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Contrasting batch fecundity estimates of albacore (*Thunnus alalunga*), an indeterminate spawner, by different laboratory techniques

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ABSTRACT

A range of methods can be applied to estimate the batch fecundity of species with an indeterminate reproductive strategy. The traditional Hydrated Oocyte (HO) method based on direct counts of hydrated oocytes is the easiest and most accurate method but the main problem with this method is the shortage of hydrated ovaries in sampled fish such as tuna species. Batch fecundity estimates of albacore *Thunnus alalunga* resulting from counts of migratory nucleus (MG) oocytes using the application of the Weibel and Gomez (W&G), Physical Disector (PD), Oocyte Packing Density (OPD), and HO methods were compared using the last method as "control". Postovulatory follicles (POFs) were also counted using the PD method. Correction factors due to shrinkage were considered in the application of the different methods. Our results showed the highest batch fecundity estimates were obtained with the design-based PD method. The outputs from the assumption-based W&G and the theoretical OPD methods were closest to the HO method. Annotations of POFs instead of MG oocytes gave markedly lower values. The new OPD method was used to estimate batch and relative fecundity on a larger sample of fish (selected according to their length). The relationships between batch and relative fecundity estimates of albacore and the associated biological metrics (length, body weight and ovary weight) were investigated. Batch fecundity estimates ranged from 0.42 to 2.16 million oocytes with a mean relative batch fecundity of 136 oocytes per gram of body weight. The batch fecundity was shown to increase with fish size (length and weight) and gonad weight, while relative batch fecundity (g^{-1}) was related only to gonad weight.

1. Introduction

Fecundity is a biological parameter of interest to fisheries researchers as a basic aspect of population dynamics (Hunter et al., 1992). The estimation of fecundity for species with an indeterminate reproductive strategy ("indeterminate species" from now on) is particularly complicated because annual fecundity is not fixed prior to the start of the spawning season (Hunter et al., 1992). In these species, potential annual fecundity should be estimated from the number of eggs spawned per batch (batch fecundity) and the number of spawning events per season (Hunter et al., 1985; Murua et al., 2003). Nowadays a variety of methods can be applied to estimate the batch fecundity (BF) and the method chosen by researchers may depend upon the available resources (see Ganias et al., 2014; Kjesbu, 2009; Murua et al., 2003).

The most common method used for estimating the BF in indeterminate species is the Hydrated Oocyte (HO) method developed by Hunter et al. (1985), not only because of its simple application, but also because only ordinary laboratory facilities, e.g. a stereomicroscope, are needed. Initially the procedure for the application of the HO method only included ovaries that contained hydrated oocytes as the most advanced group of oocytes (and no POFs) because these oocytes are easily distinguished from other oocytes and should properly reflect the "batch of eggs" soon to be spawned (Hunter et al., 1985). Later, ovaries that contained migratory nucleus (germinal) (MG) oocytes (the final stage of oocyte maturation before hydration) were also considered for BF estimations (Hunter et al., 1992; Schaefer, 1996). The background for this being that specimens with hydrated ovaries, such as tuna species, are seldom found in the catch (Schaefer, 2001). Batch fecundity estimates obtained by applying methods that required histological sections are also seen in marine laboratories. Although histology is both time-consuming and expensive, microscopic examination of the ovarian tissue makes possible to accurately distinguish the

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different oocyte developmental stages, as well as the presence of postovulatory follicles (POFs) and atretic oocytes and thereby, allow an unequivocal characterization of the reproductive phase. The earliest and the most widely used stereological method for estimating oocyte numbers is the assumption-based method of Weibel and Gomez (1962) (W&G). This method requires assumptions to be made regarding particle shape and size distribution. Nevertheless, it has been successfully used to estimate fecundity for many fish species (Coward and Bromage, 1998; Emerson et al., 1990; Haslob et al., 2013; Knapp et al., 2014; Medina et al., 2002, 2007). Two decades later Sterio (1984) introduced the physical disector (PD) method, i.e. a stereological method for design-based particle number estimation, which so far has been in limited use among fish reproductive biologist because of the large work load involved (Aragón et al., 2010; Bucholtz et al., 2013; Kraus et al., 2008). The PD method has, however, recently been applied to reflect realized batch fecundity from counts of postovulatory follicles in bluefin tuna (*Thunnus thynnus*), an indeterminate species (Aragón et al., 2010; Aranda et al., 2011, 2013). Note that these true stereological methods, W&G and PD, do not directly give the absolute number of particles but the numeric density within a reference volume. Finally, Oocyte Packing Density (OPD) theory (Kurita and Kjesbu, 2009) has been used for estimating the numbers of different stages of oocytes per gram of ovary in indeterminate species (Korta et al., 2010; Kurita et al., 2009; Kurita and Kjesbu, 2009; Saber et al., 2015a; Schismenou et al., 2012). This newer method which is strictly speaking not a true stereological method, only a part of it, i.e. the estimation of volume fraction, can, as with the two others, be scaled (by including ovary size) to give batch or potential fecundity (Ganias et al., 2014; Schismenou et al., 2012).

Only a few studies comparing fecundity methods have been published throughout time (Aragón et al., 2010; Cooper et al., 2005; Coward and Bromage, 2002; Emerson et al., 1990; Kjesbu et al., 1998). Before the use of the PD method in fisheries laboratories and the introduction of OPD theory, the most relevant paper was written by Emerson et al. (1990). These authors compared estimates of the batch fecundity using three methods and described the advantages of the novelty of the stereological method in that moment, the W&G method, over the volumetric and automated particle counter ("fish egg counter") methods, while the recent study of Aragón et al. (2010) evaluated the differences in the estimated number of oocytes in each developmental stage obtained by applying the W&G and PD methods.

The present study used albacore *Thunnus alalunga*, a batch spawner with asynchronous oocyte development and indeterminate fecundity (Otsu and Uchida, 1959) to contrast a defined variety of suitable fecundity methods. More specifically, the aim of this study was to compare batch fecundity estimates of spawning albacore resulting from counts of MG oocytes from the W&G, PD, OPD and HO methods. In addition, batch fecundity estimations carried out from counts of POFs using the PD were also compared. Corrections factors due to shrinkage were considered in the application of the different methods and benefits and limitations of the methods applied were discussed. Another objective of this study was to further test the application of the OPD method for estimating batch and relative fecundities for female albacore selected according to their size and then, to investigate the relationships between these reproductive traits and the associated biological metrics (length, body weight and ovary weight).

2. Material and methods

Albacore (*T. alalunga*) ovaries were collected from fish caught by commercial and recreational fisheries in the western Mediterranean Sea between 2005 and 2012 (Saber et al., 2015b). Fork length (cm), fresh total body weight (kg) and gonad weight (GW, g) were recorded. As part of this large sampling program, histology was applied for detailed maturity staging finding that 27% of the females showed MG oocytes as the most advanced group of oocytes within their ovaries, for more detail see Saber et al. (2015b). In the current study 61 out of these 157 females were selected according to their fork length (*FL*) to estimate batch fecundity (BF), i.e. the total number of eggs (here substituted by MG oocytes) released in a single spawning event, using advanced oocyte packing density (OPD) theory (Kurita and Kjesbu, 2009). Prior to initiating this work, a total of 12 ovaries, selected out of the 61 ovaries, were used to directly compare these theoretical results with those given from the application of the classical Weibel and Gomez (W&G) method (Weibel and Gomez, 1962) and the modern physical disector (PD) method (Sterio, 1984), both being fully stereological in nature. Given that small POFs, which represent the follicular remains in the ovary after the eggs are *de facto* spawned, coexisted with MG oocytes in all ovaries, the PD method was also applied to estimate the BF based on counts of POFs. Hence the PD method was split into two: PD_{MG} and PD_{POF}. The traditional Hydrated Oocyte (HO) method (Hunter et al., 1985) was also applied to estimate the number of MG oocytes. For proper comparisons, the HO method was defined as "control" since this method is based on direct counting of oocytes in subsamples using gravimetric principles to indicate raising factor of gonad weight to analyzed subsample weights. An additional sample of 16 fresh ovaries, all subsequently classified histologically as "spawning", was collected in the same area in July 2013 to estimate loss in ovarian tissue weight and volume during histological processing (see below).

The following sections give more background information on methods used in the detailed comparative analysis on fecundity estimation for the 12 specimens but also address the subsequent OPD study on the 61 specimens.

2.1. Approaches and methods used to enumerate oocytes using histology

Assuming a homogeneous distribution of oocytes within and between ovarian lobes, as documented earlier for tunas (Otsu and Uchida, 1959; Stéquert and Ramcharrun, 1995), a 2 – 3 cm wide cross-section from the central part of one of the lobes was fixed in Bouin's fluid for four hours and then preserved in 70% ethanol. Then, a representative subsample (from the ovarian wall to the lumen) was taken from each of preserved ovary, dehydrated through increasing concentrations of ethanol series, cleared, and embedded in paraffin. Sections were cut at 10 μm and stained with either Mallory's trichrome stain or haematoxylin-VOF (Gutiérrez, 1967). All images were taken on a Nikon photomicroscope with either ×4 (W&G and OPD methods) or ×2 (PD method) objective magnifications. Significant differences in the distribution of oocytes among the centre, mid-region and periphery of the same albacore ovarian section has been found (Otsu and Uchida, 1959). Thus, in order to avoid biased estimations due to these transversal structural differences affecting oocyte distribution, the set of micrographs of each ovary was taking randomly but representing the whole ovarian section. The resulting area of each micrograph was 3.302 mm^2 and 13.552 mm^2 for ×4 and ×2 objective magnifications, respectively.

2.1.1. Assumption-based stereological method: Weibel and Gomez

The numerical density (N_v) of the particles of interest, i.e. the number of MG oocytes per unit volume, was calculated according to principles first developed by Weibel and Gomez (1962) but using here the further modified formula in Weibel et al. (1966). The W&G stereological method includes "the Delesse principle", which states that the fractional volume (V_i) of a component *i* in a given tis-

sue (here fish ovary) is proportional to its fractional cross-sectional area (A_i) (Emerson et al., 1990) (note that the same principle is also applied in the below OPD theory). The resulting main W&G formula reads:

$$N_v = \frac{k}{\beta} \times \frac{{N_a}^{3/2}}{{V_i}^{1/2}} \quad (1)$$

where k is the MG oocyte size distribution coefficient, β the MG oocyte shape coefficient, N_a the average number of MG oocytes per unit of area, and V_i the average volume fraction occupied by MG oocytes. The parametric value of k (range: 1.001–1.004) was determined (Emerson et al., 1990) for each ovary separately. The factor β was parameterized using the function provided by Weibel and Gomez (1962) for ellipsoidal particles and was calculated from the 12 ovary samples (ten MG oocytes of each ovary, i.e. 120 oocytes) and found to be typically 1.445 (range: 1.394–1.540). The same information was given by extending this analysis to include all the 61 ovaries (ten MG oocytes of each ovary, i.e. 610 oocytes). Migratory nucleus oocytes were measured from micrographs using ImageJ software (http://rsb.info.nih.gov/ij) and the plugin ObjectJ (http://simon.bio.uva.nl/objectj/). Ten micrographs were used for the estimation of the V_i and N_a values (Coward and Bromage, 2002; Knapp et al., 2014; Medina et al., 2007). A Weibel grid, short linear probes of 107.9 μm (128 lines, i.e. 256 points), embedded in a frame with an area of 2.578 mm^2, was overlaid on each of the ten micrographs for estimating the parametric value of V_i, using again ImageJ. For the estimation of the parametric value of N_a, the number of MG oocytes located within this frame and those overlapping the right and top borders of this frame were counted.

2.1.2. Design-based stereological method: the physical disector

The physical disector, a stereological unbiased method used for counting particles by means of randomly selected counting pairs, was first described by Sterio (1984). In practical operation it consists of two consecutive sections with a known distance apart, referred to as "reference section" and "look-up section". The particles are counted if they appear in the counting frame on the reference section, but not in the look-up section and subsequently, the look-up section serves the function as reference section and vice versa, i.e. the counting pairs were presently used in both directions (Sterio, 1984). As mentioned for W&G (N_a) above, any particles which touch the left or bottom borders of the counting frame (forbidden lines) were not counted (Howard and Reed, 2010; Sterio, 1984). The numerical density (N_v) of particles, here not only referring to the number of MG oocytes (cf. W&G) but also to POFs (that is, the follicular layers that remain in the ovary after the release of the egg) per unit volume was calculated according to the following formula:

$$N_v = \frac{\Sigma Q^-}{(\Sigma P \times a/f \times h)} \quad (2)$$

where ΣQ^- is the total number of particles counted, ΣP is the total number of disector counting frames, a/f is the area per counting frame, and h is the distance between sections (disector height). Similar to other tuna species studies (Aragón et al., 2010; Aranda et al., 2011, 2013), the total of counting frames per ovary was set to be 24, and the area of the frame of each disector was 10.594 mm^2. The sections were separated by a distance of about 1/4 to 1/3 of the smallest particle size (Andersen, 2003). In the case of a POF, the whole structure was considered as the counting particle (Aragón et al., 2010; Aranda et al., 2011, 2013). For MG oocytes the nucleus was used as the counting unit (Aragón et al., 2010; Aranda et al., 2011; Bucholtz et al., 2013). The proper distance between sections was determinate measuring 25 POFs (using the 10 μm consecutive sections) and a hundred nuclear sizes of MG oocytes (using the ImageJ software). The resultant h was 40 μm for MG oocytes as well as for POFs.

2.1.3. Theoretical oocyte packing density (OPD)

The oocyte packing density (OPD) was estimated theoretically for MG oocytes based on the formula developed by Kurita and Kjesbu (2009):

$$\log(OPD_{MG}) = \log[V_i \times (\frac{1}{\rho_o}) \times \left\{\frac{(1+k)^3}{(8 \times k)}\right\}] + 12.28 - 3 \times \log(cOD_{Vi}) \quad (3)$$

where we presently used the specific term OPD_{MG} to reflect the number of MG oocytes per gram of ovary, V_i is the volume fraction of MG oocytes, ρ_o is the ovarian density, k is the shape factor (ratio of short and long diameter of MG oocytes), and cOD_{Vi} is the volume-based mean oocyte diameter of MG oocytes (calculated from individual oocyte diameter corrected for shrinkage) (see Korta et al., 2010; Saber et al., 2015a; Schismenou et al., 2012). The total of 610 individual MG oocyte diameters measured in histological sections (see above) ranged between 393 and 556 μm (mean ± SD = 464 ± 29 μm). These individual oocyte diameter measurements were then corrected for shrinkage using the regression line given in Saber et al. (2015a). As in the W&G method, Delesse principle was used for estimating the volume fraction of the target cells. Hence, the resultant volume fraction estimations obtained from the ten counting frames used for the W&G method were also included. The ovarian density of ovaries in the migratory nucleus gonad subphase was taken to be 1.069 g cm^{-3} (Saber et al., 2015a).

2.2. Estimation of batch fecundity

Batch fecundity (BF) was defined as the total number of MG oocytes or POFs per spawning batch. As the OPD method gave the number of oocytes per gram of ovary, BF was estimated by multiplying OPD_{MG} with gonad weight. For W&G and PD methods the given number of oocytes per unit volume, N_v, was multiplied by the whole ovarian volume. Using Scherle's method (1970) the 16 ovaries collected in 2013 showed a mean loss in tissue volume of ≈4.5% during preservation in 70% ethanol, but all together 12.4% when thereafter dehydrated in 99.6% ethanol. Hence, ovarian volume following processing shrinkage (sV_o) was standardized as:

$$sV_o = 0.876 \times fV_o (r^2 = 0.99, p < 0.001) \quad (4)$$

where fV_o is initial, fresh volume. These standardized ovarian volume values were thereafter incorporated in the estimations of batch fecundity for the W&G (Knapp et al., 2014; Medina et al., 2007) and for the PD (Aranda et al., 2011, 2013) methods.

In order to properly compare the BF estimations based on the above fecundity methods, the Hydrated Oocyte method (Hunter et al., 1985) was selected as control. Following the procedure described by Hunter et al. (1985) BF was estimated for each female. Three subsamples of 0.07–0.1 g (±0.00001 g) were cut transversely from each preserved ovarian tissue, including tissue from the outer surface of the ovary to the centrally located lumen. The number of MG oocytes present in each subsample was counted manually under a stereomicroscope. Migratory nucleus oocytes could be effectively distinguished from secondary vitellogenic oocytes not only because they were more transparent in appearance but also because they were consistently larger in size (Saber et al., 2015a; Schaefer, 1996). As ethanol-preserved ovarian samples are lighter than fresh ovarian samples, the effect of preservation on measured weight was determined. The ovaries of the 16 females collected in 2013 were used to estimate the per cent difference between the weight of the fresh gonad and the weight of the preserved gonad in 70% ethanol ($GW_{ethanol}$), which resulted in values ranging from

8.9 to 15.6% (mean± SD = 12.9 ± 1.8%). Therefore, the values of the fresh gonad weights of the 12 females used for BF comparisons were converted to preserved gonad weights (Chen et al., 2010) using the following relationship:

$$GW_{ethanol} = 0.877 \times GW(r^2 = 0.99, p < 0.001) \quad (5)$$

Then the three estimates of BF per female were calculated by multiplying the number of MG oocytes in the subsample by the raising factor (preserved gonad weight divided by preserved subsample weight). If the coefficient of variance (CV) between the three fecundity estimates exceeded 10%, additional subsamples were counted in order to reach this level of precision (Zudaire et al., 2013).

The difference in BF between the four methods (W&G, PD_{MG}, PD_{POF}, OPD) and the Hydrated Oocyte method were expressed as a percentage (Z) according to the formula: $Z = 100 \times (BF_i - BF_{HO})/BF_{HO}$, where BF_i is the batch fecundity obtained by one of the four fecundity methods and BF_{HO} is the batch fecundity from the application of the Hydrated Oocyte method.

The relative batch fecundity (BF_{rel}) was calculated by dividing the batch fecundity by the body weight of the fish.

2.3. Statistical analyses

An analysis of covariance (ANCOVA) was used to test for homogeneity of slopes of the relationship between batch fecundity and female length among the different methods (ANCOVA, $F_{(4,50)} = 0.587$, $P = 0.674$). As a result, ANCOVA model with a common slope for the linear relationship between batch fecundity and length for all methods was then used to test the differences in the batch fecundity estimates by method. Post-hoc pairwise comparisons were accomplished by means of Tukey's HSD test to evaluate differences in batch fecundity estimations among methods. In addition, using the HO method as control, post-hoc comparisons were accomplished by means of Dunnett test.

The relationships between batch fecundity and relative batch fecundity and biological metrics, such as length, body weight and ovary weight were assessed by standard regression analysis. Both a linear model (untransformed response and explanatory variables) and a power model (log-transformed response and explanatory variables) were fitted to the data. In addition, generalized linear model (GLM) and generalised additive model (GAM) techniques were used to analyze (1) the relationships among BF, oocyte size and gonad weight, and (2) the relationships among BF, female length and gonad weight. For statistical significance, a 5% significance level ($\alpha = 0.05$) was assumed in all tests. All statistical analyses were conducted using R statistical software (R Core Team, 2015).

3. Results

3.1. Comparison of batch fecundity estimates among methods

Except for one female, the highest values for batch fecundity (BF) were obtained with the PD_{MG} method (Table 1). Conversely, the lowest values of BF were seen with the associated method, the PD_{POF} method (Table 1). The W&G, OPD and HO methods provided marginally different results when comparing the estimates of the total number of MG oocytes within the same specimen (Table 1). Analyses of covariance (ANCOVA) indicate that batch fecundity estimates varied significantly among methods (ANCOVA, $F_{(4,54)} = 24.330$, $P < 0.001$) (Fig. 1). Highly significantly lower values of BF were obtained with the PD_{POF} method in comparison with the other methods applied (Tukey test, $P < 0.001$). Statistically significant differences in BF estimates between the PD_{MG} method and the other methods were also found (Tukey test, $P < 0.05$).

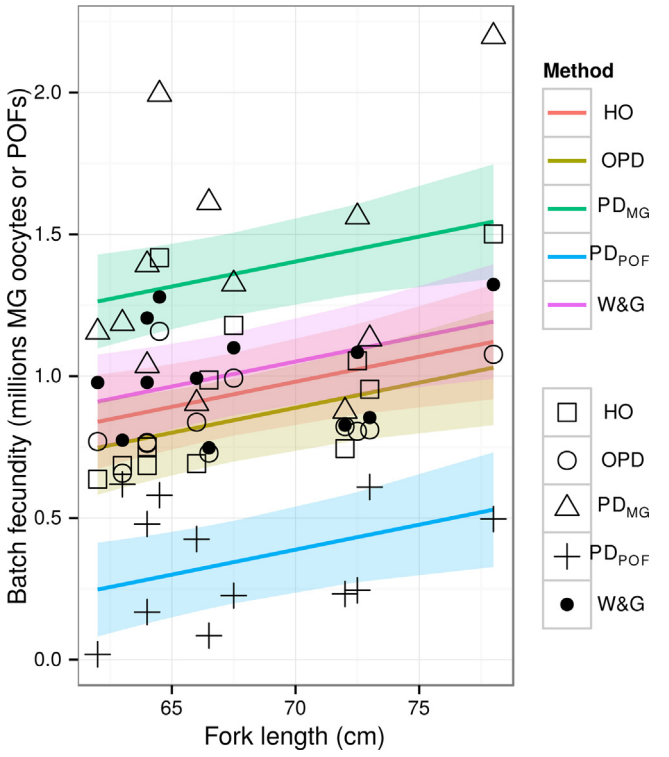

Fig 1. Albacore batch fecundity estimates by different laboratory methods regressed on fork length and tested by ANCOVA. W&G, Weibel and Gomez method; PD_{MG}, Physical Disector method used for counting MG oocytes; OPD, Oocyte Packing Density method; PD_{POF}, Physical Disector method used for counting postovulatory follicles; HO, Hydrated Oocyte method. Shaded region for each laboratory method refers to ±95% confident interval.

3.2. Comparison of the hydrated oocyte method with the other methods

The estimates of the total number of MG oocytes from the PD_{MG} method were consistently higher than those found by the HO method, with individual differences ranging between 12% and 103% (Table 1); these differences were statistically confirmed (Dunnett test, $P < 0.05$). In contrast, the PD_{POF} method resulted in much lower BF estimates than the HO method (Dunnett test, $P < 0.001$). The total number of MG oocyte estimated by the HO method was lower than for the W&G and OPD methods in 5 and 7 out of 12 females, respectively (Table 1). No statistical differences were found between the results from the HO method and those from the OPD and W&G methods (Dunnett test, $P > 0.05$). In essence, the estimates of BF from the PD_{POF} and PD_{MG} methods were on average 0.39 (CV = 0.68) and 1.48 (CV = 0.19) times the values obtained by the HO method, respectively. The W&G and OPD methods showed intermediate values, i.e., on average 1.14 (CV = 0.27) and 0.95 (CV = 0.19) times the HO results, respectively. Hence, the total number of MG oocytes given by the OPD and W&G methods was closer to those given by the HO method than was the case for the PD_{MG} method (Fig. 2). Note here that the OPD and HO methods both use ovarian weight for the calculations of the total number of oocytes in the whole ovary, while the other methods use ovarian volume.

Table 1
Batch fecundity (BF) estimations, where differences (%) between methods were found by setting the Hydrated Oocyte (HO) method as control. W&G, Weibel and Gomez method; PD_{MG}, Physical Disector method used for counting MG oocytes; OPD, Oocyte Packing Density method; PD_{POF}, Physical Disector method used for counting postovulatory follicles.

	BF (millions of MG oocytes/POFs) determined by each method					Differences (%)			
n Female	W&G	PD_{MG}	OPD	PD_{POF}	HO	W&G vs HO	PD_{MG} vs HO	OPD vs HO	PD_{POF} vs HO
1	0.98	1.16	0.77	0.02	0.64 ± 0.06	54	81	21	−97
2	0.77	1.19	0.66	0.62	0.69 ± 0.07	13	73	−4	−10
3	0.98	1.04	0.77	0.48	0.75 ± 0.06	30	38	2	−36
4	1.20	1.39	0.76	0.17	0.69 ± 0.07	76	103	11	−75
5	1.28	1.99	1.16	0.58	1.42 ± 0.06	−10	41	−18	−59
6	0.99	0.90	0.84	0.43	0.69 ± 0.07	43	31	21	−39
7	0.75	1.61	0.73	0.08	0.99 ± 0.05	−24	63	−26	−91
8	1.10	1.32	0.99	0.23	1.18 ± 0.12	−7	12	−16	−81
9	0.83	0.88	0.82	0.23	0.74 ± 0.01	11	18	10	−69
10	1.08	1.56	0.81	0.24	1.05 ± 0.09	3	48	−24	−77
11	0.85	1.13	0.81	0.61	0.95 ± 0.05	−10	19	−15	−36
12	1.32	2.20	1.08	0.50	1.50 ± 0.09	−12	46	−28	−67
Mean ± SD	1.01 ± 0.19	1.36 ± 0.41	0.85 ± 0.15	0.35 ± 0.21	0.94 ± 0.30				

3.3. Fecundity estimates, and its relationships with length, body weight and ovary weight

Individual BF estimates of the 61 *T. alalunga*, ranging from 57.2 to 85.5 cm *FL*, varied from 0.42 (61 cm *FL*) to 2.16 million of MG oocytes (79 cm *FL*), with a corresponding mean BF of 0.98 ± 0.40 (mean ± SD) million of MG oocytes. Batch fecundity was associated with a high variability at length (CV = 0.41). The corresponding relative fecundity (BF_{rel}) averaged 136 ± 36 (mean ± SD) ranging from 259 to 81 oocytes per gram of body weight at 67.6 and 83.0 cm *FL*, respectively.

The relationships between BF and the three biological metrics (length, body weight and gonad weight) were characterized by means of linear regression and power function, and all of them were statistically significant ($P < 0.05$) (Fig. 3a–c). Both types of models showed similar coefficients of determination (r^2) (Table 2). The highest r^2 was found for the relationship BF vs. gonad weight. However, the relationships between BF_{rel} and length and body weight were insignificant ($P > 0.05$) (Fig. 3d,e). Only a weak relationship existed between BF_{rel} and gonad weight ($P < 0.05$, $r^2 = 0.18$) (Fig. 3f). All relationships, coefficients of determination and *P*-values are listed in Table 2.

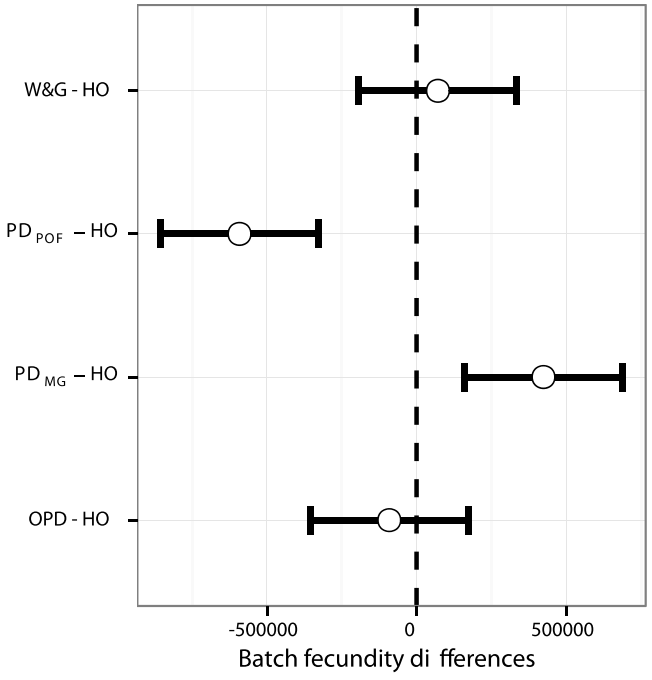

Fig. 2. Comparisons of albacore batch fecundity estimates between the Hydrated Oocyte (HO) method used as "control" and the other laboratory methods (mean differences and 95% confident interval, dashed line reflects no difference). W&G, Weibel and Gomez method; PD_{POF}, Physical Disector method used for counting postovulatory follicles; PD_{MG}, Physical Disector method used for counting MG oocytes; OPD, Oocyte Packing Density method.

3.4. Relationships between batch fecundity, gonad weight, oocyte size and fish length

A weak negative correlation existed between MG oocyte size and BF (Pearson's $r = -0.255$; $P = 0.047$) while no evidence existed for a relationship between MG oocyte size and gonad weight (GW) (Pearson's $r = 0.070$; $P = 0.593$). However the relationship between GW and BF was strong and positive (Pearson's $r = 0.902$; $P < 0.001$). Based on the results of the GLM analysis including two regressors (mean MG oocyte size and GW) and BF as the response, the relative importance of each explanatory term (based on partial eta squared statistic, i.e. correlation ratio) was 0.549 and 0.910 for mean MG oocyte size and GW, respectively. Predictions based on a semiparametric model (GAM) with BF as the response, and both GW and mean MG oocyte size modelled by means of a thin plate regression spline (edf = 6.226 and 1.001, respectively) showed that the fitted model accounted for a high amount of the variance in batch fecundity estimates (adjusted $r^2 = 0.924$; deviance explained = 93.4%) but this was due to the strong influence of GW. Hence, lower mean MG oocyte size values will not necessarily be indicative of higher fecundity while large ovaries positively influences the number of oocytes to be released (Fig. 4).

As expected gonad weight was related to female length (Pearson's $r = 0.852$; $P < 0.001$). Based on the GLM analysis including *FL* and GW (regressors) and BF (response), the relative importance of each explanatory term, based on partial eta squared (correlation ratio) statistic, was 0.030 and 0.614 for *FL* and GW, respectively. Likewise, predictions based on the a semiparametric model (GAM) with BF as the response, GW modelled by means of a thin plate regression spline (edf = 6.484) and *FL* modelled as a parametric term (edf = 1), which accounted for much of the variance in BF estimates (adjusted $r^2 = 0.85$; deviance explained = 86.6%), suggested that both small and large females can attain high fecundity values (Fig. 5).

4. Discussion

Based on counts of MG oocytes, the mean BF estimates, from highest to lowest, were obtained with the PD_{MG}, W&G, HO and OPD method. The PD_{MG} method gave the highest individual BF val-

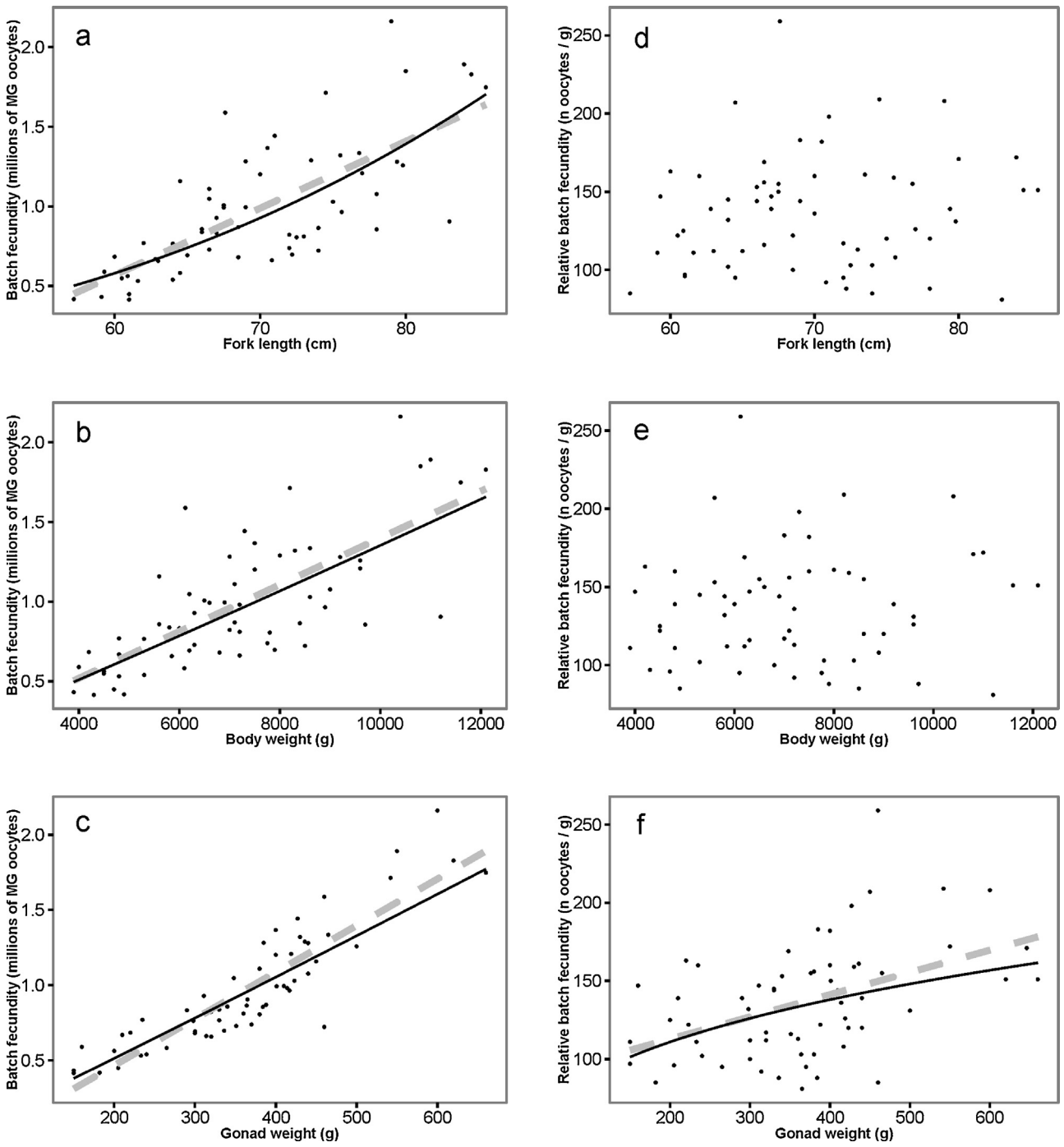

Fig. 3. Relationship between batch fecundity and fork length (a), body weight (b) and gonad weight (c); and relationship between relative batch fecundity and fork length (d), body weight (e) and gonad weight (f) for 61 albacore *Thunnus alalunga* from the western Mediterranean Sea. The dashed line represents the relationship fitted to linear regression and the solid line represents the relationship fitted to power function.

Table 2
Relationships between batch fecundity (BF) (millions of MG oocytes) and relative batch fecundity (BF_{rel}) (MG oocytes per gram of body weight) and the following biological metrics: *FL*, fork length (cm); BW, body weight (g) and; GW, ovary weight (g).

	Linear regression	Power function
BF versus:		
Length	$BF = -1.9495 + 0.04197\ FL$ $r^2 = 0.53$ ($P < 0.001$, df = 59)	$BF = 2.2968\ 10^{-6}\ FL^{3.0384}$ $r^2 = 0.58$ ($P < 0.001$, df = 59)
Body weight	$BF = -0.0687 + 146.8\ 10^{-6}\ BW$ $r^2 = 0.54$ ($P < 0.001$, df = 59)	$BF = 74.330\ 10^{-6}\ BW^{1.065}$ $r^2 = 0.58$ ($P < 0.001$, df = 59)
Gonad weight	$BF = -0.1538 + 3100\ 10^{-6}\ GW$ $r^2 = 0.81$ ($P < 0.001$, df = 59)	$BF = 2097.5\ 10^{-6}\ GW^{1.038}$ $r^2 = 0.81$ ($P < 0.001$, df = 59)
BF_{rel} versus:		
Length	$P = 0.4323$, df = 59	$P = 0.4392$, df = 59
Body weight	$P = 0.5548$, df = 59	$P = 0.5902$, df = 59
Gonad weight	$BF_{rel} = 84.3354 + 0.1423\ GW$ $r^2 = 0.21$ ($P < 0.001$, $df = 59$)	$BF_{rel} = 20.90524\ GW^{0.315}$ $r^2 = 0.18$ ($P < 0.001$, df = 59)

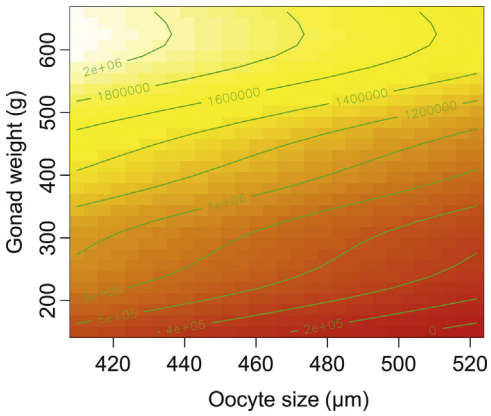

Fig. 4. Contour plot of the fitted GAM model for batch fecundity as a function of gonad weight and migratory nucleus oocyte size.

ues, except for one female. Also Aragón et al. (2010), who examined different bluefin tuna oocytes stages (lipid stage, vitellogenic and MG oocytes) found that the PD method generally gave higher values than the W&G method, but with significant differences only for vitellogenic oocytes. Overall the BF estimates from the W&G, and OPD methods are comparable with the standard HO method outputs in terms of quantification of MG oocytes. It is important to note that by simply multiplying the results obtained by the HO and OPD methods with ovarian weight, the total number of oocytes is estimated whereas the W&G and PD methods by themselves do not estimate the total number of oocytes (N) but rather the number density of oocytes (N_v). Hence, here the estimation of N requires the estimation of ovarian volume. Consequently, ovarian volume shrinkage should be measured in order to obtain proper BF estimates. In the present study the estimates of ovarian volume shrinkage turned out to be generally 12.4%, while for bluefin tuna ovaries, fixed in formalin instead of presently Bouin's fluid, the same type of figure is about 28% (Medina et al., 2007). Thus, as Bouin's fluid is a potent and rapid fixative, this could cause lesser tissue retraction. Oocyte shrinkage rates differ among oocyte developmental stages (Schismenou et al., 2012), so not only the effect of fixative on ovarian volume shrinkage may be influencing, but also the proportional volume of each oocyte within the ovary. Alternatively, the physical disector can be combined with a hierarchical scheme, the fractionator, that allows for estimating the number of particles directly, with no need for assumptions, corrections factors or gonad volume shrinkage estimation, although this would require complete sectioning of the organ. This method has been successfully used by Bucholtz et al. (2013) for Baltic herring (*Clupea harengus*), but its use is unfeasible for large organs such as tuna ovaries of the genus *Thunnus*. So, a remarkable advantage of the OPD and HO methods in respect to the W&G and PD methods is that both use ovarian weight for the calculations of the total number of oocytes in the whole ovary.

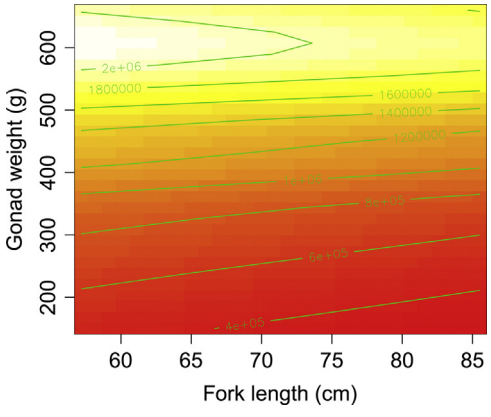

Fig. 5. Contour plot of the fitted GAM model for batch fecundity as a function of gonad weight and fork length.

The quantification of irregular particles should preferably be performed with the PD method because, unlike the W&G and OPD methods, the PD method does not require any assumptions about particle shape and size distribution. Hence, the PD has been used as an appropriate method to estimate the number of atretic oocytes and postovulatory follicles (Aragón et al., 2010; Aranda et al., 2011, 2013; Kjesbu et al., 2010; Kraus et al., 2008; Kurita et al., 2003). However, in the present study highly significant lower values of BF were obtained with the PD_{POF} method. Lower means of BF estimates from the counts of POFs have been also reported by Aragón et al. (2010) and Aranda et al. (2013) for bluefin tuna caught in the western Mediterranean Sea, but in these cases with no statistical difference between BF estimates obtained from counts of POFs and MG oocytes. Our result could indicate that when POFs coexist with MG oocytes in the ovaries of our target species the majority of them have already been reabsorbed (or are too small to be differentiated from other structures). It is known that spawning of tuna species occurs when sea surface temperature exceeds 24 °C (Schaefer, 2001) and at this temperature albacore POFs do not persist for more than 24 h after ovulation (Farley et al., 2013). Although both albacore and bluefin tuna spawn around the Balearic Islands (western Mediterranean Sea), the peak albacore spawning season occurs later in the summer season when the water temperature is higher (Alemany et al., 2010), so the rate of reabsorption of POFs could be faster for albacore than for the bluefin tuna. Thus, counting the POFs in present albacore ovaries might not well represent the actual number of eggs released, at least when these structures coexist with MG oocytes.

For the application of the methods currently considered, the necessary equipment should be widely available in virtually every routine histology lab. Then, in order to choose the best method for estimating BF in an indeterminate species, such as the present one, the advantages and disadvantages in terms of time-cost associated with each method, the number of samples needed for a certain study and the level of accuracy required in that particular study should be evaluated. The HO method, based on counts of hydrated oocytes (big and translucent oocytes) is the easiest and most accurate method (Hunter et al., 1985). However the main problem with this method is the scarceness of hydrated ovaries in sampled fish. So for species such as tuna, ovaries with MG oocytes are also included in BF estimates. From our point of view, although migratory nucleus oocytes may be distinguished from other oocytes under the stereomicroscope because they are less opaque than vitellogenic ones (Schaefer, 1996); the time required to count hydrated oocytes should be shorter than the required for counting MG oocytes because hydrated oocytes are quickly distinguished from other oocytes. Moreover, the HO method is supposed to be the quickest method, but usually oocytes from three pieces of ovary are counted (Alejo-Plata et al., 2011; Ewing and Lyle, 2009; Motos, 1996; Schaefer 1996; Zudaire et al., 2013; Rodrigues et al., 2015). The advantages of the W&G method are the conceptually easy implementation as it is well documented, and a relative short time is needed using free image analysis software such as ImageJ. The disadvantage of this method is that assumptions about the size, shape and orientation of the particles being counted are required. The important feature of the PD method is that its operation involves no prior assumptions about geometry of the objects. However, the major disadvantage of the PD method is that it is much more time-consuming (cf. serial sectioning of the ovary and alignment of the sections) in comparison with the others methods used in this study, and therefore not efficient for the considerable

number of females that normally are required in fecundity studies. An advantage of the OPD method is that BF is simply estimated by multiplying the result given in grams (OPD_{MG}) with the fresh gonad weight (see above). Moreover, five counting fields per ovary should in principle be sufficient for reliable volume fraction estimations (Korta et al., 2010; Saber et al., 2015a), i.e. time-cost could be reduced. However, unlike the other methods, this is a new method that has only been used for estimating BF in a previous study of albacore (Saber et al., 2015a). Therefore, other indeterminate species should be tested as well in order to examine its efficiency in BF studies on a more general basis.

A major advantage of methods that use histological sections for BF estimation is that the oocyte stages are classified by highly precise intra-cellular criteria and not merely oocyte size. The HO method is relatively less expensive and time-consuming than the methods that quantify particles from planar sections; however those methods might be reproducible and verified at any time by different laboratories. Moreover, slides can be stored for years without requirements of additional preservation procedures, so historical samples can be used for quantitative comparisons between past and present reproductive features, allowing us to track trends or changes in the reproductive status of a given stock (Aranda et al., 2013). However, one of the limitations that affect methods that use histological sections is related to the problem of tissue shrinkage. It is important to note that our ovarian samples were in paraffin, which is known to cause higher degree of tissue shrinkage than when resin is used (Dorph-Petersen et al., 2001). Consequently, if the weight and volume of the organ is found from the fresh gonad, any changes during fixation, embedding and sectioning should be considered (Andersen, 2003). Regarding the PD and W&G methods, a shrinkage correction factor is applied to the estimated ovarian volume to get the correct BF estimates. As volume fraction of a given type of oocytes is lower in paraffin than in resin sections due to a higher degree of shrinkage in the former than in the latter embedding medium (Saber et al., 2015a), the OPD formula should in principle result in lower fecundity estimates for paraffin than for resin. On the other hand, in cases where the oocytes under study are also equally affected, this should in theory cancel out any effect on the resulting enumeration, as seen for present MG oocytes but not so for the corresponding hydrated oocytes, which collapse during the histological process of dehydration (Saber et al., 2015a). Note that the shrinkage correction factor in the OPD method refers to the level of sectioning, correcting from fresh to embedded MG oocyte size.

Only a limited number of studies comparing teleost fecundity estimates between different methods has been published, here the Emerson et al. (1990) paper being, from our perspective, the most relevant one for fish fecundity works. In contrast, in the biomedical field a large number of researchers are interested in evaluating the performance of the different available methods for counting particles (Geuna, 2005; Lemley et al., 2013; Von Bartheld, 2002; West, 1999). In this field most authors consider the design-based methods to be unbiased (Howard and Reed, 2010) but this view is challenged by limited use of proper validation tests (Baddeley, 2001; Delaloye et al., 2009; Kaplan et al., 2012; Popken and Farel, 1996; Von Bartheld, 2002, 2012). Although nowadays many studies use the design-based methods (i.e. both the physical and optical disector, or the physical disector in combination with the fractionator), the fact is that only a small percentage of studies (16.7%) use these methods in comparison with conventional assumption-based methods (71.9%) (from a survey carried out by Geuna and Herrera-Rincon, 2015). Authors that prefer design-based methods declare that these methods should be the first choice and the use of assumption-based methods should only be applicable when the first choice is not possible (Geuna and Herrera-Rincon, 2015). Nevertheless, several researchers raise questions about how much the estimates obtained from conventional methods actually differ from those obtained with the design-based methods. For instance, in the study of White and Bilous (2004), similar values for epithelial cell numbers were provided by design-based and assumption-based models. Based on the present ground- truthed comparison, no method should be dismissed for fecundity estimation purposes. However, if we consider a reasonable trade-off between workload/economic cost and data quality, the OPD or W&G methods stand out as the best candidates, though the latter being more time consuming due to the estimation of N_a. If hydrated ovaries could be commonly collected, we would argue for the HO method, exploring the possibility of using automatic counting procedures in whole mounts, i.e. freely available image analysis software.

Batch fecundity estimates of *T. alalunga* from the Western Mediterranean Sea were found to increase with length. The length of the specimens used in this study ranged from 57.2 to 85.5 cm *FL*. Within this size range a high variability in BF was noted among specimens but also for specimens at similar length. These results are in line with those found in other tuna species (Chen et al., 2010; Farley and Davis, 1998; Thorogood, 1986; Zudaire et al., 2013). Several studies have reported that this variation in BF can be due to inter-annual, geographic, nutritional or fish condition factors (Kjesbu et al., 1991, 1998; Schaefer, 2001; Witthames et al., 2013). For the size range of the individuals of this study, both regression models (linear and power) gave similar r^2 and trend. Positive relationships between BF and body weight and gonad weight were also found. Gonad weight was found to be the most powerful predictor of BF, indicating that fish with larger ovaries produce more eggs per batch. In support of this, the GAM analysis showed that not only large females but also small ones are capable of spawning a high number of oocytes. Moreover, the only significant relationship between the BF_{rel} and the target biological metrics was found for gonad weight. Our estimate of mean BF was similar to other albacore stocks (Chen et al., 2010; Farley et al., 2013; Otsu and Uchida, 1959) supporting that BF estimates applying the OPD method can be used in indeterminate species. In contrast, BF_{rel} for albacore from the western Mediterranean Sea was much higher than those estimates for oceanic albacore populations. The reason for that result may be found in differences in minimum size at maturity, i.e. the albacore from the Mediterranean Sea reaches maturity at smaller size (Akayli et al., 2013; Arena et al., 1980; Saber et al., 2015b).

In summary, batch fecundity from counting MG oocytes can be estimated using different methods. In the present study the BF estimates given by the OPD and W&G methods were the closest to those given by the HO method. In general, the variation between individuals (the biological variation) was the major source of total variance. Thus, the most convenient way to reduce total variation is to add as many individuals as possible. Then, in routine works the less time-consuming methods should be used, and preferably resin as embedding medium to minimize the level of shrinkage during histological processing. More labour-intensive methods, i.e. the design-based methods, should be used in experimental studies but also when the particles of interests are irregular in shape. Batch fecundity estimates using the OPD method provided good fits to body length, body weight and gonad weight. Therefore, the OPD method appears as a promising future candidate for estimating batch fecundity in indeterminate species.

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