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Stereological calibration of the profile method to quickly estimate atresia levels in fish

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ABSTRACT

The (physical) ‘disector method’, a frequently cited stereological technique, has so far received little attention within fish fecundity studies, although it can be used to provide unbiased, assumption-free data on levels of atresia (resorption of vitellogenic oocytes). In comparison, traditional simple counting to estimate the numerical ratio of normal to atretic cells is biased because the smaller atretic cells have a lower chance of being transected in histological sections. These problems are circumvented by the disector method as it operates in three dimensions, i.e., uses consecutive pairs of slides. However, the high labour costs involved prevent regular usage of this method in population studies where large numbers of ovarian samples are processed. In the present paper we assess the corresponding bias of the traditional profile method, analysing developing ovaries of Atlantic cod (*Gadus morhua*) and herring (*Clupea harengus*), i.e., in a relatively broad and narrow oocyte frequency situation, respectively. A highly significant but non-linear relationship (r^2 = 0.975, $P < 0.001$, df = 154) was found between the relative intensity of atretic vitellogenic oocytes (A_{RI}) estimated by the disector and profile method. Both species fitted well to this polynomial model. The degree of underestimation of atretic oocytes still containing yolk (i.e. the alpha stage) was at maximum (11.6 %) at intermediate levels (A_{RI} = 50% (disector)) but, logically, no such bias existed at the extreme ends (A_{RI}: 0 and 100% (disector)). The practical use of this simple, fast approach designated *the stereo-profile method*, including the additional use of image analysis for further refinements, is successfully demonstrated on field samples.

Keywords: Atresia, Fecundity, Vitellogenic oocytes, Disector method, Profile counting

1. Introduction

Resorption of vitellogenic oocytes (atresia) is observed in all types of fish ovaries, although the prevalence and intensity might vary considerably (Trippel and Harvey, 1990; Ganias et al., 2008; Kraus et al., 2008). Assessment of the level of atresia is essential when aiming to predict realised fecundity (number of eggs spawned) from potential fecundity (number of developing oocytes) (Murua et al., 2003; Kjesbu, 2009) but also in studies of trade-offs between fecundity and egg size (Wootton, 1998). Thus, for these and other reasons (such as effect of chemical contamination) insight into the process of atresia has long been sought in fish biology (see Lambert, 1970 and references therein; Santos et al., 2008). Today the practical diagnostic characteristics of the different stages of atresia are well in place (Hunter and Macewicz, 1985), although with special focus on oocytes sequestering vitellogenin and restricted to the so-called alpha (α) stage (Kjesbu et al., 1991; Witthames and Greer Walker, 1995; Bromley et al., 2000). One main reason for this is that atretic oocytes become significantly smaller in size as the cellular content disappears and therefore are more difficult to assess but later stages might also be confused with old post-ovulatory follicles (Witthames et al., 2009). According to Witthames and Greer Walker (1995) smaller developing oocytes seem more inclined to become atretic than the corresponding larger ones. Thus, a clear size hierarchy can exist within the ovary between degenerating and healthy oocytes complicating proper quantification by common, simple methods such as profile counts from histological slides (Andersen, 2003).

Procedures adopted so far to estimate the total number of α-atretic oocytes in the fish ovary can be listed as: 1) the use of stereological, assumption-based methods (Weibel and Gomez, 1962 (theory); Emerson et al., 1990 (implementation); Witthames and Greer Walker, 1995 (result)), 2) the use of profile counts in combination with gravimetric counts (Ma et al.,

60 1998), 3) the use of stereological, assumption-free methods in combination with gravimetric
61 (Kurita et al., 2003) or automated counts (Thorsen et al., 2006; Kennedy et al., 2007), or cell
62 size along with 'Delesse principle' (saying that area fraction equals volume fraction (Howard
63 and Read, 1998)) (Kraus et al., 2008). In the last case the introduction of the so-called
64 (physical) 'disector method' by Sterio (1984) has proven to be a major breakthrough in
65 stereology (Mayhew and Gundersen, 1996); because there is no longer any requirement to
66 assume particle shape, size and orientation. To date, the amount of data produced by this
67 method in fish fecundity laboratories is still, however, very limited due to the high work load
68 involved. The standard estimation of total number of particles consists of two parts, which are
69 united by multiplication: 1) number of particles per unit volume, and 2) the reference volume
70 in question. Potential problems include non-uniform shrinkage, physical distortion or
71 expansion of the reference volume, e.g. growth in gonad size causing a fall in the volume
72 fraction of previtellogenic oocytes but not necessarily in their number (Andersen, 2003)), the
73 latter called the 'reference trap problem' (Howard and Reed 1998; Andersen, 2003; Ganias et
74 al., 2008). Thus, any disector results should not be considered as unbiased per definition as
75 often done (Geuna, 2005). If possible, it is apparent that many of these difficulties can be
76 negated by excluding any volumetric considerations, i.e., strictly limiting the disector
77 analysis to the estimation of relative intensities (without unit) and finding the reference
78 volume and thereby the total number of particles by other methods. In the latter respect the
79 recent successful introduction of digital image analysis in biological research has opened up a
80 new world: e.g. hundreds of vitellogenic oocytes can now be counted and measured within
81 seconds in whole mounts (Thorsen and Kjesbu, 2001). Thus, the total number in the ovary
82 can be estimated from packing density formulae and the size of the ovary, represented
83 normally by its total weight. This 'auto-diametric method' procedure is, however, primarily
84 designed for determinate spawners (without *de novo* vitellogenic oocyte recruitment during

spawning) rather than indeterminate spawners (with *de novo* vitellogenic oocyte recruitment during spawning), although it has been used with some success on the last category (Kurita and Kjesbu, 2009; Witthames et al., 2009). Nevertheless, as there is still no accurate quantitative method to discriminate between degenerating and healthy oocytes using morphology in whole mounts (Witthames et al., 2009), any estimation of their relative intensities still requires histology, and probably also will do so in the future, at least during validation. Therefore, in addition to the fast estimation of total number of vitellogenic oocytes by the image analyser there should, at least ideally, also exist a fast way to estimate which ones are atretic in histological sections.

One potentially useful idea would be to calibrate the quick profile method by the disector method to handle the foreseen problem of underrepresentation of the intensity of atresia by the former one. Logically such a calibration curve between the two methods in question should be 'anchored', i.e., showing identical values, at the point of origin (no atresia) and at the final point (total atresia) when plotted against each other while between these extremes less atresia should be seen in the profile method than in the disector method for the same samples. Consequently, our present null hypothesis was 1) the disector and profile method give similar outputs in terms of level of atresia. If rejected, we aimed at testing the next null hypotheses: 2) the level of atresia can be predict from atretic profile counts with a reasonable level of certainty both at the group level and 3) at the individual level following proper calibration by the disector method. As this study was intended to be of general, practical value in the laboratory, we focused on two main species in the North Atlantic ecosystem, Atlantic cod (*Gadus morhua*) and herring (*Clupea harengus*) showing widely different widths of their vitellogenic oocyte distribution, i.e., from relatively large (Kjesbu et al., 1990) to small standard deviation (Kurita and Kjesbu, 2009). Atresia was estimated in both

prespawning and spawning cod, since cod is a multiple batch spawner but only in prespawning herring, since herring is a total spawner.

2. Material and methods

The potential source of errors in the profile method was considered to be related to: a) the level of atresia; b) the size of atretic oocytes; c) the 'patchiness' (heterogeneity) of atretic oocytes in the ovary and d) the size of the reference space (in this case the size of healthy vitellogenic oocytes). Point a), b) and c) were addressed using histology/stereology, i.e., studying sectioned oocytes, while d) was addressed by image analysis of whole mounts, i.e., studying fixed but intact oocytes. In the case of b) all measurements undertaken were considered biased because of orientation problems, i.e., no nucleus (see below). As an alternative, the atretic process was detailed including studies of at which oocyte size the chorion is believed to start showing cracks, i.e., the first step in the atretic process.

The ovarian samples studied were basically of three types: method samples, validation samples and test samples. The method samples were used to examine the previous four points and to establish a model for the unbiased estimation of atresia, the validation samples were included to 'ground truth' the model output, and, finally, the test samples should clarify the operational usefulness in a realistic situation. In the last case the realism of the output was checked against additional relevant information on the fish (such as liver index) and previous validated outputs.

2.1. Method samples

Subsamples were taken from developing ovaries of Northeast Arctic (NEA) cod and Norwegian spring-spawning (NSS) herring and fixed in 3.6% phosphate-buffered formaldehyde (Bancroft and Stevens, 1996) for examination of oocyte frequency distribution and atresia. A few supplementary samples from Coastal cod (CC) were included in pilot studies. Otoliths were used to characterise cod into stock type while herring were characterised based on scale readings in combination with length-at-age data (A. Slotte, IMR, personal communication).

The ovarian samples of NEA cod originated from two sources: 1) fish captured in the Barents Sea and transported to the IMR laboratory in Bergen, where they were maintained for several months before being 'biopsied' repeatedly during natural spawning (Kjesbu et al., 1996) in February-March 2000-2002, and 2) fish processed shortly after capture in the Barents Sea – Lofoten area, Northern Norway in February-April 2003-2004. In total 137 subsamples from 93 cod females in prespawning or spawning status were selected for further method development following detection of atretic oocytes in histological sections (see below). During this pre-screening process care was taken to maximise the atresia intensity range in the individuals selected for calibrating the two methods.

Post-spawning herring were seined west of Bergen in March 2002 and transported to the IMR Research Station Matre where they were held in circular tanks (diameter: 5 m) until sampling in February 2003 (i.e., just prior to the upcoming spawning season). Nineteen females were selected for further analyses following the same pre-screening process as for cod.

2.2. Validation and test samples

Data were collected from prespawning NEA cod up to two months prior to the likely start of spawning for subsequent method validation. These individuals were taken by sampling commercial catches a few hours post mortem (≈ 5 ºC) at Andenes, Northern Norway in late February/early March 2003 (n = 48) and 2004 (n = 51) as an integral part of the traditional IMR fecundity time series (Kjesbu et al., 1998). Following proper method validation, the established model (see Eq. 1 in Results Section) was tested for operational use on an additional set of material from the same type of fish collected in early March 2005 (n = 44), mid-February 2006 (n = 39) and early March 2006 (n = 42). The mid-February sample was labelled as 'extra' to identify it from the other samples collected a couple of weeks later.

As both the validation and test data referred to fish that had been sampled and processed randomly, ovaries with and without atresia were equally considered in the analysis to produce overall, unbiased basic statistics. Fish and liver size data were extracted from the general data base for further use. All laboratory protocols were identical to those described in the previous section.

2.3. Chorion thickness

Chorion thickness was measured in cod and herring oocytes to track development as a function of oocyte size. These data were subsequently included in the overall framework to better understand at which oocyte size atresia is initiated as the quick disappearance of the nucleus in this type of cells complicated proper orientation during histological sectioning and thereby adequate oocyte size measurements.

Five specimens of cod (Lofoten) and herring (Matre) in different phases of maturity were selected studying in each case seven normal oocytes of typical size sectioned equatorially,

i.e., close to the central part of the nucleus. Light microscopy measurements (200 X) were undertaken with image processing software (ImageJ) at a precision of ± 0.1 µm.

2.4. Image analysis

The vitellogenic oocyte diameter frequency distribution of each individual was described using automated image analysis (Thorsen and Kjesbu, 2001). Typically 100 vitellogenic oocytes were measured in whole mounts, excluding previtellogenic oocytes (< 250 µm) and any hydrated and ovulated oocytes. For spawning cod, oocytes in final maturation 'budding off' from the right hand side of the distribution were removed (Kjesbu et al., 1990). In addition to the routinely collected data on mean (OD) and standard deviation ($SD_{vit.}$), the mean diameter of the 10% smallest oocytes, labelled as smallest cohort (SC) diameter, was included for cod to better characterise the left hand side of this rather broad oocyte distribution. This region in the oocyte frequency distribution has been hypothesised in sole (*Solea solea*) as the most likely origin of atretic cells and is associated with hiatus development between the previtellogenic and vitellogenic oocyte populations (Witthames and Greer Walker, 1995).

2.5. Profile and disector method

Standard histological protocols were used to produce 4 µm-thick resin (Technovit®) sections stained with 2% toluidine blue and 1 % tetraborate. The same experimenter worked up all samples during the following investigations.

Before the actual compilation of any histological sections for further analysis by the disector method (DM), a pilot test was run to make sure that parallel sections would be

206 separated by a vertical distance of about 1/3 of the smallest ‘particle’ size (see Andersen,
207 2003). As a start, the image analysis data on SC diameter for cod and on mean diameter for
208 herring were consulted to indicate size of relevant particles. Thus, any likely subsequent
209 shrinkage during the histological processing (Bancroft and Stevens, 1996) was ignored. This
210 approach appeared satisfactory as the adopted separation height between successive pairs of
211 sections was less than the size of any of the smallest, relevant oocytes, including the presently
212 studied atretic ones (Fig. 1), i.e., all had an equal probability of being sectioned, an essential
213 requirement (Sterio, 1984) (Fig. 2). Thus, this sectioning protocol was consistently followed.
214 Next, the total number of oocytes (including atretic ones), which should be counted in DM
215 to get a reliable assessment of the relative intensity of atresia (A_{RI}, see definition below) was
216 tested in three females showing from ‘low’, ‘medium’ and ‘high’ A_{RI} values using standard
217 approaches (Howard and Read, 1998). Due to the labour-intensive work, the maximum
218 oocyte count was initially set to175 based on earlier, relevant cost-benefit analyses. The
219 deviation from the normalised mean fluctuated at low counts but stabilised with increasing
220 counts (Fig. 3). Based on these results and the present prerequisite that DM records should as
221 far as possible reflect true values, about 150 transected oocytes from typically 11 histological
222 sections were counted from each female in the main study (Table 1).
223 In the case of the profile method (PM) special care was taken that the same oocyte was not
224 counted twice, i.e., using sufficiently, large separation heights. For the sake of standardisation
225 with DM, the same order of oocytes was also counted in this method but from two sections
226 only.
227
228 *2.6. Definitions*
229

Here atresia refers to the alpha stage as noticed in histological sections (Hunter and Macewicz, 1985). Thus, we concentrated on the process of oocytic (incl. yolk) degeneration prior to the degeneration of granulosa and theca cells. The alpha stage was divided into three phases of progressive degeneration: Early Alpha (EA), Late Alpha Residual Chorion (LARC) and Late Alpha No Chorion (LANC) (Fig. 1), motivated by approaches taken in Witthames and Greer Walker (1995). The EA phase shows cracks in the chorion, which is located peripherally. For the LARC phase the chorion remnants are dislocated towards the centre of the sectioned profile. No chorion is detected in LANC profiles but vacuoles along with (small) yolk granules staining poorly are seen. This classification system was adequate for both species (cod: Fig.1; herring: Kurita et al., 2003). All atretic disector data used in method development, except for the first experimental season of cod (27 samples), were subdivided into these phases. The same was done for the corresponding profile data for cod.

Relative intensity of atresia (A_{RI}) was defined as $A_{RI} = 100\times$(number of atretic oocytes)/(number of atretic and normal oocytes). This expression was preferred to the other option where the denominator contains only normal oocytes (Kjesbu, 2009) as the data produced by the image analyser was expected to include normal as well as the present three phases of atretic oocytes (A. Thorsen, personal communication; see also Kurita and Kjesbu, 2009). The number of females with atresia in relation to total number of females, designated prevalence, was reported for cod field samples.

As the profile data were obtained from two sections only, labelled as Section 3 and 9, and atresia might be sporadically located in the ovary (Kraus et al., 2008), i.e., atretic heterogeneity, a simple test was included to evaluate the consequences of this ‘patchiness’ on model performance: Patchiness = $\left|(A_{RI\_3} - A_{RI\_9})/A_{RI}\right|$. Thus, the absolute difference between the two sections was divided by the overall value, excluding cases where $A_{RI} = 0\%$.

In a few cases the portion of the total number of eggs spawned (PES) was added to the cod data base to indicate where the experimental female resided in the spawning cycle at the time of sampling (Kjesbu et al., 1990). More specifically, PES (%) was calculated as 100×cumulative number of eggs spawned when the ovarian biopsy was taken/total number of eggs collected from this particular female during the whole season (all egg batches). Total length (TL) was reported to the nearest 1 cm below and HSI (hepatosomatic index) was 100×liver weight (in g)/whole body weight (in g). Any stomach content was excluded.

3. Results

3.1. Characterisation of method samples

Profile (PM) and disector method (DM) analyses of these samples specially selected for method development confirmed that they all contained atretic oocytes, although sometimes at extremely low numbers. In one case for cod no atretic oocytes were detected in DM while a few appeared in PM. Generally the herring sections showed higher relative intensities of atresia (A_{RI}) than those of cod, 32 vs. 13% (DM). Both data sets covered the complete range in A_{RI}; each species was represented by individuals with A_{RI} at ≈ 0% and at 100%. In the tested material on cod, prespawning ovaries showed indications of less atresia than spawning ovaries, typically 10 vs. 15% (DM).

The EA phase appeared more often in the studied samples of herring than of cod, i.e., 84 vs. 28 % (DM). Hence, several ovaries contained only LARC and LANC phases. For both methods (cod) and species the EA fraction fell with increasing A_{RI} (Fig. 4). Therefore, LARC and LANC phases dominated at high A_{RI}. Field and experimental samples showed very much

the same pattern, judged for cod. As a consequence of these findings, i.e., to properly reflect the whole atretic situation in the ovary, all three phases were pooled in the further work.

Basic whole-mount statistics showed that the width of the vitellogenic oocyte distribution ($SD_{vit.}$) usually was about three times broader for cod than for herring, 101 vs. 33 µm, but also more diverse, 13 – 220 vs. 21 – 48 µm. The oocytes were typically about 25 % smaller in cod than in herring, 622 vs. 827 µm, but again with large variations, 314 – 771 vs. 529 – 1161 µm. As above, due to the heterogeneous nature of these samples no explicit statistical tests were performed.

3.2. Chorion thickness

Analysed samples of herring and cod showed a comparable chorion thickness, i.e., around 5-25 µm, but highly different trajectories in relation to oocyte size (Fig. 5). There were indications that the chorion thickness of cod increases rather linearly during vitellogenesis while for herring the chorion barely grows in thickness during a long phase but then enters a period with fast growth up to spawning.

3.3. Relationship between profile and disector results

The relationship between relative intensity of atresia as observed by the disector method (A_{RI_DM}, %) and the profile method (A_{RI_PM}, %) could be best described by a polynomial formula (Fig. 6):

$$\hat{A}_{RI\_PM} = 0.5379(SE\pm0.0248)\times A_{RI\_DM} + 0.0046(SE\pm0.003)\times(A_{RI\_DM})^2 \ (r^2 = 0.975,\ P < 0.001,\ df = 154) \quad (1)$$

Note that a 'hat' is added to the estimated variable to keep it separate from the observed value, A_{RI_PM}. Data from both herring and cod were included, supported by no significant differences in their residuals, i.e., $\Delta A_{RI\_PM} = A_{RI\_PM} - \hat{A}_{RI\_PM}$ ($P = 0.904$, Wilcoxon signed-rank test). A similar conclusion, although not so strong, was reached when splitting the cod data further into 'prespawners' and 'spawners' ($P = 0.146$, Wilcoxon signed-rank test). Replacement of Eq. (1) with a power function resulted in less explanatory power ($r^2 = 0.788$) and trends in residuals.

Only about 5% of the absolute variation in ΔA_{RI_PM} could be explained by the phenomenon of patchiness (atretic heterogeneity) (cod: $r^2 = 0.055$, $P = 0.146$, $df = 38$), i.e., an insignificant effect. The noted difference in atretic values between the two PM sections could, however, in extreme cases be three times larger than the combined value. The mean CV of patchiness was 25 %. Further analyses including patchiness in multiple regressions supported that this variable could presently be taken out.

Although Eq. (1) explained a major part of the variation, there were underlying patterns in the data, especially for cod. More specifically, ΔA_{RI_PM} (percent point) of cod fell significantly with increasing mean oocyte size (OD) as reported in whole mounts (Fig. 7):

$$\Delta\hat{A}_{RI\_PM} = 8.552(SE\pm1.962) - 0.0139(SE\pm0.0031)\times OD \quad (r^2 = 0.134,\ P < 0.001,\ df = 129) \qquad (2)$$

The corresponding regression for herring behaved comparably but was insignificant ($r^2 = 0.086$, $P = 0.253$), likely due to the much lower degrees of freedom ($df = 15$). The same message as gathered from Eq. (2), although less strong, was received when replacing OD with smallest cohort oocyte diameter (SC) ($r^2 = 0.059$, $P < 0.006$, $df = 124$), tested on cod,

while $SD_{vit.}$ had no clear impact (cod: $P = 0.235$; herring: $P = 0.654$). Likewise, the fraction of EA phase was unimportant in these respects (cod: $P = 0.654$; herring: $P = 0.810$). As eight out of the ten points found outside the prediction bands in Fig. 7 belong to spawners, the analysis was rerun with prespawners only:

$$\Delta\hat{A}_{RI\_PM} = 8.100(SE\pm2.384) - 0.0136(SE\pm0.0041)\times OD \ (r^2 = 0.186, P = 0.002, df = 48) \quad (3)$$

Although the output was only slightly different from Eq. (2), i.e., about 0.3 percent points below, this regression line crossed $\Delta A_{RI\_PM} = 0\%$ at OD = 595 µm instead of 615 µm as calculated from Eq. (2). The ΔA_{RI_PM} of samples with OD above and below this threshold value should therefore, when relevant, be corrected upwards and downwards, respectively. A standard method of estimation practice was developed (Table 2) and applied in all further atresia assessment.

3.4. Validation

The Andenes samples (prespawning NEA cod) collected in 2003 and 2004 showed a prevalence of atresia of 33 and 55%, respectively. Most specimens were between 75 and 90 cm in total length, while the full range was 55–117 cm in 2003 and 63–121 cm in 2004.

Focusing on only those females with atresia, i.e., $A_{RI\_PM} > 0$, observed PM and DM A_{RI} values were clearly different (2003: $P = 0.001$; 2004: $P < 0.001$), while the latter did not deviate from the corresponding estimated values (Eq. 1) (2003: $P = 0.234$; 2004: $P = 0.539$) (Wilcoxon signed-rank test) (Table 3). Defining the observed DM data as ‘true values’, both the observed PM and estimated DM values were generally underestimates, while the

353 ‘corrected’ version of the last, found by iteration (Eq. 3; Table 2), in most cases was an
354 overestimate.
355 In more detail, the mean (95 % CI interval) residual between estimated and observed DM
356 values, $\Delta\hat{A}_{RI\_DM}$ ($\Delta\hat{A}_{RI\_DM} = \hat{A}_{RI\_DM} - A_{RI\_DM}$), was -0.77 (-1.95 – 0.40) and -0.33 (-1.26 –
357 0.61) percent point for 2003 and 2004, respectively. However, individual examples of
358 residuals up to ± 5 percent points appeared (Fig. 8). Residuals were uncorrelated with
359 observed atresia (A_{RI_DM}) (2003: $P = 0.202$ (excluding one observation with large leverage);
360 2004: $P = 0.296$). Thus, in relative terms the present estimation practice appears more
361 accurate for individuals with high intensities than low intensities.
362
363 *3.5. Testing*
364
365 In this part of the analysis only observed PM A_{RI} values were available to simulate the new
366 situation in the laboratory following introduction of the above practice (Table 2). The females
367 collected in 2005, ‘2006, extra’ and 2006 (Table 3) were comparable in length with those
368 used in the above method validation. The prevalence of atresia was also very similar (2005:
369 43%; 2006, extra: 33%; 2006: 26%). Within the ‘2006, extra’ sample individuals with atresia
370 showed a lower liver index (HSI) than expected, i.e., in relation to the rest of the fish taken in
371 that year (Fig. 9).
372
373 *3.5.1. Intercalibration using HSI*
374 The corrected DM estimates of mean A_{RI} for the test samples appeared reasonable when
375 regressed on mean HSI and compared with previous validated results (Fig. 9). The combined,
376 fitted trendline for atretic + non-atretic (‘all’) samples was clearly significant ($r^2_{adjusted} =$
377 0.941, $P = 0.004$), while the more restricted analysis on females ‘with atresia’ only showed

that the ‘2006, extra’ sample formed an outlier (Studentized residual: -3.027) resulting in an insignificant relationship ($r^2_{adjusted}$ = 0.544, P = 0.096). Exclusion of this point restored the fit ($r^2_{adjusted}$ = 0.898, P = 0.035).

3.5.2. Influence of maturity stage on atresia levels

The ‘2006, extra’ sample, taken a few weeks earlier in the year than the other samples, showed less developed (smaller) oocytes (Table 3). Consequently, the cause for the weaker fit of the ‘with atresia’ data from ‘2006, extra’ was explored by studies on atretic patterns in relation to oocyte size (Fig. 10). As noticed, atresia generally peaked around an OD of 600±75 µm, while the extra sample showed individuals with atresia predominately around or to the left side of this mean.

4. Discussion

In this study we have introduced a new method, named *the stereo-profile method*, which is calibrated by unbiased stereological (disector) results to effectively handle the problem of significant but systematic underestimates in the traditional profile method. Although our main aim has been to quickly quantify the level of atresia in fish in a reliable way (as a step to predict realised fecundity) any discrete ‘particles’ could in principle be candidates for this method to estimate relative proportions, i.e., of interest to a broader audience than fish biologists. Our approaches are based on the reality that modern image analysis continuously changes the working situation in fecundity laboratories and thereby directly or indirectly reduces the dependence upon traditional stereology. So the normal routine would be to use the image analyser to count and measure oocytes in whole mounts followed by histological sectioning and then eventually stereological assessment. We believe that the present outline is

403 a more robust alternative to the practice in recent articles containing methodology in the grey
404 zone between simple profile counting and unbiased stereology (see Introduction). Although
405 there exist relevant examples of thorough studies (e.g. Emerson et al., 1990 (fecundity);
406 Kraus et al.; 2008 (atresia)), fish biology has generally lagged behind development in this
407 field of research and journals within other disciplines, such as medicine, promptly reject
408 manuscripts which only contain profile counts as the basis for estimation (Andersen, 2003).
409 The present equations should be a way forward to produce unbiased atretic oocyte counts
410 within applied fisheries reproductive biology at low labour costs. The key equation, Eq. 1, is
411 somewhat special in that it 1) is anchored at the origin (both methods with no atresia) and at
412 the final point (both methods with total atresia), and 2) contains very few points > 50%
413 (A_{RI_DM}), despite examinations of a high number of ovaries. The last point indicates that these
414 fish with high levels of atresia either down-regulate the fecundity to a certain minimum level,
415 if energetically required, or switch to complete resorption of developing oocytes (Hunter and
416 Macewicz, 1985; Kennedy et al., 2010). Actually, nearly all Northern anchovy (*Engraulis*
417 *mordax*) with > 50% (A_{RI_PM}) skip spawning (Hunter and Macewicz, 1985). Thus, we foresee
418 that any potential bias in the estimation associated with high atretic intensities will have little
419 impact due to likely few examples of such ovaries (although some caution should be
420 exercised during interpretation). Even so, the modeled curve should be a good approximation
421 also for intensities falling between 50-100% as the same fundamental principle of
422 underrepresentation of smaller objects (atretic oocytes) in relation to larger objects (healthy
423 oocytes) should still apply.

424 The stereo-profile method worked exceptionally well at the group level, validated and
425 tested on cod. The approaches taken appeared also adequate for herring, characterised by a
426 very narrow oocyte frequency distribution compared to cod. More specifically, observed and
427 estimated disector method (DM) values were statistically similar while the traditional profile

method (PM) gave clear underestimates. Estimated DM values were found by iteration using Eq. (1). The more straightforward way would be to use the DM data as the dependent (response) variable instead of the PM data. This would, however, be in conflict with sound statistical principles stating that the covariate within Model I regression is to be measured without error (Sokal and Rohlf, 1981). Here we assumed that the observed DM values were not subject to any errors. This might not be the case as further collections of histological section pairs could have changed the picture to some extent but this exercise appears unrealistic in terms of the extra work load involved (Kraus et al., 2008). However, application to atresia, or any other particle, assessment, where the size differential between the two classes is greater than found in cod or herring would require further verification using the approach adopted in this paper. Although cod and herring showed similar residuals with respect to Eq. (1), there were patterns within the cod data related to the broad oocyte size distribution leading to the additional estimation of a corrected DM version. These values appeared extremely close to observed DM values (differences of 0.1-0.3 percent points) favouring this approach in future analyses when working on species like cod. Assuming that an oocyte becomes atretic at a given size (see below), the underlying principle may relate to the fact that the probability of sectioning a particle is proportional to its height (see Andersen, 2003). Thus, as maturity progresses the chances of hitting an atretic cell in relation to a growing oocyte falls, as noticed. In the case of herring the difference in size between atretic and normal oocytes was probably too small to have any impact. Note that image analysis measurements were used to possibly reflect the oocyte size distribution as found in histology. Thus, any hydrated or ovulated oocytes were ignored as these cells collapse during histological processing. Taken together we have developed routines which now make it possible to rapidly produce highly reliable average figures on atresia for fish with different reproductive styles, although so far only for determinate spawners.

453 The predictive power of the stereo-profile method was much less convincing at the
454 individual level, seeing deviations from actual values of ± 5%. This variance is considered
455 acceptable in relation to the whole individual range in atretic values (0-100%), but would
456 certainly complicate any detailed explanatory analyses, as done successfully with liver index
457 at the group level. Atretic heterogeneity (patchiness) apparently did not form a solid
458 background explanation, at least statistically, but the associated CV was clearly on the high
459 side: 25%. Hence, an obvious recommendation would be to include more PM sections in
460 future studies, provided the focus is on individual variations. This would of course imply
461 some extra work but PM counts are extremely quick to gather compared to DM counts.
462 Within the two studied species atresia seems to start when the chorion reaches a thickness
463 of about 10-15 µm. Thus, the surrounding follicle cells apparently do not take on the job of
464 breaking down the chorion (Santos et al., 2008) when the thickness doubles towards the end
465 of the vitellogenic period. Likewise, this process seems less common early on in the maturity
466 cycle when there are not yet any constrains on available energy resources for oocyte
467 development (Kurita et al., 2003). However, atresia has been seen in all classes of oocytes
468 from cortical alveoli to advanced oocytes producing cysts (Witthames et al., this monograph).
469 Despite this complicating reality, the present critical chorion thickness of 10-15 µm, seen
470 when correlated with normal vitellogenic oocyte size during the main 'atretic window', gives
471 somewhat useful information in the following settings. We found that atresia in prespawning
472 cod peaks at a mean diameter (OD) (±SD) of 600 ± 75 µm while Kurita et al. (2003) showed
473 that this happens at OD = 900 ± 100 µm for herring. Although different OD's, this
474 corresponds to similar chorion thicknesses due to different chorion development trajectories.
475 Thus, this type of knowledge is valuable to consider when suspect atretic values appear,
476 presently for '2006, extra'. There is evidence to suggest that the reason for the unexpected

low relative intensity of atresia (A_{RI}) in relation to HSI in the 'with atresia' females from this sample was caused by oocytes still recruiting to the atretic window.

Any thorough prediction of realised fecundity (F_R) of an individual (for use in e.g. the Annual Egg Production Method to estimate spawning stock biomass (Armstrong et al., 2001)) should be based on the following five rules of thumbs: 1) proper definition and use of atretic phases, 2) unbiased relative intensity of atresia (A_{RI}), 3) correct atretic turnover rate (duration), i.e., for the species, the oocyte classes in the atretic stage and environmental temperature in question (T) ($A_{T,\alpha\text{-stage}}$), 4) relevant atretic period (in days) (e.g. main atretic window or spawning duration) (D), and 5) appropriate fecundity reduction formula using potential fecundity (F_P) as initial value. For the last point, there exist two candidates: a) the standard formula: $F_R = F_P - F_A$, where $F_A = F_P \times A_{RI} \times D/A_{T,\alpha\text{-stage}}$, and 2) the revised formula: $F_R = F_P \times (1 - A_{RI})^{D/AT,\alpha\text{-stage}}$. The two formulae were reviewed by Kjesbu (2009), influenced in the last case by approaches in Kurita et al. (2003). In short, the standard formula is considered to be conceptually insufficient as it does not take into account the instantaneous decline in the standing stock of oocytes. However, as mean A_{RI} typically is a few percent only (Kraus et al., 2008), the corresponding two figures on F_R will in most cases deviate only slightly, i.e., provided there are no examples of large errors associated with large A_{RI} values (Fig. 11). Biologically speaking, the revised formula indicates that a female can sustain a high level of atresia and still spawn quite a few eggs, provided the other parametric values are realistic (Fig. 11). Switching to Point 1, this article tells that a restricted focus on the early atretic phase would have an unwanted effect as it would favour data from samples with low A_{RI} values due the dominance of later phases at higher A_{RI} values. In other words, such a practice may result in an underestimation of the whole atretic situation in the ovary. Point 2, referring to unbiased A_{RI} values, is highlighted above. Unfortunately, in terms of Point 3 actual information on atretic turnover rates exists so far only for a limited number of

502 species (Witthames et al., this monograph). It should be emphasised that these published rates
503 refer to the whole α-phase in ovaries at late stages of maturation – early spawning
504 (Witthames et al., this monograph), strengthening once more that early and late phases should
505 be reported jointly. Following the introduction of the concept of 'down-regulation' a few
506 years ago (see Kjesbu, 2009) an increasing number of articles are showing that the potential
507 fecundity (or more correctly the standing stock of oocytes) is markedly reduced during the
508 length of vitellogenesis (see updates in Kennedy et al., 2009). Down-regulation is believed to
509 be a natural process but accelerated when the fish is in poor condition (Kjesbu, 2009). Such
510 data, found by manual counting or automatically, can be used to validate A_{RI} data found
511 elsewhere. According to Thorsen et al. (2006) down-regulation of NEA cod amounts to 27%
512 between an OD of 500 and 700 µm. Using the above revised fecundity reduction formula,
513 this corresponds to an overall A_{RI} of 5%. Narrowing the atretic window to present 525-
514 675µm, as the atretic information in Thorsen et al. (2006) is limited, the resulting A_{RI} equals
515 7%. These findings are in good agreement with A_{RI_DM} values for females 'with atresia'
516 (Table 3). However, several females did not show any atresia at the time of sampling
517 implying that the atretic value for 'all' was roughly half of this. Conversely, all studies on
518 down-regulation strongly indicate that every female reduces its fecundity. Therefore, the
519 concept of prevalence of atresia seems somewhat hollow (but still needed in calculations at
520 the population level). This implies that we are left with three possibilities for why some
521 ovarian samples, in conflict with expectations, do not show any atresia at all, i.e., A_{RI} = 0%:
522 1) the fish was collected outside the main atretic window, 2) atresia takes place elsewhere in
523 the ovary and 3) the individual atretic window is much shorter than anticipated. The first two
524 arguments are generally not supported, cf. Fig. 10 and data on 'patchiness', respectively. The
525 last argument gains some support: maximum A_{RI} values in Thorsen et al. (2006) and this
526 study for similar type of samples (Andenes), around 20-25 %, are comparable with the above-

mentioned degree of down-regulation. The aging of the 'atretic pulse', which is probably partly explained by a shorter life time of the EA phase than the LARC and LANC phases, points to the same. Hopefully, future research will address these questions.

In summary this method paper gives access to new practices to quickly report levels of atresia in fish ovaries. The output can be considered accurate and precise at the group level supported by a series of various types of successful validations. On the individual levels there is clearly room for improvements in terms of precision. Here the simple answer seems to increase the number of analysed slides due to indications of patchiness (atretic heterogeneity). As these additional profile counts can be collected with little costs, we foresee that the stereo-profile method also has a future at the individual level.

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647

FIGURE CAPTIONS

Fig. 1. Representative examples of normal vitellogenic oocytes (a) and the successive resorption of such oocytes through the process of atresia in Atlantic cod: Early Alpha (b), Late Alpha Residual Chorion (c) and Late Alpha No Chorion (d) in resin sections stained with toluidine blue. Arrow points at the chorion. Horizontal bar is 100 µm.

Fig. 2. Separate frequency distributions of histologically sectioned diameter of normal vitellogenic oocytes (V) ($n \approx 50$) and atretic vitellogenic oocytes ($n \approx 50$) characterised as Early Alpha (EA), Late Alpha Remaining Chorion (LANC) and Late Alpha No Chorion (LANC) phase, examining three cod sampled at Andenes, Northern Norway on 3 March 2003: a) 5 year-old, 61 cm CC cod: A_{RI_DM} = 15 %; SC diameter = 331 µm, b) 8 year-old, 82 cm NEA cod: A_{RI_DM} = 28 %; SC diameter = 275 µm, and c) 5 year-old, 61 cm CC cod: A_{RI_DM} = 29 %; SC diameter = 372 µm, where A_{RI_DM} refers to relativity intensity of atresia (all three phases combined) found by the disector method (DM), and SC diameter to the smallest cohort oocyte diameter in whole mounts (image analysis). Diameter is average of short and long axis. DM section separation height was 110, 95 and 125 µm in a), b) and c), respectively, i.e. to left of the presented frequency distributions.

Fig. 3. Deviation from normalised mean relative intensity of atresia (A_{RI_DM}) as a function of total number of normal and atretic oocytes examined by the disector method (DM) for three experimental NEA cod showing low, medium and high values of A_{RI_DM}. Low: Portion of eggs spawned (PES) = 0% (18 days prior to start of spawning), TL: 77 cm; Medium: PES = 13%, TL: 79 cm; High: PES = 34%, TL: 71 cm. All three individuals were in moderately good condition, i.e., with a Fulton's K around 1.

673

674 **Fig. 4.** Variation in the fraction of EA phase with increasing relative intensity of atresia (A_{RI})
675 as observed in the disector method (DM) (herring and cod) and profile method (PM) (cod).
676

677 **Fig. 5.** Mean chorion thickness (± SD) of cod and herring as measured in histological
678 sections in relation to mean developing oocyte diameter (OD) in whole mounts.
679

680 **Fig. 6.** Relationship between relative intensity of atresia from the disector method (A_{RI_DM})
681 (defined as true values) and in the profile method (A_{RI_PM}) (indicated values) for cod and
682 herring and the estimated, combined polynomial curve (Eq. 1).
683

684 **Fig. 7**. Residual value of relative intensity of atresia, ΔA_{RI_PM}, as a function of mean whole-
685 mount oocyte diameter (OD), where $\Delta A_{RI\_PM} = A_{RI\_PM} - \hat{A}_{RI\_PM}$, using in the last case Eq.
686 (1). 95% confidence (short dashed line) and prediction (long dashed line) bands are inserted,
687 while the horizontal line ('Reference') refers to full match between observed and estimated
688 atretic values.
689

690 **Fig. 8.** Calculated difference (residual) between estimated DM values of relative intensity of
691 atresia and corresponding observed values ($\Delta\hat{A}_{RI\_DM} = \hat{A}_{RI\_DM} - A_{RI\_DM}$), studying NEA cod
692 from Andenes caught in 2003 and 2004. 'Reference' shows no error, i.e., $\Delta\hat{A}_{RI\_DM} = 0$.
693

694 **Fig. 9.** Mean atretic intensity of atresia (A_{RI_DM}) plotted versus mean hepatosomatic index
695 (HSI) for samples used either for method validation (2003 and 2004) or operational testing
696 (2005, 2006, extra and 2006). Encircled points showed pairs of observed and estimated,
697 corrected disector method (DM) values. The trend lines refer to samples 'with atresia' (dotted

line) and ‘all’ (atretic+non-atretic) samples (dashed line) using estimated, corrected DM values as dependent variable.

Fig 10. Appearance of atresia, reported as relative intensity (A_{RI_DM}), in relation to whole-mount mean oocyte diameter (OD), grouped into observed (2003 and 2004) and estimated (2005 and 2006) atretic DM values, showing for the last year the data points of ‘2006, extra’ separately.

Fig. 11. Development in realised fecundity with increasing relative intensity of atresia (A_{RI}) calculated by the standard fecundity reduction formula and the revised fecundity reduction formula using a 70-cm NEA cod as an example setting its potential (initial) fecundity to 1 million (Kjesbu et al., 1998), the atretic window to 43 days (OD: 525 – 675 µm (Fig. 10) corresponding to an increase of 150 µm, which was divided by an oocyte growth rate of 3.50 µm·day-1 at 4.5 ºC (Kjesbu et al., unpublished data)) and the atretic turnover rate (duration) to 9.7 days at 4.5 ºC (Witthames et al. this monograph).

TABLE CAPTION

Table 1

Present laboratory protocol developed for the disector method.

Table 2

Routines used to estimate disector method values of relative intensity of atresia from data produced by the profile method, first at the individual level and then at the group level.

723

724 **Table 3**

725 Overview of output data from prespawning NEA cod samples used either for method
726 validation (2003 and 2004) or operational testing of the established model (2005, 2006, extra
727 and 2006). The samples were collected at Andenes, Northern Norway in mid-February (2006,
728 extra) or late February/early March (all other samples). Oocyte diameter (OD) was measured
729 in whole mounts using image analysis while the relative intensity of atresia (A_{RI}) was
730 observed either by the profile method (PM) or the disector method (DM). For DM both
731 uncorrected (Eq. 1) and corrected values (Eq. 3) are presented (Table 2). For each sample the
732 atretic information is first given for 'all' females and then for females 'with atresia' only.

733

Figure 1

Click here to download high resolution image

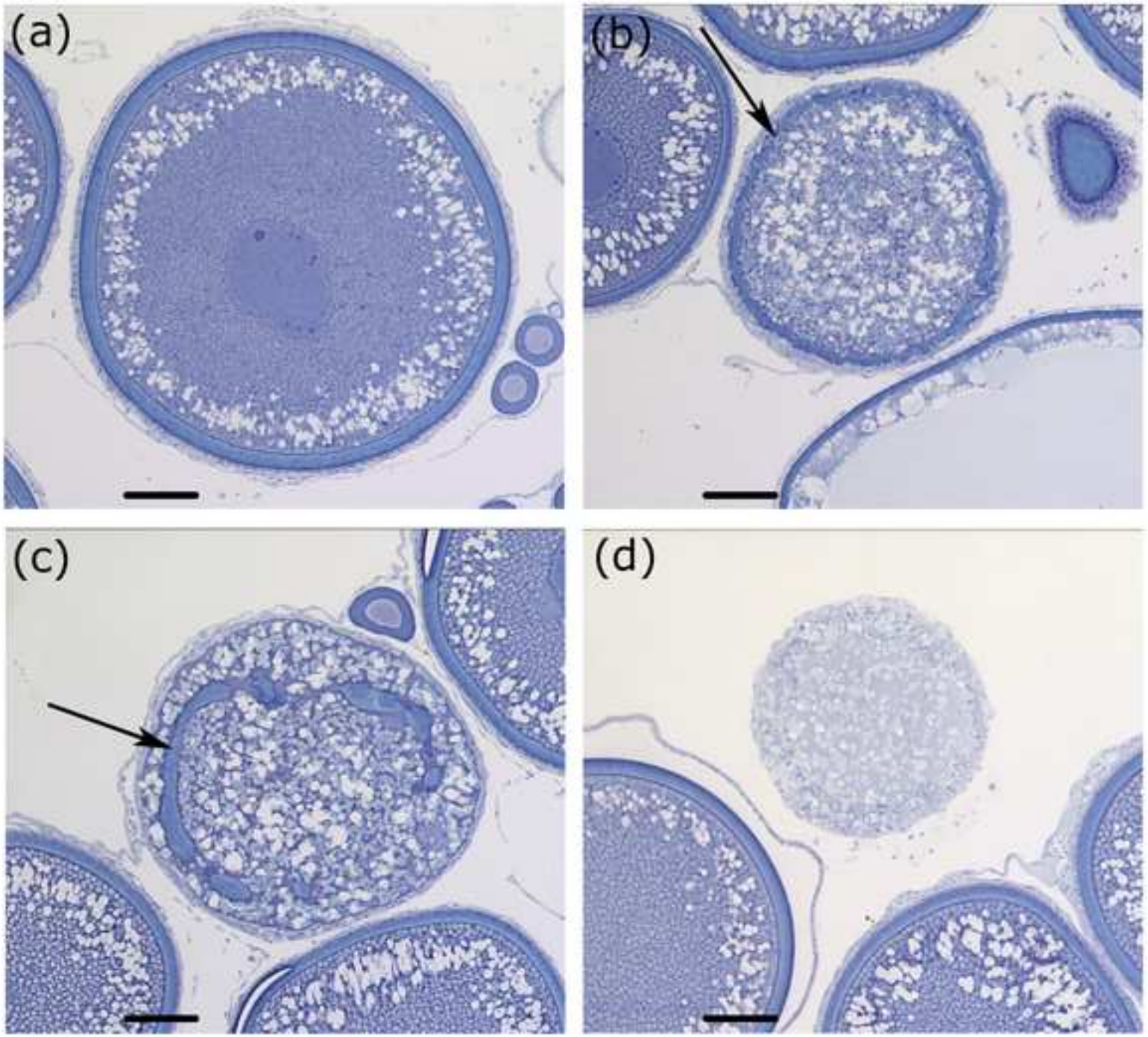

Figure 2

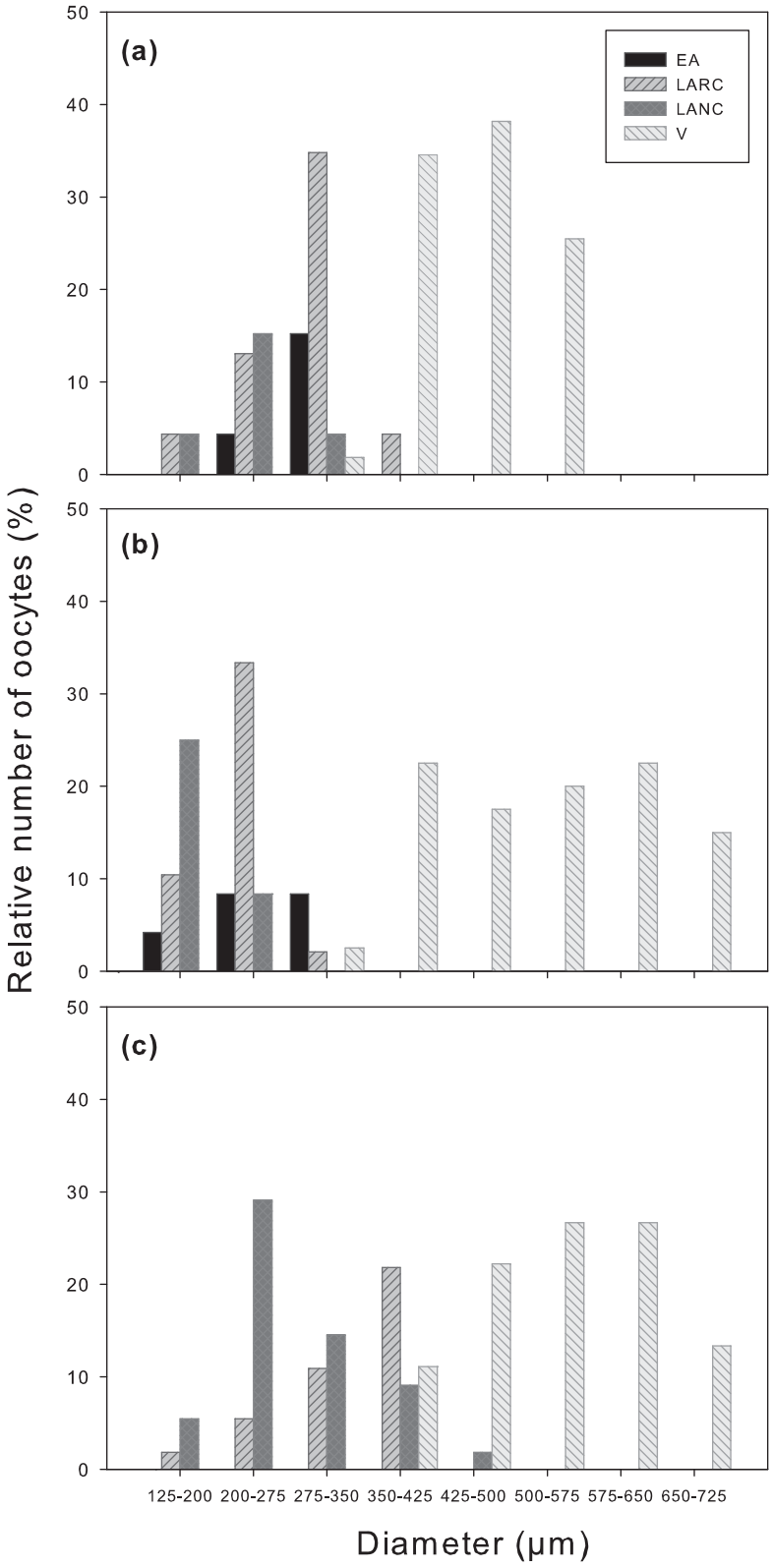
(a)
(b)
(c)
EA
LARC
LANC
V
Relative number of oocytes (%)
0
10
20
30
40
50
125-200
200-275
275-350
350-425
425-500
500-575
575-650
650-725
Diameter (μm)

Figure 3

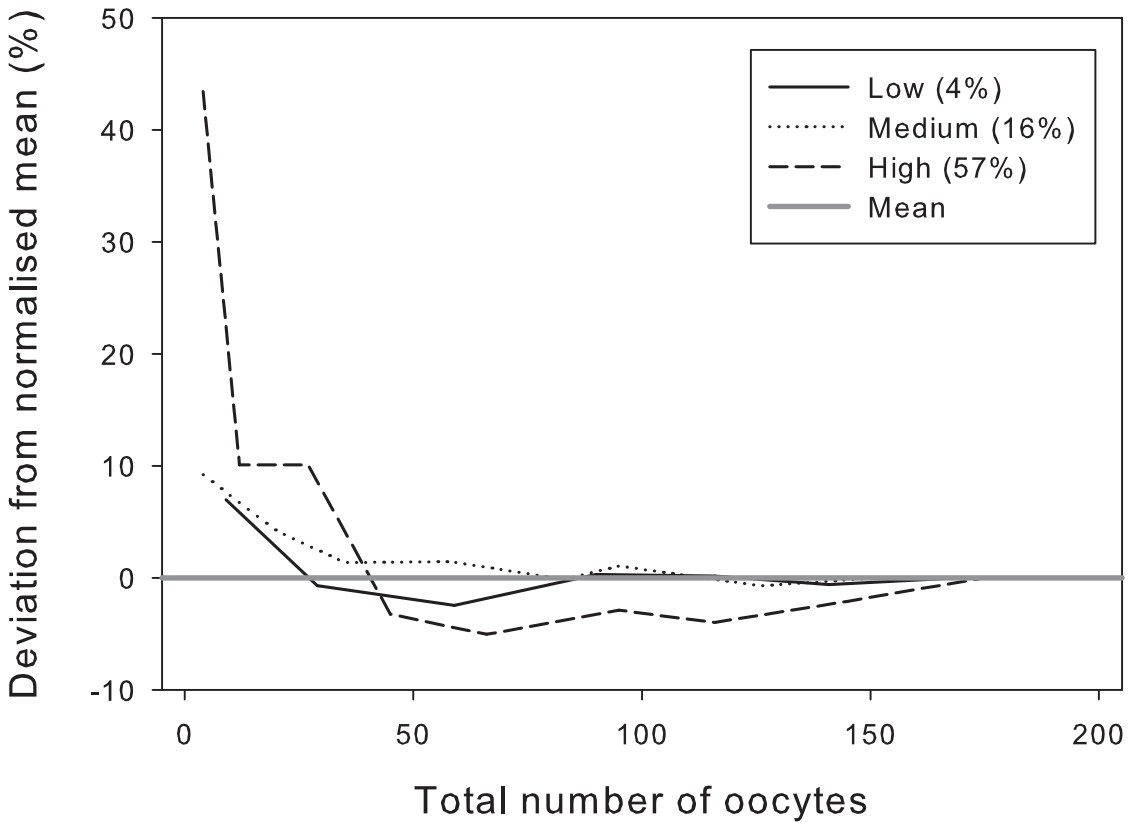
Deviation from normalised mean (%)
50
40
30
20
10
0
-10
0
50
100
150
200
Total number of oocytes
Low (4%)
Medium (16%)
High (57%)
Mean

Figure 4

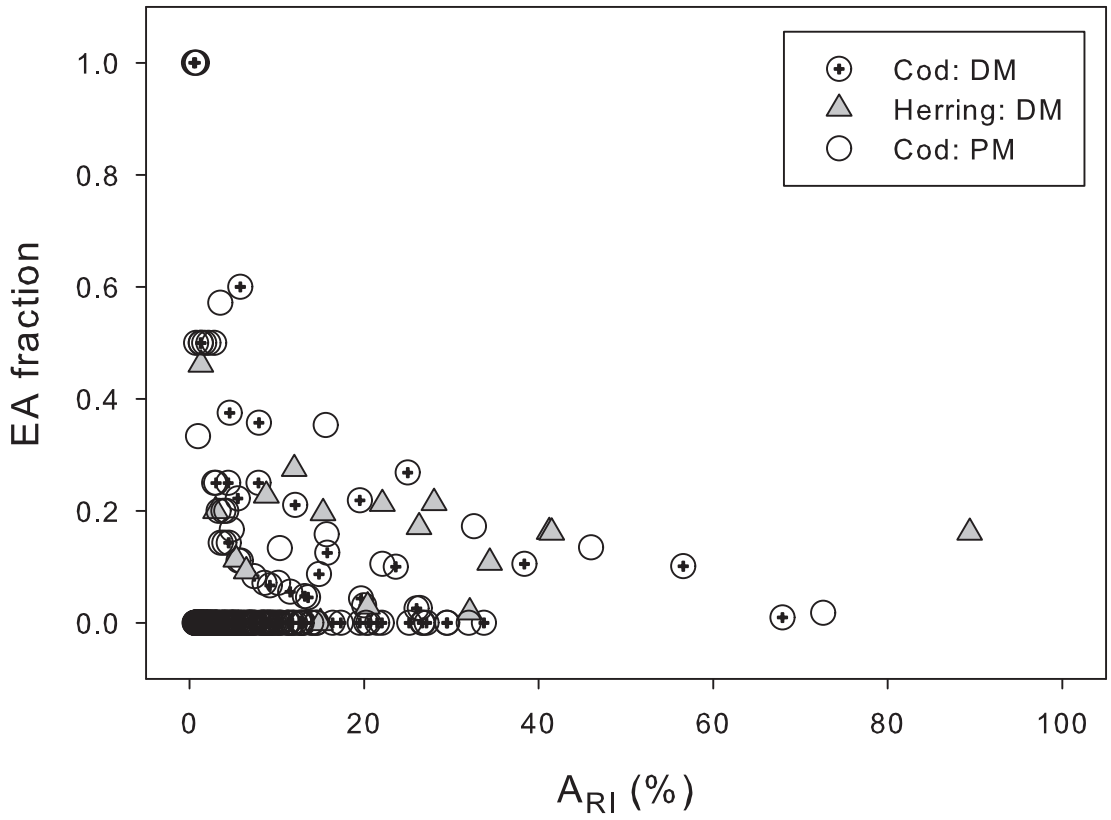
Cod: DM
Herring: DM
Cod: PM
EA fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
A_{RI} (%)

Figure 5

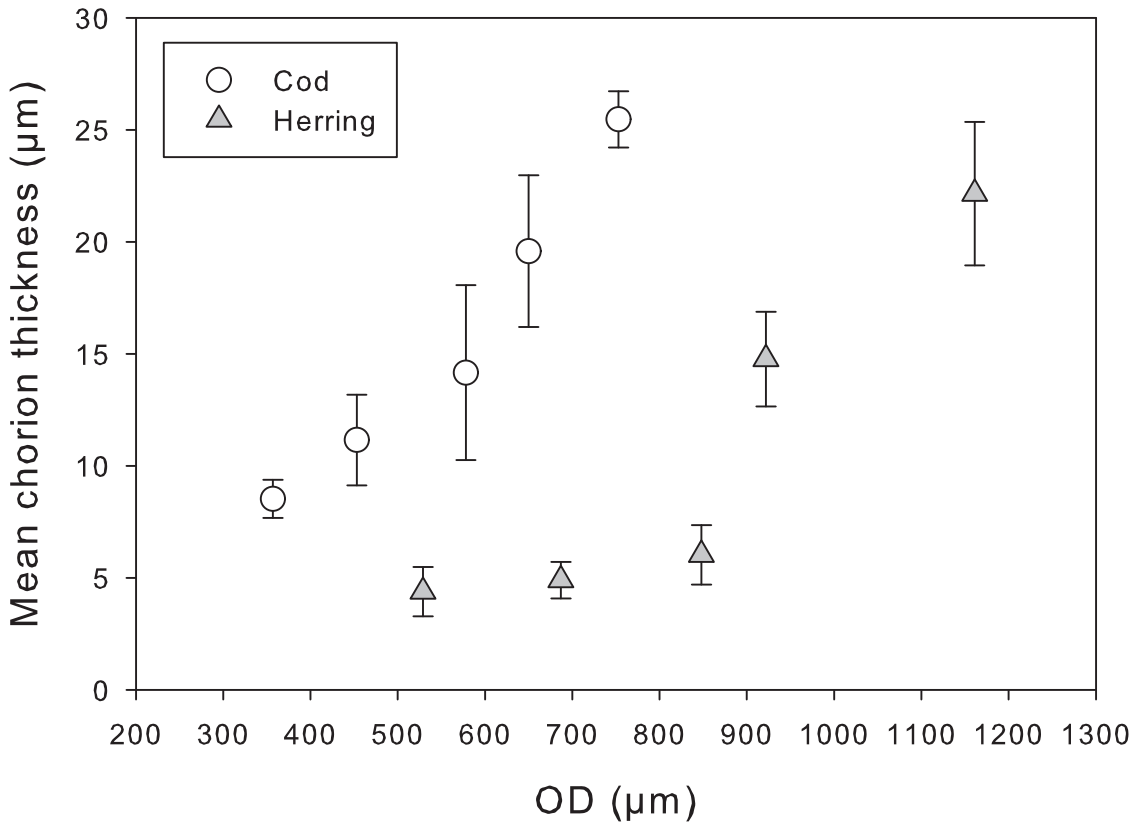
Cod
Herring
Mean chorion thickness (µm)
OD (µm)
0
5
10
15
20
25
30
200
300
400
500
600
700
800
900
1000
1100
1200
1300

Figure 6

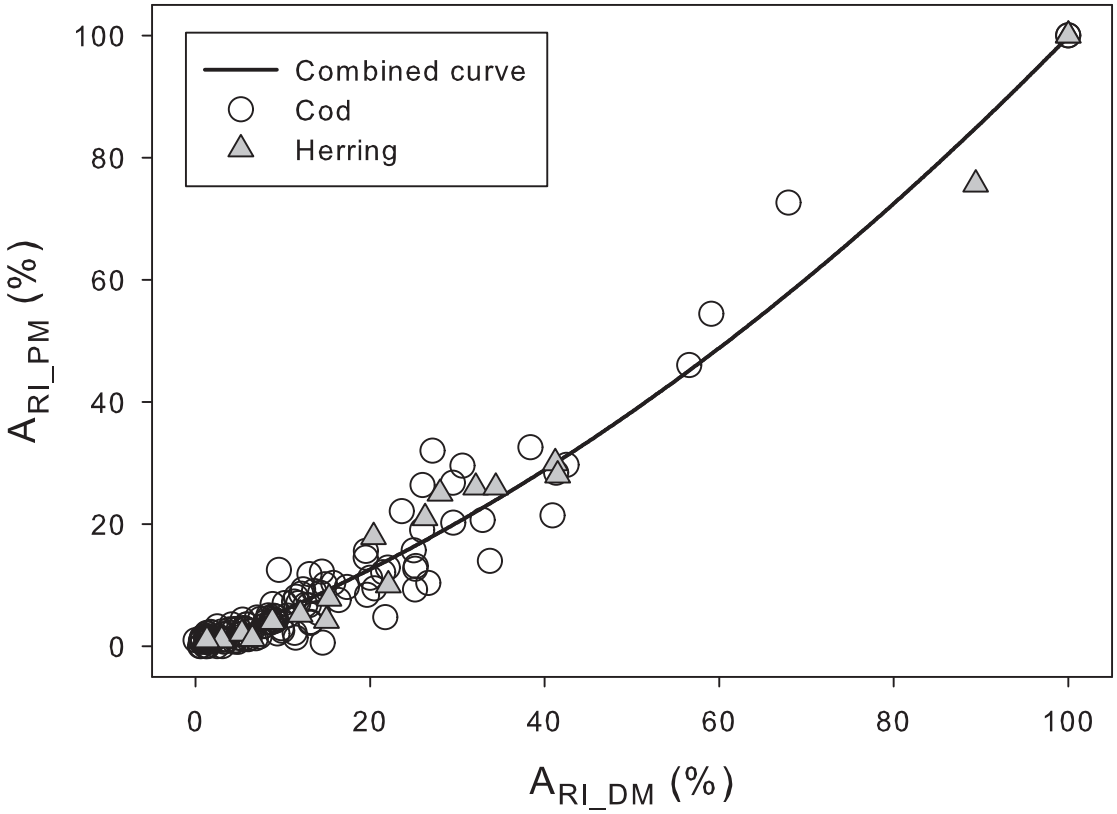
Combined curve
Cod
Herring
100
80
60
40
20
0
0
20
40
60
80
100
A_{RI_PM} (%)
A_{RI_DM} (%)

Figure 7

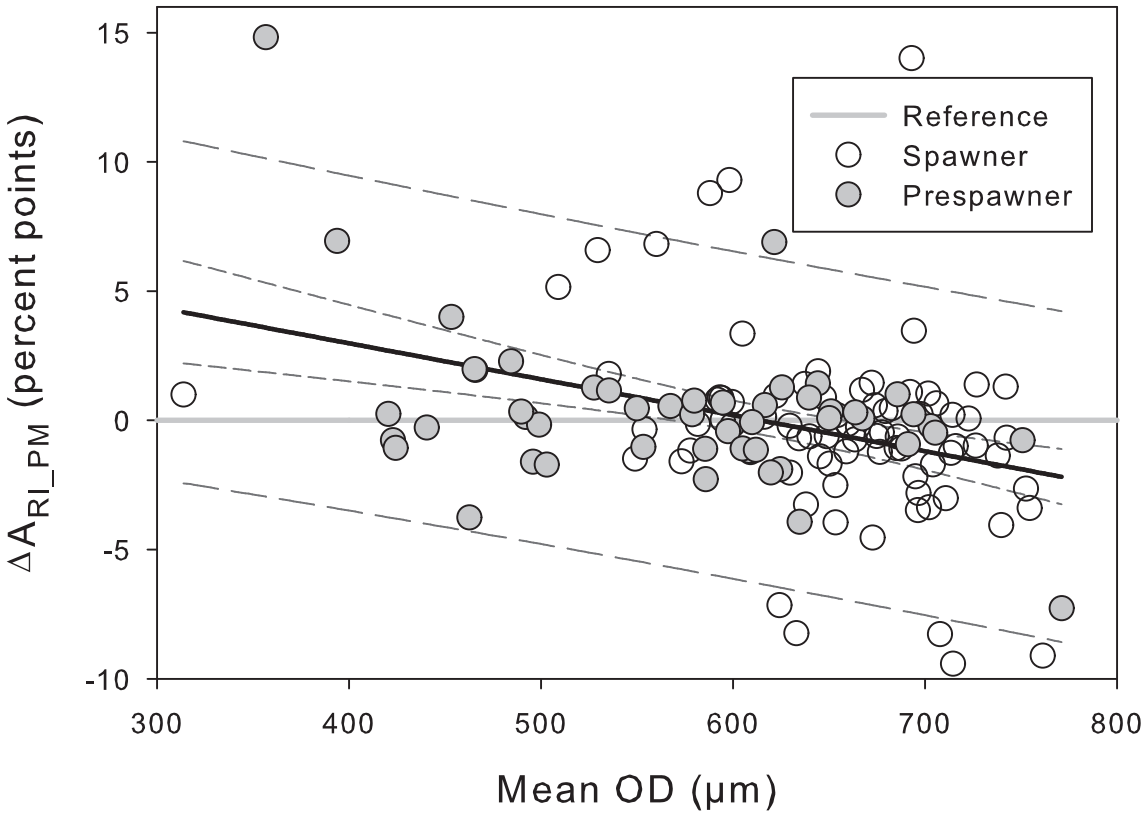
15
10
5
0
-5
-10
300
400
500
600
700
800
ΔA RI_PM (percent points)
Mean OD (μm)
Reference
Spawner
Prespawner

Figure 8

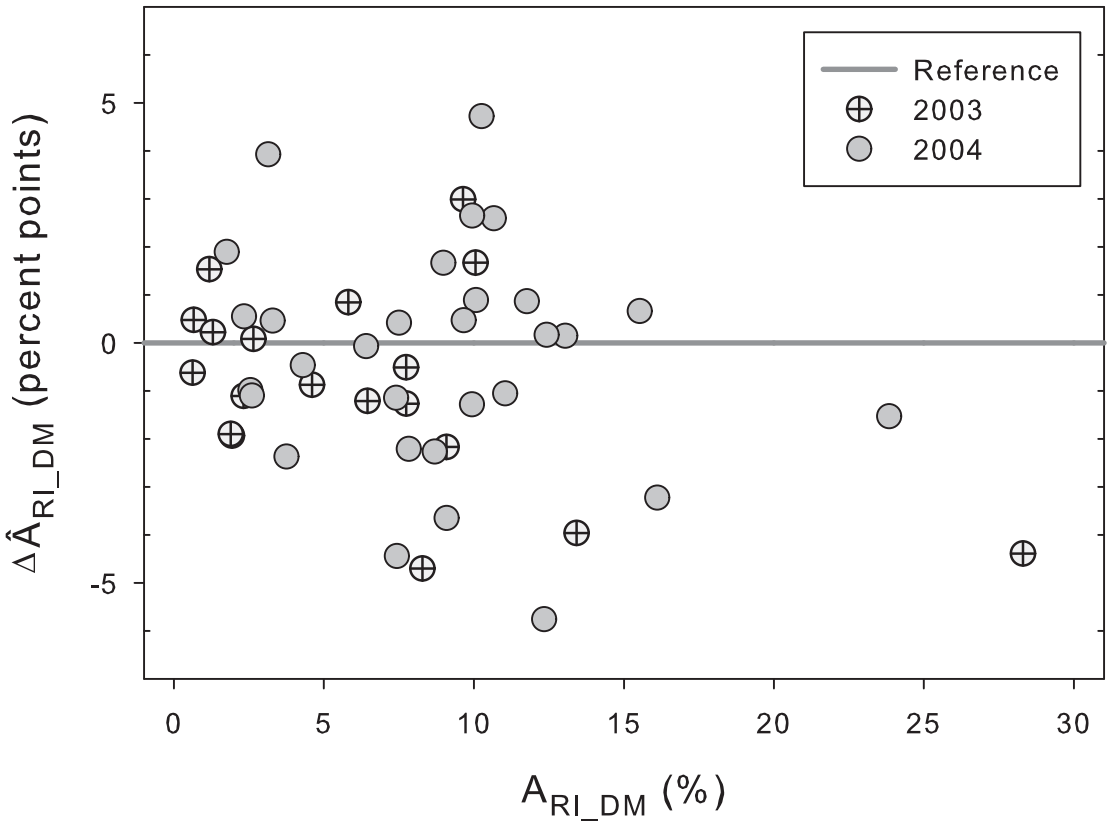
Reference
2003
2004
5
0
-5
0
5
10
15
20
25
30
$\Delta \hat{A}_{RI\_DM}$ (percent points)
A_{RI_DM} (%)

Figure 9

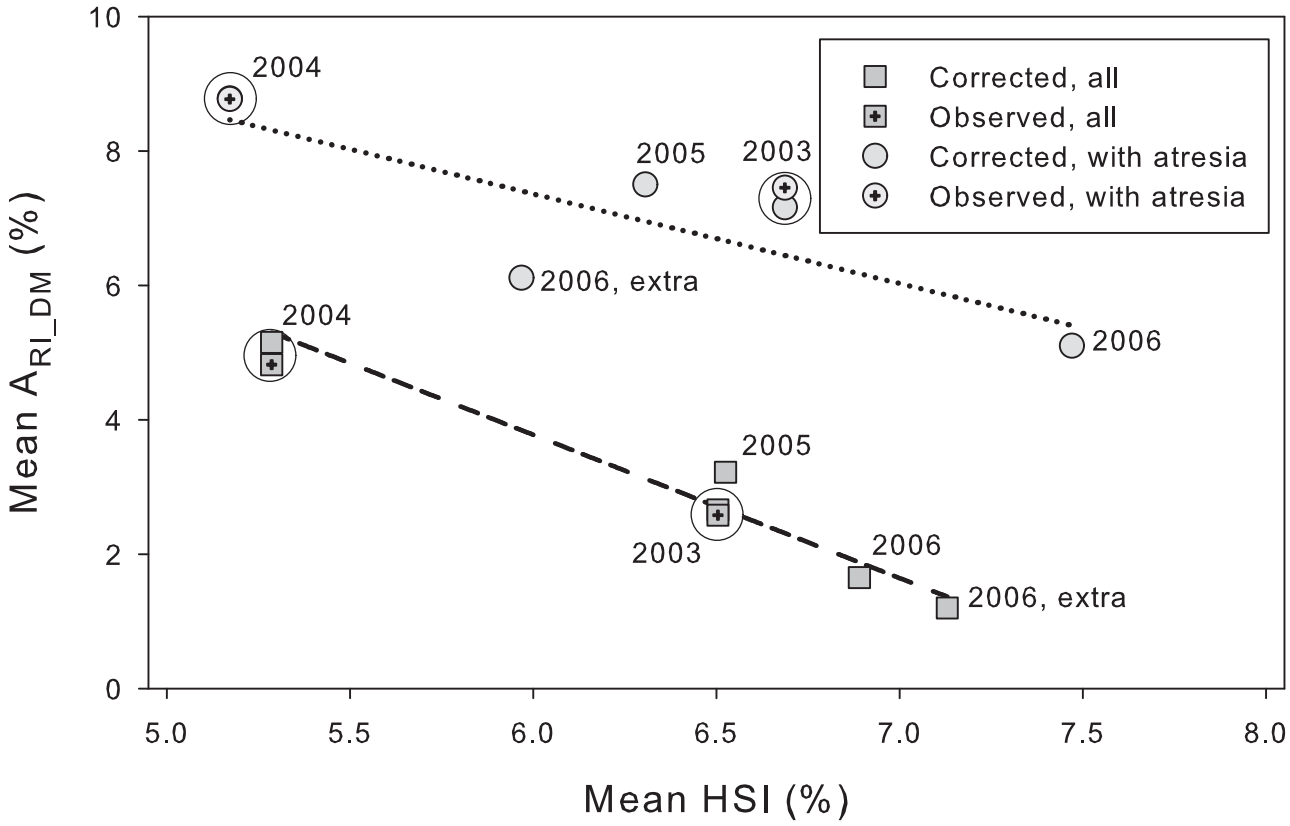
Corrected, all
Observed, all
Corrected, with atresia
Observed, with atresia
2004
2005
2003
2006, extra
2006
2004
2005
2003
2006
2006, extra
Mean A_{RI_DM} (%)
Mean HSI (%)
0
2
4
6
8
10
5.0
5.5
6.0
6.5
7.0
7.5
8.0

Figure 10

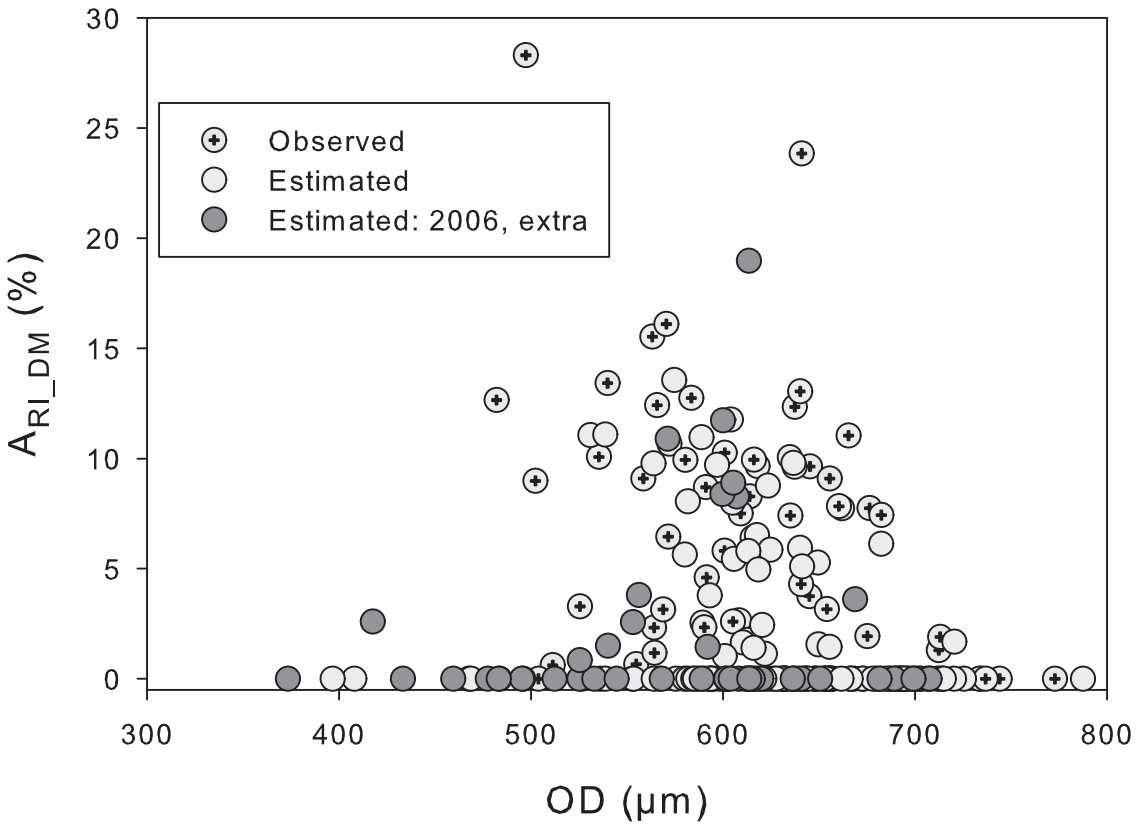
Observed
Estimated
Estimated: 2006, extra
A_{RI_DM} (%)
OD (μm)
0
5
10
15
20
25
30
300
400
500
600
700
800

Figure 11

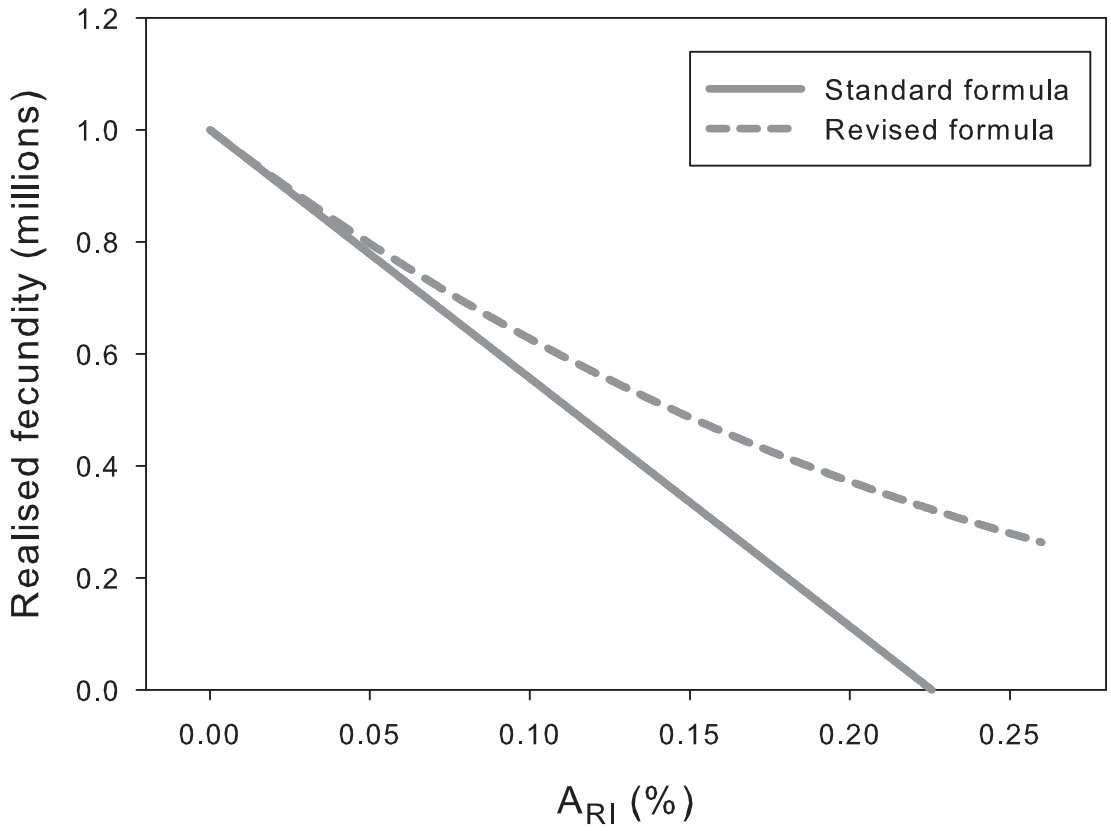
Realised fecundity (millions)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.00
0.05
0.10
0.15
0.20
0.25
A_{RI} (%)
Standard formula
Revised formula

Table 1.

Step	Procedure
1	Cut, stain and mount 4-µm serial sections from the block.
2	The distance between the serial sections should be 1/3 of the average diameter of the smallest cohort (SC) of vitellogenic oocytes. This SC diameter is given from the image analysing system.
3	The sections should fit into the field of a video camera attached to a binocular microscope (magnification: 7X).
4	A picture of each section is printed out: Section 1 as a paper sheet, Section 2 as a transparency sheet. Continue to alternate between paper and transparency sheets also for the other sections.
5	Bring the sections and the printouts (sheets) to a light microscope.
6	Look through the section under the microscope to locate any atretic cells.
7	Marked the atretic cells in your two printouts. Use a good permanent marker on the transparency sheet.
8	If required mark the Early Atretic (EA), Late Atretic With Residual Chorion (LARC) and Late Atretic No Chorion (LANC), separately
9	Cells to be be counted appear in only one of the two printouts: a) Take the transparency and paper sheet and locate them to overlay each other. b) Start to mark the atretic cells that appears in both sheets with a black ring. These will **not** be counted. c) Mark the atretic cells that appear in only one of the sheets with a red ring. These will be counted. d) Mark the normal cells that appear in both sheets with a black cross (on both sheets). These will **not** be counted. e) Mark the normal cells that appear only in one of the sheets with a red cross. These will be counted.
10	Sheet 1 in the first pair is now done. Sheet 2 in the first pair is also done, but you need it for the next step.
11	Find Sheet 3 and locate it to overlay with Sheet 2. You may have to turn the transparency up-side down to make it fit.
12	Mark Sheet 3: Black ring/cross if the respective cells are in both sheets and red if the respective cells appear only in Sheet 3.
13	Count the red rings/crosses in each sheet and fill the numbers in a form.
14	Continue doing these procedures until you have at least totally 150 cells (atretic + normal oocytes).

Table 2.

Step	Estimation practice
Individual level	
1	Analyse 100 vitellogenic oocytes by the image analyser to produce basic whole-mount statistics including mean diameter (OD), and to classify the female as either prespawner or spawner
2	Count about 150 histological profiles (normal and alpha-atretic oocytes) from the same sample under the light microcope; the Profile Method (PM)
3	Calculate observed relative intensity of atresia (100×atretic oocytes/(normal and atretic oocytes)); A_{RI_PM} (%)
4	Estimate the corresponding Disector Method (DM)-based A_{RI} ($\hat{A}_{RI\_DM}$) by iteration (e.g. in Excel) using Eq. (1)
Group level	
5	Estimate the general residual $\Delta\hat{A}_{RI\_PM}$ from grand mean OD using either Eq. (2) (prespawners and spawners) or (3) (prespawners only)
6	Correct $\hat{A}_{RI\_PM}$ by the expression: $\hat{A}_{RI\_PM} = A_{RI\_PM} - \Delta\hat{A}_{RI\_PM}$, all mean figures
7	Correct the matching $\hat{A}_{RI\_DM}$ by iteration (Eq. 1)

Table 3

Year	Category	n	Grand mean OD (SD) (µm)	Mean observed A_{RI} values (SD) (%)		Mean estimated A_{RI} value (SD) (%)	
				PM	DM	DM, uncorrected	DM, corrected
2003	all	48	626 (71)	1.31 (2.83)	2.58 (5.15)	2.23 (4.57)	2.66 (—)
	with atresia	16	606 (57)	3.94 (3.74)	7.45 (6.68)	6.68 (5.80)	7.16 (—)
2004	all	51	627 (51)	2.73 (3.36)	4.82 (5.60)	4.64 (5.53)	5.15 (—)
	with atresia	28	603 (43)	4.97 (3.05)	8.77 (4.71)	8.45 (4.83)	8.78 (—)
2005	all	44	619 (69)	1.73 (2.42)	—	2.99 (4.14)	3.22 (—)
	with atresia	19	617 (45)	4.00 (2.11)	—	6.93 (3.49)	7.50 (—)
2006, extra	all	39	572 (79)	1.26 (2.59)	—	2.14 (4.29)	1.20 (—)
	with atresia	13	573 (60)	3.77 (3.32)	—	6.42 (5.35)	6.11 (—)
2006	all	42	630 (54)	0.70 (1.55)	—	1.23 (2.69)	1.65 (—)
	with atresia	11	610 (27)	2.67 (2.01)	—	4.68 (3.43)	5.10 (—)