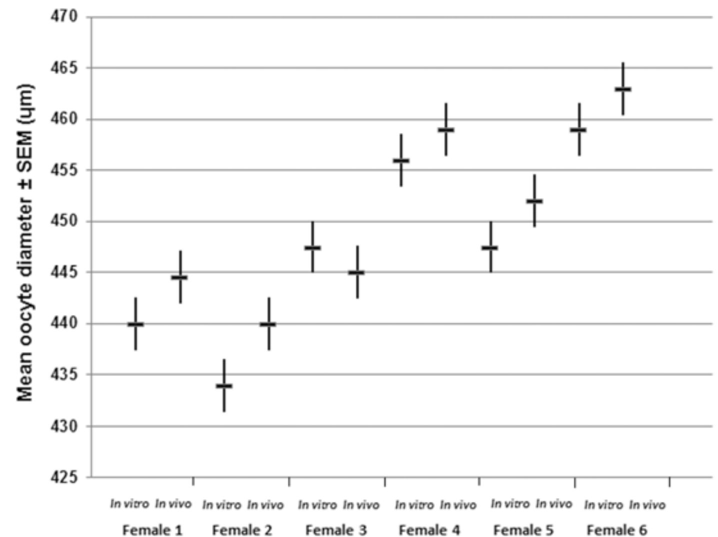


Figure 3. Oocyte measurements (mean oocyte diameter and 95% confidence intervals, represented by horizontal and vertical bars, respectively) of 50 late vitellogenic oocytes of paired ovarian samples obtained *in vitro* and *in vivo* from six individual females of spotted rose snappers, *Lutjanus guttatus*.

DISCUSSION

The presence of oocytes at all development stages, except for final maturation, verified that an asynchronous development pattern characterize snapper species (Grimes, 1987), confirming previous studies with spotted rose snapper (Cruz-Romero *et al.*, 1996). A variety of procedures have been used to observe and measure fish oocytes. Procedures include unpreserved oocytes (Thomas *et al.*, 1994), but most studies used various fixatives, usually 1 to 10 % formalin solutions, although some studies used physiological solution (Markmann and Doroshov, 1983). Ibarra-Castro and Duncan (2007) introduced the spotted rose snapper oocytes directly into the clearing solution and oocyte diameters were measured afterwards. The choice of the proper fixation procedure is very important, and must be statistically validated as fixatives can induce swelling or shrinkage (Garcia, 1989; Harvey and Carolsfeld, 1993). The present study demonstrated that a fixation solution of 1 % formalin in saline solution acts as an adequate preservative without inducing significant distortion in oocytes within 36 h. These results are in agreement with Shehadeh et al. (1973) and Franscescon *et al.* (1992). However, Larsson *et al.* (1997) reported a 12 % average swelling of yellowtail

flounder *Pleuronectes ferrugineus* oocytes after 24 h with the same solution.

The fact that the right and left ovary lobes were not significantly different in their sexual development, as shown in this study, demonstrate that biopsy samples are representative of the ovary development, since in live females it is impossible to know from which ovary lobe was the *in vivo* sample was taken.

An understanding of how the sexual development of oocytes occurs along the length of the ovaries also requires standardization in each species, so as to select a representative sample. In some species, there are no significant differences (Franscescon *et al.*, 1992; Coward and Bromage, 2001) while oocytes at the posterior ovarian region are larger in other species (Beumer, 1979; Alvarez-Lajonchère *et al.*, 1991), as was found in the present study.

The size at which vitellogenesis is complete is a species-specific characteristic (Mayer *et al.*, 1988). In gilthead seabream, Franscescon *et al.* (1992) reported mean diameters of 550-600 µm for oocytes at the end of vitellogenesis. There is a disagreement on this point, because there is a change in size with older, larger fish and certainly there is a variation depending on the amount of yolk that is deposited under various levels of nutrition (W. L. Shelton, Oklahoma State University, 2011, personal communication to L.A.-L.). Hence, it is important to estimate the diameter of the largest vitellogenic oocytes (Franscescon *et al.*, 1992; Mylonas *et al.*, 1996, 1997), on which hormones will have the strongest effect (Rottmann *et al.*, 1991; Thomas *et al.*, 1994). The site for *in vivo* oocytes sampling in the spotted rose snapper should be the posterior region, as demonstrated in the present study. Reports on precision of measurements oocytes diameter showed variations from 50 µm (Shehadeh *et al.*, 1973), to 5-12 µm (Garcia, 1989; Franscescon *et al.*, 1992). Oocyte measurements to the nearest 10 µm may have positively influenced the general precision in this study, with a low coefficient of variation (Alvarez-Lajonchère *et al.*, 2001; Ferraz *et al.*, 2004).

As in other sampling procedures a test for sample size, based on the variability of the parameter, must be made to obtain reliable estimates. In previous reports sample size varied from a range of 8-20 (Franscescon *et al.*, 1992; Berlinsky *et al.*, 1996; Mylonas et al. 1996, 1997), to 100 or more oocytes (Shehadeh *et al.* 1973; Alvarez-Lajonchère *et al.*, 1983; Tamaru *et al.*, 1988). The sample size of 50 late vitellogenic oocytes was calculated for this study, as in Ferraz *et al.* (2004), which is 150 % larger than the number of oocytes measured in previous studies with the spotted rose snapper (Ibarra-Castro and Duncan, 2007), and precision increased more than 2.5 times.

Here was demonstrated that *in vivo* samples were representative of the oocytes from the biopsy sampled region, in agreement with similar tests carried out in other species (Shehadeh *et al.*, 1973; Franscescon *et al.*, 1992; Alvarez-Lajonchère *et al.*, 2001; Coward and Bromage, 2001; Ferraz *et al.*, 2004).

Late vitellogenic oocyte diameters have been found to correlate with their development in many species. Hence, it has been used as an objective estimate of the most receptive stage of maturity for induced spawning and for breeder selection (Harvey and Carolsfeld, 1993). However, large differences between species have been reported. The critical initial oocyte diameter in channel catfish, *Ictalurus punctatus* (Rafinesque, 1818), is 2.3 mm (Markmann and Doroshov, 1983), 850 µm in striped bass, *Morone saxatilis* (Walbaum, 1792), (Mylonas *et al.*, 1997), 700 µm in white bass, *Morone chrysops* (Rafinesque, 1820), (Mylonas *et al.*, 1996), 600 µm in striped mullet, *Mugil cephalus* Linné, 1758, (Kuo *et al.*, 1974), 500 µm in several marine species (Francescon *et al.*, 1992; Thomas *et al.*, 1994; Berlinsky *et al.*, 1996), and > 400 µm for mangrove red snapper, *Lutjanus argentimaculatus* (Forsskål, 1775), (Emata, 2003) and Asian sea bass, *Lates calcarifer* (Bloch, 1790), (Schipp *et al.*, 2007). Oocyte frequency distribution can also directly influence induced spawning success in some species, such as in milkfish (Tamaru *et al.*, 1988).

Asynchronous development and batch spawning in an extended season (Cruz-Romero *et al.*, 1996) results in the high variability of rose spotted snapper oocyte. Consequently, a rigorous gonad biopsy sampling procedure is required to assure the high accuracy and precision of the estimates on which the selection of broodstock for hormone induced therapy could be based. The standardization of the biopsy procedure as a result of the present study has improved the estimation of intra-ovarian oocyte development, decreasing sampling error and preventing variations between females for spawning experiments, which should not be considered statistically or biologically homogeneous (Ibarra-Castro and Alvarez -Lajonchère, 2011).

The current biopsy procedure was experimentally applied during induced spawning (Ibarra-Castro and Alvarez-Lajonchère, 2009, 2011). The new procedure enabled unbiased and precise estimates of mean oocyte diameter, which made it possible to estimate the relationship between the effective dose of GnRHa EVAc implants and oocyte diameter, improving the reliability on spawner selection and induced spawning procedures compared to the previous biopsy method applied at the same laboratory (Ibarra-Castro and Duncan, 2007).

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