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Report of the Workshop on Mackerel and Horse Mackerel Egg Staging and Identification (WKMHMES)

23–27 October 2006

Lowestoft, UK

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Contents

Executive summary

The Workshop on Mackerel and Horse Mackerel Egg Staging and Identification [WKMHMES] met in Lowestoft, England, UK, from 23–27 October 2006 to address six Terms of Reference (Section 1.2).

Highlights

- A number of excellent presentations were given prior to the practical aspects of the Workshop commencing. These included the use of image analysis systems for the automatic measuring of fish egg and oil globule diameters. This imaging technology is advancing rapidly, and participants were encouraged to maintain and develop an interest in this area for its potential use as an aid in the identification and staging of fish eggs. Other presentations described a DNA technique for the identification of fish eggs to species, the apparent dramatic increase in the population of Snake Pipefish (*Entelurus aequoreus* (L.)) and the standardisation of Bongo nets for use on the mackerel and horse mackerel egg surveys (See Section 6 for abstracts).

- The 'spray technique' for the removal of fish eggs from preserved plankton samples was again tested for efficiency, following the preliminary trials conducted at the 2003 Workshop (ICES, 2004). The results were encouraging, particularly once initial problems had been discussed and addressed.

- The majority of the time at the Workshop was spent identifying and staging mackerel, horse mackerel and similar eggs. The results promoted discussion and highlighted specific problem areas. These discussions led to the further development of standard protocols, and enhancements to the species and stage descriptions. The results were very re-assuring and similar to those obtained at the 2003 workshop. There was a slight under-estimate of stage 1 mackerel eggs (stages 1a and 1b combined) during the first round of analysis (−2%) and a slight over-estimate (2%) during the second round. The results for stage 1 horse mackerel eggs were similar with under-estimates of −2% and −1% respectively. This is particularly re-assuring as it is this stage on which the egg production estimates are based.

- Whilst the egg workshop was being conducted some histology training and inter-calibration took place under the instruction of Mr Peter Witthames (Cefas). This proved beneficial to all concerned and as a result enhancements were made to the WGMEGS survey manual (Annex 5) and an adult fish sampling and fecundity estimation manual was produced (Annex 6).

Terms of Reference and outcomes

ToRs a) and b) referred to the comparative plankton sorting and egg staging trials.

These trials followed the same procedures as those conducted during the 2003 workshop (ICES, 2004). The sorting trials were designed to assess the effectiveness of the 'Spray Technique'. If this technique proved effective then WKMHMES would recommend this procedure as the preferred method for removing mackerel and horse mackerel eggs from plankton samples. The staging trials were carried out exactly as in the previous two workshops in 2000 and 2003. However, for the first time, all the eggs used were of known species as they had come from artificial fertilisations or had been naturally spawned in large tanks.

ToRs c) and e) referred to the production of standard pictures and descriptions to aid identification of eggs to species and allocation to development stages.

Standard pictures and descriptions of mackerel and horse mackerel eggs and those of other fish eggs, which can be confused with these two target species, were reviewed during the course of the workshop. The descriptions of the various stages of fish egg development were also reviewed and updated as appropriate. These descriptions are presented in Sections 3.2.2 and 3.3.2 of this report.

ToR d) referred to inter-calibration of fecundity determination to harmonise the analysis and interpretation of fecundity samples.

This work took place under the instruction of Mr Peter Witthames (Cefas Laboratory, Lowestoft). Discussion took place on recent changes of procedures and an updated fecundity manual was produced (Annex 6). An inter-calibration exercise was then conducted on images and slides of mackerel ovaries, which were 'scored' by each participant. Comparisons were made between unstained and stained (using various stains) images and slides.

Tor f) referred to a review of any other egg identification procedures being tested by participating laboratories, particularly DNA probes.

Two working documents were presented which addressed this term of reference. One described the use of 'image J' processing software to automatically measure fish egg and oil globule diameters to determine species identification. The other presented the work of Marineggs which was an EU project designed to enable the identification of fish eggs using species specific DNA markers. Abstracts of both these working documents are given in Section 6.

1 Introduction

1.1 Background

In preparation for the 2007 international ICES coordinated mackerel and horse mackerel egg survey, a workshop was held at CEFAS laboratory, Lowestoft, England (23–27 October 2006) for the majority of plankton analysts who would be involved with the 2007 survey. The aims of the workshop were to standardise procedures and produce definitive criteria for the identification and staging of mackerel and horse mackerel eggs. The workshop would also investigate the reasons for individual differences in the identification and staging of mackerel and horse mackerel eggs and attempt to harmonise these. In addition, further evaluation of the 'spray' technique for removing fish eggs from plankton samples, was carried out.

To enable the calculation of the numbers of spawning female fish in a stock by using the Annual Egg Production Method (AEPM. Lockwood *et al.*, 1981, Armstrong *et al.*, 2001) it is essential to correctly identify (both in terms of species and age) the number of freshly spawned eggs, i.e. the eggs in development stages Ia and Ib, and to distinguish these from eggs in later stages of development. It is therefore vital that the analysts involved with sorting, identification and staging of mackerel and horse mackerel eggs from the tri-ennial egg surveys (ICES, 2006) are able to accurately identify and stage the eggs of each of the target species. These workshops (WKMHMES) were designed to bring the analysts together to develop consistent criteria for the identification and staging of the eggs, and to discuss how to overcome the practical problems encountered whilst doing so.

Previous workshops (ICES, 2001 and ICES, 2004) have developed a comprehensive set of criteria for both mackerel and horse mackerel egg identification and staging. These criteria were to be expanded and developed during the 2006 workshop. In addition, a few inexperienced analysts would be involved for the first time, and it was critical that they became fully aware of the procedures and criteria in advance of the 2007 plankton samples being collected.

1.2 Terms of Reference

At the ICES Annual Science Conference in Aberdeen, Scotland, in September 2005 it was decided that (C.Res. 2005/2/LRC16) a Workshop on Mackerel and Horse Mackerel Egg Staging and Identification [WKMHMES] (Chair: S. Milligan, UK) will be held in Lowestoft, UK from 23–27 October 2006 to:

a) carry out comparative plankton sorting trials on typical survey samples. This should follow the pattern of trial – analysis – retrial – identification of problem areas;

b) carry out a comparative egg staging trial for mackerel and horse mackerel eggs following the pattern used in the 2003 egg staging workshop;

c) produce a set of standard pictures and descriptions for species ID and egg staging;

d) carry out inter-calibration work on fecundity determination and harmonise the analysis and interpretation of fecundity samples;

e) provide a review of any available documentation on identifying eggs to species and define standard protocols;

f) provide a review of any information available on other egg ID procedures – particularly DNA probes.

1.3 Participants

A list of participants can be found in Annex 1 of this report.

2 Adoption of the agenda

An agenda was distributed to all participants a few weeks before the workshop. This agenda, which can be found at Annex 2 of this report, was agreed prior to the workshop commencing.

3 Materials and methods

3.1 Egg sorting trials (referring to ToR 'a')

As a result of the egg sorting trials conducted during the 2003 WKMHMES workshop, several participating institutes were now using the 'spray technique' for routinely removing fish eggs from plankton samples. The technique has also been refined, and a draft paper (Eltink, 2006) describing the enhanced procedures was available for participants to refer to.

In an attempt to evaluate the effectiveness of the 'spray technique' three plankton samples (typical plankton from the 2004 survey) were prepared, each containing a total of 500 mackerel and horse mackerel eggs. As many participants as possible were asked to undertake the following procedure to remove and count the eggs from the prepared samples.

The formaldehyde was rinsed from the sample in a 270µm mesh sieve. The plankton was then washed into a plastic funnel, fitted with a tap, with a little seawater. A normal garden spray pump was used to 3/4's fill the funnel with pressurised water. The spray jet was rotated around the sides of the funnel to limit damage to the plankton. The fine, pressurised spray caused aeration of the sample with many fine bubbles, which gave the sample a cloudy appearance. The sample was then left to stand for one to two minutes whilst the air bubbles became trapped in the parts of the plankton that had projections (legs, antennas etc). The aerated plankton floated to the surface and all smooth particles, including the fish eggs, sank to the bottom. The fish eggs were then drained from the bottom of the funnel, by opening the tap, and collected in a small beaker. The spraying was then repeated until very few eggs were removed from the bottom of the funnel (a maximum of 8 times). It is recommended that the waiting time is increased for each subsequent spraying to allow the more buoyant eggs time to settle out from the rest of the plankton. The sample was then fully sorted using a binocular microscope, to remove any remaining eggs from the plankton.

The numbers of eggs removed after each spraying and those eggs remaining in the plankton were counted, and the results recorded in Table 4.1.1.

3.2 Egg staging (referring to ToR's 'b'and 'c')

3.2.1 Egg staging trials

A total of 400 mackerel, horse mackerel, hake (*Merluccius merluccius*, L.) and megrim (*Lepidorhombus whiffiagonis,* Walbaum) eggs were placed in 16 small, Perspex trays. Each tray contained 50 small wells but only the first 25 wells were used to hold one egg each. Each tray was numbered and placed on the stage of a stereo-zoom microscope. The rows and columns of each tray were labelled so that the position of each individual egg could be identified.

In contrast to previous workshops, all of the eggs used were validated (of known species from artificial fertilisations or from natural spawning of captive fish). The eggs were mainly those of mackerel and horse mackerel with a few eggs of hake and megrim, which are morphologically similar to those of the two target species. It was hoped that these definitive eggs, of known parentage, would enable participants' species identification to be judged more consistently than in previous workshops (see Section 4.3). The eggs were selected at random with the intention of providing the full range of egg stages, but with greater emphasis on stage 1 eggs on which the estimates of SSB are based. The mackerel, hake and megrim eggs in each tray were staged to Ia, Ib, II, III, IV, V and the horse mackerel were staged to Ia, Ib, II, III, IV,

as horse mackerel larvae hatch before the eggs reach stage V. Due to the fact that computers can only calculate with numeric values, stage Ia was changed to 0 and stage Ib to 1 in the result tables.

Each participant moved from one microscope to another in order to complete the staging and identification of all 400 eggs. In this way, the results of the egg stage readers were not affected by differences in the quality of the microscopes. Unlike the workshop held in 2000, most of the microscopes were modern and provided good optical quality. There were, however, limitations to the amount of transmitted light provided by some microscopes and only a few were fitted with eyepiece graticules.

Once each participant had staged and identified each of the eggs and the results had been entered into a result spreadsheet, a full discussion on egg staging and identification took place. From the analysis of the first set of results it became apparent which individual eggs had resulted in high or low agreement of allocated stage. Low agreement amongst participants indicated problems in allocating an egg consistently to one developmental stage. These eggs were then placed under a microscope equipped with a video camera and displayed on a large screen. Discussions then took place on the diagnostic features visible in the egg, which generally led to an agreement on the most likely developmental stage and/or species involved. In this way, the egg staging criteria (ICES, 2004) were revised (see Section 3.2.2).

During the course of the first round of analysis several eggs became damaged, or were moved, from one cell to another in the trays. It was not, therefore, possible for all participants to always stage or identify each egg. Before the second round of analysis began, another set of eggs was randomly placed in the trays. This provided a different mix of species and stages and prevented a direct comparison between the first and second round of results. However, the lessons learnt during the first round of analysis and subsequent discussions would, hopefully, still be reflected in the second round results.

3.2.2 Egg stage criteria

As a result of discussions following the first round of egg staging the participants decided upon the following definitions of the developmental stages for mackerel, horse mackerel, hake and megrim. The primary characteristics are based on those presented in Lockwood *et al*. (1977) for mackerel (Figure 3.2-1.), but now include some other (secondary) characteristics, which the participants thought were crucial in determining egg stage.

Stage Ia

Primary characteristics: From fertilisation until cleavage produces a cell bundle in which the individual cells are not visible.

Secondary characteristics: There are no signs of a thickening of cells around the edge of the cell bundle. **NB**. In preserved eggs the edge of the cell bundle can sometimes fold over giving the appearance of a ‘signet ring’ seen in a stage Ib.

Stage Ib

Primary characteristics: Formation of the blastodisc, visible as a ‘signet ring’ and subsequent thickening a one pole.

Secondary characteristics: The cell bundle has thickened around the edge giving a distinct ring appearance. Cells in the centre of the ring form a progressively thinner layer and eventually disappear. **NB**. At the end of this stage the ring can become very indistinct as it spreads towards the circumference of the egg.

Stage II

Primary characteristics: From the first sign of the primitive streak until closure of the blastopore. By the end of this stage the embryo is half way round the circumference of the egg. However, the tail still tapers to end flattened against the yolk, in this stage.

Secondary characteristics: Early in this stage the primitive streak can be difficult to see, only appearing as a faint line in the surface of the yolk. Late in this stage the head is still narrow and the eyes are not well formed.

Stage III

Primary characteristics: Growth of the embryo from half way to three-quarters of the way around the circumference of the egg. The end of the tail has thickened, becoming bulbous in appearance.

Secondary characteristics: Widening of the head and development of the eyes. Pigment spots develop on the embryo, usually close to the posterior end.

Stage IV

Primary characteristics: Growth of the embryo from three-quarters to the full circumference of the egg.

Secondary characteristics: Eyes continue to develop and the lenses become visible. Development of the marginal fin and the tail begins to separate from the yolk. Pigmentation of the body increases.

Stage V

Primary characteristics: Growth of the embryo until the tail has reached the nose.

Secondary characteristics: Pigmentation develops in the eye.

NB

The preservation of eggs can cause shrinkage and distortion of the embryo. Therefore care should be taken when assessing the length of the embryo, as they do not always remain around the full circumference of the yolk. They may also become distorted giving a false impression of development stage.

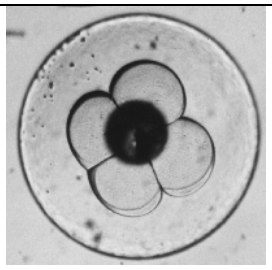
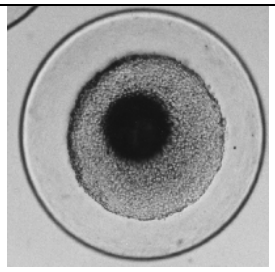
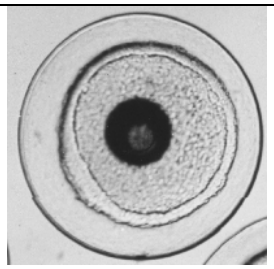
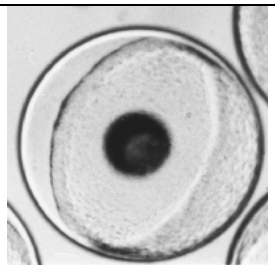
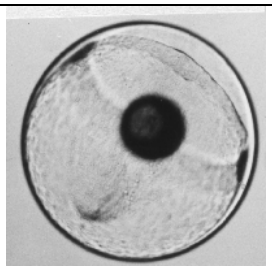
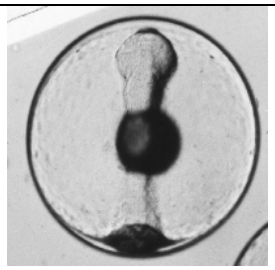
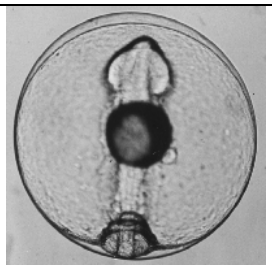
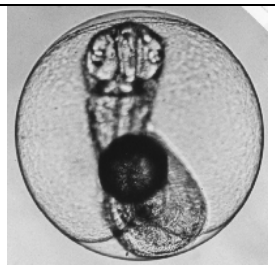
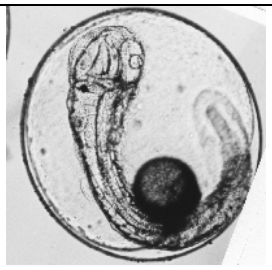
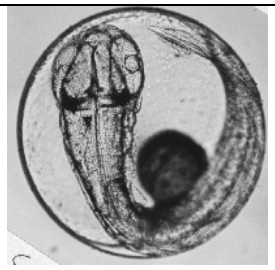
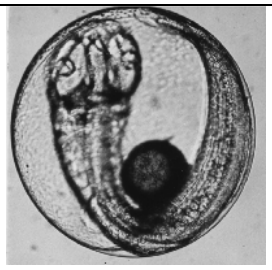
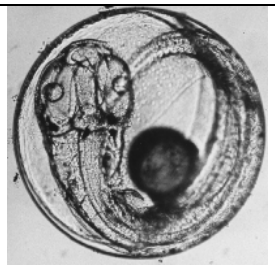
Early stage	Late stage
IA 	
IB 	
II 	
III 	
IV 	
V 	

Figure 3.2-1. Mackerel eggs at the beginning and end of the six development stages.

3.3 Egg Identification (referring to ToR's 'c' and 'e')

3.3.1 Egg identification trials

The same trays of fish eggs (described in Section 3.2 above) were also used for the egg identification exercise. As each participant moved from microscope to microscope they were asked to provide a species identification for each egg, in addition to a development stage. A short presentation on the main features of mackerel and horse mackerel eggs was delivered before the first round of analysis. This provided some useful preliminary information on egg identification to less experienced participants and enabled the more experienced analysts to discuss the criteria they used to identify mackerel and horse mackerel eggs.

The results of the first round of egg identifications were collated and input into spreadsheets at the same time as the results for egg staging. The results were presented and eggs with low agreement in species identification were displayed on a large screen (as described in Section 3.2 above). A discussion then took place until a consensus was reached on the most likely species identification for each of these eggs. As a result of these discussions and before the second round of analysis was begun, a review of the egg identification criteria produced by previous WKMHMES participants was carried out.

3.3.2 Egg identification criteria

Table 3.3-1 summarises published descriptions of mackerel, horse mackerel and other species of eggs with similar morphological features. It particularly concentrates on egg and oil globule sizes, which may vary through the spawning season and from area to area. A complete reference list is given at the end of this report.

In addition to the published descriptions given in Table 3.3-1, various other criteria are used by participants to help with egg identification based their own knowledge and experience. These criteria can be regarded as secondary characteristics and are described for each species below. Photographs of known horse mackerel eggs from the southern area are shown in Figure 3.3-1 for comparison with mackerel eggs shown in Figure 3.2-1.

Mackerel *(Scomber scombrus)*. (See Lockwood *et al.*, 1977)

- Oil globule often orientated to the top of the egg during analysis with the embryo following the circumference of the egg.

Horse Mackerel (*Trachurus trachurus*). (See Pipe and Walker, 1987)

- Oil globule easily broken into several smaller pieces. This seems to be more common in eggs found in the southern area, particularly in eggs from the Portuguese coast.
- Some early stage eggs from the southern area also lack colour in the yolk, which is unusual, as horse mackerel eggs normally have a darker yolk than mackerel.
- The oil globule migrates to the head of the embryo after stage 2.
- In stages 3 and 4 the embryo shows very strong pigmentation.

Hake (*Merluccius merluccius)* (See Coombs, 1982)

- Pigmented oil globule.
- Towards the end of it's development the embryo begins to show the characteristic postanal pigmentation of three bars.
- Positive surface adhesion test (SAT) is also used to identify hake eggs (Porebski, 1975) and (Coombs, 1994).

Megrim (*Lepidorhombus whiffiagonis*)

- Striated punctuate appearance of egg membrane.
- Oil globule is closer to egg membrane than in mackerel.

- Embryo thinner than a mackerel embryo, with less pigmentation.
- Yolk unsegmented and the egg has a small perivitelline space.
- Pigmentation on yolk from stage II onwards.

Longspine snipefish (*Macrorhamphosus scolopax*)

- Egg spherical and transparent.
- Membrane is light amber with grainy reflections.
- Yolk with rose or violet halo depending on viewing light.
- Oil globule is amber / rose in colour.

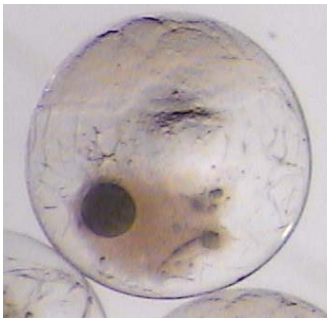
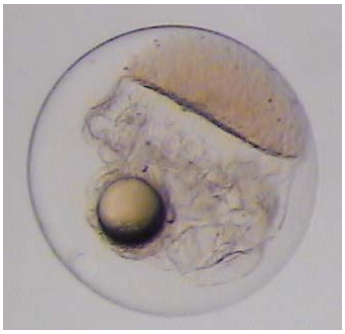
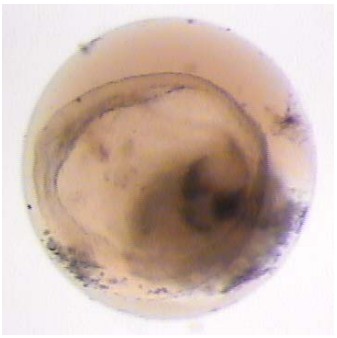
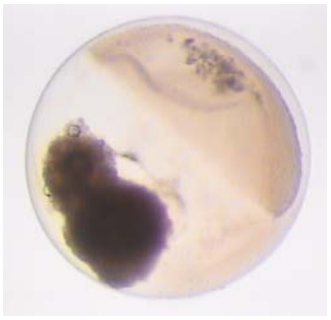
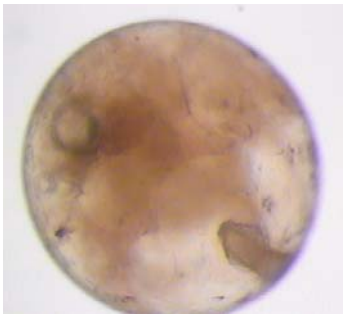
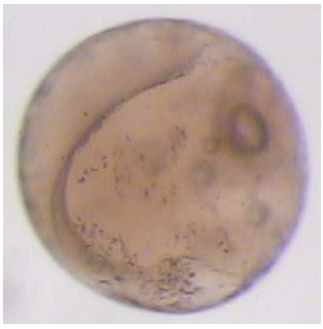
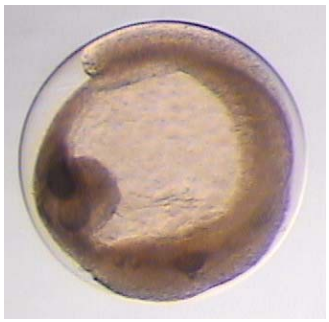
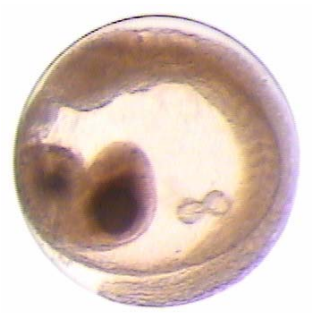
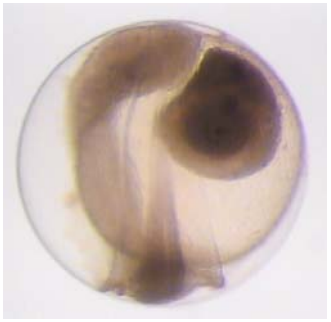
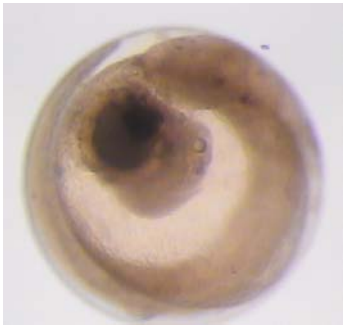
Stage IA	Stage IA	Stage IB
		
Stage II	**Stage II**	**Stage II**
		
Stage III	**Stage III**	**Stage IV**
		
Stage IV	**Stage IV**	
		

Figure 3.3-1. Horse mackerel eggs in each of the five development stages.

Table 3.3-1. Comparison of the Characteristics of Mackerel, Horse Mackerel, Megrim, Hake and Snipefish Eggs (Details of fixative and concentration unknown).

Species	Diameter (mm)		Other Features Noted	Area	Reference
	Egg	Oil Globule			
Mackerel (*Scomber scombrus*)	1.0-1.38	0.28-0.35	Unsegmented yolk	North Sea, English Channel	Russell, 1976
	1.09-1.36	0.26-0.37	Homogenous yolk	N.W. Atlantic	Fahay, 1983
	0.97-1.38	0.25-0.35		Irish Sea, North Sea	Ehrenbaum, 1905–1909
	1.071-1.193	0.285-0.360		Mediterranean	D'Ancona *et al*., 1956
	0.97-1.38	0.22-0.38	Perivitelline space approx 0.05mm	Mid-Atlantic Bight	Development of Fishes of the Mid-Atlantic Bight, 1978
	1.0-1.38			North Atlantic	
	0.86-1.04			Mediterranean	
	0.97-1.38	?		Isle of Man	Johnstone, Scott and Chadwick, 1934
	1.21-1.33	~0.32		West of Ireland	Holt, 1893
	0.9-1.4	?		NE Atlantic	Froese and Pauly, 2003
Horse Mackerel (*Trachurus trachurus*)	0.81-1.04	0.19-0.28	Segmented yolk	North Sea, English Channel	Russell, 1976
	1.03-1.09	0.26-0.27	Segmented yolk	North Sea	Holt, 1898
	0.81-0.93	0.22-0.23		Plymouth	
	0.84-1.04	0.19-0.24	Totally segmented yolk	North Sea, English Channel	Ehrenbaum, 190–1909
	0.81-1.04	0.19-0.24	Segmented yolk	North Sea, English Channel	D'Ancona *et al.*, 1956
	Max. 0.84	0.24-0.26	Granular yolk	English Channel	Holt, 1893
	0.76-1.07	0.19-0.29	Segmented yolk	Europe	Froese and Pauly, 2003
Megrim (*Lepidorhombus whiffiagonis*)	1.02-1.22	0.25-0.30	Striated membrane. Pigment on oil globule as larva develops	North Sea, Irish Sea	Russell, 1976
	1.07-1.22	0.25-0.30	Fine "meshwork" on inside of membrane. Pigment on oil globule as larva develops	North Sea	Ehrenbaum, 1905–1909
	1.07-1.13	0.30	Striations on inside of membrane	West of Ireland	Holt, 1893
	1.08-1.30	0.29-0.34	Striated membrane	Celtic Sea	Milligan *et al.*, In prep.
	1.02-1.22	0.25-0.3	Slight ridges on inside of membrane	Europe	Froese and Pauly, 2003
Hake (*Merluccius merluccius*)	0.94-1.03	0.25-0.28	Pigmented oil globule	North Sea, English Channel, Mediterranean	Russell, 1976
	0.94-1.03	~0.27	Black and yellow pigment on oil globule	North Sea, English Channel, Mediterranean	Ehrenbaum, 1905–1909
	0.94-1.03	~0.27		?	D'Ancona *et al*., 1956
	1.10-1.16	0.27-0.35		Celtic Sea	Shaw, 2003
	0.94-1.03	0.25-0.28		Europe	Froese and Pauly, 2003
Longspine Snipefish *Macrorhamphosus scolopax)*	1.00	0.2	Amber/rose single oil globule Membrane is light amber with grainy reflections	Europe	Development of Fishes of the Mid-Atlantic Bight, 1978

3.4 Fecundity and atresia determination

3.4.1 Methodology for fecundity estimation

Since the 2001 Triennial assessment, the methodology to determine mackerel and horse mackerel fecundity has changed in order to discontinue the use of Gilson fixative. This has entailed inter calibration of fecundity estimates produced from Gilson fixed and formaldehyde fixed ovaries using the gravimetric and stereometric methods applied to mackerel and horse mackerel respectively (ICES, 2005). A detailed review was carried out during this Workshop to provide an updated fecundity manual for both species (Annex 6) based on the manual produced after the Fecundity Workshop held at Lowestoft in December 2000. The text table below summarises the changes made since the manual was produced in December 2000.

Mackerel 2000	2006
On board ovaries were collected whole and fixed in Gilson's fluid (for potential fecundity) and formaldehyde solution (for to assess spawning status and atresia).	On board ovaries are weighed and pipette sub-samples of known volume and weight taken and fixed in formaldehyde solution, remainder of ovary used for atresia.
Potential fecundity: Count follicles > 130 µm after Gilson digestion	Gravimetric fecundity estimation. Sub samples preserved in 3.6% buffered formaldehyde. F = O * C * S (F = fecundity, O = Ovary weight, C = count follicles > 185 µm in sub-sample, S = sub-sample weight) (Hunter *et al*., 1989)
Atresia: Stereometric method sections stained with PAS	Stereometric method sections stained with H&E -PAS – Toluidine blue
Horse mackerel 2000	2006
Potential fecundity: Stereometric method sections stained with H&E	Gravimetric fecundity estimation as mackerel. Sub samples preserved in 3.6% buffered formaldehyde. F = O * C * S (F = fecundity, O = Ovary weight, C = count follicles > 175 µm in subsample, S = subsample weight) (Hunter *et al*., 1989)

3.4.2 Standardisation of potential fecundity analysis

Images were prepared from either an unstained whole mount sample of mackerel ovary tissue or after staining with Rose Bengal or Periodic acid Schiffs (PAS). Each analyst attending the meeting scored these images to count the number of normal, atretic and post ovulatory follicles in each preparation prior to and after discussion. The results are presented in Section 4.4.1.

3.4.3 Standardisation of mackerel atresia assessment

The quantification of each early alpha atresia stage follicle class (yolk vesical, yolk vesical – yolk granule and yolkgranule) stained with heamotoxylin and eosin (H&E) PAS Mallory (PM) or Toluidine blue (TB) was discussed. The atretic follicle classification criteria was based on the mackerel / horse mackerel fecundity methods manual produced following the Workshop held at Lowestoft in December 2000.

Serial sections were produced from 6 mackerel ovary samples and stained with either H&E, PM or TB and scored by AZTI, CEFAS, IMARES and IMR for early alpha atresia in the 3 follicle classes.

4 Results

4.1 Results of the egg sorting exercise

The results of the egg sorting exercise using the 'spray technique' are given in Table 4.1-1. Three plankton samples were prepared with 500 fish eggs (a mix of mackerel and horse mackerel eggs) present in each. There were widely fluctuating results in determining egg numbers and increasing damage to the eggs whilst using the first prepared sample. After four participants had used the first sample, it was decided to use a second pre-prepared sample until a spillage prevented its further use. Three participants then used the 'spray technique' to remove eggs from a third pre-prepared sample. The results from the second and third samples were much more consistent than those from the first sample, as participants discussed the technique and began to resolve the practical problems encountered.

Table 4.1-1 shows the numbers and percentage of eggs removed by each use of the spray technique, and the numbers of eggs remaining in the plankton sample. In the first sample, the percentage of the original egg numbers (500) removed by the spray technique was apparently between 78% and 105%. After some improvements to the technique and increased care when spraying, this improved to between 100% and 102% of the eggs removed from the second sample and 94% to 100% for the third sample. On all occasions very few eggs were left in the plankton sample after spraying.

Table 4.1-1 Evaluation of the 'Spray method' by estimating the percentage of eggs removed from the remaining eggs and by estimating the cumulative percentage of eggs removed by separation, by participant and for all participants combined

NUMBER OF EGGS REMOVED AT EACH SEPARATION

Sample number	1	1	1	1	2	2	3	3	3	TOTAL
Actual No. of eggs	500	500	500	500	500	500	500	500	500	4500
Egg sorter	Por CV	Net CvD	Ire BOH	Ger SK	Ire BOH	Eng JP	Nor JdL	NorEH	Spain	
1st separation	390	361	375	364	427	420	465	471	458	**3731**
2nd separation	2	24	68	18	58	58	6	0	41	**275**
3rd separation	14	2	45	4	17	14	6		3	**105**
4th separation	3	12	23	2	7	6	14			**67**
5th separation	1	0	8	1	2	2	0			**14**
6th separation	2	0	5	0	0	0				**7**
7th separation	2		0	0	1	1				**4**
8th separation	2				0	0				**2**
TOTAL (spray method)	**416**	**399**	**524**	**389**	**512**	**501**	**491**	**471**	**502**	**4205**
Eggs found by hand sorting	0	0	0	0	2	2	0	1	0	**5**
TOTAL (spray and hand sorting)	**416**	**399**	**524**	**389**	**514**	**503**	**491**	**472**	**502**	
Eggs found (%) by spray and hand	83%	80%	105%	78%	103%	101%	98%	94%	100%	

PERCENTAGE OF EGGS REMOVED AT EACH SEPARATION BY SPRAYING

Sample number	1	1	1	1	2	2	3	3	3	
Egg sorter	Por CV	Net CvD	Ire BOH	Ger SK	Ire BOH	Eng JP	Nor JdL	NorEH	Spain	Mean
1st separation	78%	72%	75%	73%	85%	84%	93%	94%	92%	**83%**
2nd separation	2%	17%	54%	13%	79%	73%	17%	0%	98%	**39%**
3rd separation	13%	2%	79%	3%	113%	64%	21%		300%	**74%**
4th separation	3%	11%	192%	2%	-350%	75%	61%			**-1%**
5th separation	1%	0%	-73%	1%	-22%	100%	0%			**1%**
6th separation	2%	0%	-26%	0%	0%	0%				**-4%**
7th separation	2%		0%	0%	-9%	0%				**-1%**
8th separation	2%				0%	0%				**1%**

CUMULATIVE PERCENTAGE OF EGGS REMOVED BY SPRAY METHOD

Sample number	1	1	1	1	2	2	3	3	3	
Egg sorter	Por CV	Net CvD	Ire BOH	Ger SK	Ire BOH	Eng JP	Nor JdL	NorEH	Spain	Mean
1st separation	78%	72%	75%	73%	85%	84%	93%	94%	92%	**83%**
2nd separation	78%	77%	89%	76%	97%	96%	94%	94%	100%	**89%**
3rd separation	81%	77%	98%	77%	100%	98%	95%		100%	**91%**
4th separation	82%	80%	102%	78%	102%	100%	98%			**92%**
5th separation	82%	80%	104%	78%	102%	100%	98%			**92%**
6th separation	82%	80%	105%	78%	102%	100%				**91%**
7th separation	83%		105%	78%	102%	100%				**94%**
8th separation	83%				102%	100%				**95%**

4.2 Results of the egg staging exercise

4.2.1 Egg staging results

The results of the egg staging exercise are given in Tables 4.2-1 to 4.2-6.

Tables 4.2-1 to 4.2-3 present the results for each participant for the first round of analysis for eggs of all species (Table 4.2-1), for mackerel eggs (Table 4.2-2) and for horse mackerel eggs (Table 4.2-3). Tables 4.2-4 to 4.2-6 present the results for the second round of analysis in exactly the same way.

The original assessment of each egg, by each participant, for stage (and species), was input into a primary result table (not presented here). Once the results were available from every participant a modal stage could be calculated for each egg. This modal assessment of egg stage was presumed to be 'correct' although it does not necessarily mean that this was the true stage. In some cases, eggs were apparently mis-identified to species by a few readers before staging. When these 'mis-identified' eggs were allocated a stage by a few readers then it was not always possible for a model stage to be calculated. These eggs were then removed from the species / stage analysis in Tables 4.2-2, 4.2-3, 4.2-5 and 4.2-6.

Tables 4.2-1 to 4.2-6 summarise the results into six sub-tables labelled A-F, where the performance of each participant is judged against the modal egg stage.

Sub-tables A show the number of eggs at each modal stage that were assessed by each participant. The numbers at each modal stage will therefore be the same for all participants that read all the eggs.

Sub-tables B show the numbers of eggs at each stage as assessed by each participant.

Sub-tables C show the over / under estimation of stage 1 (1a + 1b) by each participant.

Sub-tables D show how well each participant's assessment of egg stage agrees with the numbers of eggs at each model stage.

Sub-tables E show the percentage agreement of each participant's assessment of eggs in stage 1a+1b against the modal stage 1a+1b.

Sub-tables F show the bias of each participant's egg staging against the modal stage i.e. how much their assessment of each egg stage varies from the modal stage.

By studying the results presented in Tables 4.2-1 to 4.2-6, some encouraging improvements in the consistency of egg staging between participants can be observed from the first to the second round of analysis.

The overall agreement in egg stage for all species of eggs, in all stages of development was 77% in the first round (Table 4.2-1). This increased to 84% agreement in the second round of analysis (Table 4.2-4). The overall agreement for all egg stages, for mackerel, increased from 86% (Table 4.2-2) to 90% (Table 4.2-4), and for horse mackerel from 71% (Table 4.2-3) to 77% (Table 4.2-6).

The overall agreement for stage 1 (1a+1b) eggs shows similar improvements (between 5% and 6%) from the first to the second round, but with an overall greater level of agreement (≥90%). This is very re-assuring, as it is this stage upon which the estimates of SSB for both mackerel and horse mackerel are based.

The overall agreement in the assessment of stage 1 (1a+1b) eggs of all species was 90% in the first round (Table 4.2-1). This increased to 96% agreement in the second round of analysis (Table 4.2-4). The overall agreement of stage 1 eggs, for mackerel, increased from 92%

(Table 4.2-2) to 98% (Table 4.2-5), and for horse mackerel from 90% (Table 4.2-3) to 95% (Table 4.2-6).

The percentage agreement in allocating eggs to stage 1 (1a+1b) as a percentage over or underestimation, are given in sub-tables C. Although the overall bias was reasonable, particularly after the second round of analysis, some individuals showed surprisingly high levels of bias. In the first round of analysis the overall bias was an under-estimate of 1% for eggs of all species but individual bias ranged from an under-estimate of 24% to an overestimate of 8% (Table 4.2-1). In the second round this did improve to a perfect 0%, demonstrating NO overall bias, with a range of individual bias also reduced to range between −14% to 10%.

The overall bias for stage 1 mackerel eggs (Tables 4.2-2 and 4.2-5) was -2% in the first round to 2% in the second round of analysis. However, the bias of individual participants was much greater, ranging from −41% to 10% in the first round, but improving to between −9% to 18% in the second round of analysis. The overall bias for stage 1 horse mackerel eggs (Tables 4.2-3 and 4.2-6) was −2% in the first round to −1% in the second round of analysis. However, the bias of individual participants was again much greater, ranging from −42% to 13% in the first round, but improving to between −19% and 12% in the second round of analysis.

Figures 4.2-1 to 4.2-6 show the egg stage bias plots in which the mean egg stage +/−2 standard deviations of each stage reader and all stage readers combined are plotted against the modal egg stage.

Table 4.2-1. All eggs first staging.

(A) The numbers of eggs at each modal stage read by each participant. (B) The numbers of eggs allocated to each stage by each participant.
(C) The over / underestimation of stage 1 (1a+1b) by each participant. (D) The percentage agreement by modal egg stage by each participant.
(E) The percentage agreement by modal stage 1a and 1b combined, by each participant.
(F) The bias is indicated by the percentage over or under estimation of each egg stage, as estimated by each participant, in relation to the modal stage.
For each table the combined result is also given.

A

NUMBER OF EGG STAGE READINGS BY MODAL EGG STAGE

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Stage 1a ==>	0	123	130	119	109	117	121	118	120	125	128	130	127	132	128	132	124	1585
Stage 1b ==>	1	24	25	23	24	29	32	22	29	31	24	32	32	32	30	32	25	446
Stage 2 ==>	2	61	65	63	59	59	65	57	58	61	64	65	62	65	62	64	65	995
Stage 3 ==>	3	133	134	121	119	125	126	135	141	142	138	143	142	143	142	142	138	2164
Stage 4 ==>	4	20	20	20	16	20	20	20	20	20	20	20	20	20	20	20	20	316
Stage 5 ==>	5	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	96
Total	0-5	367	380	352	333	356	370	358	374	388	380	396	389	398	388	396	378	6003

B

EGG STAGE COMPOSITION

	Stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Stage 1a ==>	0	74	76	85	94	145	139	102	88	81	156	151	125	131	111	104	69	1732
Stage 1b ==>	1	81	77	56	38	4	2	39	52	79	2	24	26	43	55	21	81	682
Stage 2 ==>	2	62	73	57	69	32	68	70	65	66	50	37	67	35	51	102	75	979
Stage 3 ==>	3	132	125	130	105	143	137	130	133	140	135	161	141	160	122	135	141	2170
Stage 4 ==>	4	12	20	16	17	27	18	10	30	19	32	18	19	17	40	29	6	330
Stage 5 ==>	5	6	9	8	10	5	6	7	6	3	5	5	8	12	9	5	6	110
Total	0-5	367	380	352	333	356	370	358	374	388	380	396	389	398	388	396	378	6003

C

OVER- / UNDERESTIMATION OF STAGE 1 (=1A+1B)

MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
1a+1b	5%	-1%	-1%	-1%	2%	-8%	1%	-6%	1%	4%	8%	-3%	6%	5%	-24%	1%	-1%

D

PERCENTAGE AGREEMENT BY EGG STAGE

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Stage 1a ==>	0	49%	54%	65%	68%	93%	85%	80%	66%	61%	97%	93%	85%	78%	69%	75%	54%	73%
Stage 1b ==>	1	50%	92%	83%	25%	3%	0%	77%	79%	97%	4%	56%	56%	44%	50%	19%	92%	51%
Stage 2 ==>	2	74%	77%	76%	80%	47%	72%	86%	72%	85%	77%	54%	84%	48%	60%	84%	85%	72%
Stage 3 ==>	3	90%	87%	98%	83%	94%	93%	86%	87%	93%	91%	95%	94%	96%	80%	89%	90%	91%
Stage 4 ==>	4	55%	65%	70%	56%	100%	60%	45%	75%	70%	95%	45%	75%	55%	80%	90%	20%	66%
Stage 5 ==>	5	100%	100%	83%	100%	83%	100%	83%	83%	33%	67%	50%	100%	100%	100%	83%	83%	84%
Weighted mean	0-5	69.2%	73.4%	79.8%	72.4%	78.9%	77.0%	81.8%	76.5%	79.4%	84.7%	81.3%	85.6%	76.6%	71.1%	77.0%	73.5%	77.4%
	RANKING	16	13	5	14	7	8	3	11	6	2	4	1	10	15	9	12	

E

PERCENTAGE AGREEMENT STAGE 1A and 1B combined

MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
1a+1b	94%	90%	93%	91%	93%	85%	93%	83%	93%	97%	95%	90%	94%	91%	71%	91%	90%
RANKING	4	13	8	10	5	14	7	15	6	1	2	12	3	9	16	11	

F

BIAS

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Stage 1a ==>	0	0.57	0.60	0.45	0.39	0.14	0.32	0.24	0.53	0.44	0.07	0.14	0.23	0.24	0.36	0.45	0.54	0.35
Stage 1b ==>	1	-0.33	0.08	0.00	-0.33	-0.97	-0.50	0.05	-0.07	0.03	-0.96	-0.31	-0.44	-0.19	-0.10	0.72	0.00	-0.20
Stage 2 ==>	2	-0.18	-0.12	-0.10	-0.15	0.14	-0.15	-0.16	-0.09	-0.13	-0.09	-0.22	-0.05	-0.18	-0.40	-0.13	-0.12	-0.13
Stage 3 ==>	3	-0.11	-0.11	-0.03	-0.08	0.02	-0.02	-0.14	0.04	-0.06	0.04	-0.01	-0.01	0.02	0.13	0.03	-0.09	-0.02
Stage 4 ==>	4	-0.60	-0.05	0.00	0.06	0.00	-0.45	-0.35	-0.15	-0.20	0.05	-0.65	-0.05	0.15	0.10	-0.10	-0.80	-0.19
Stage 5 ==>	5	0.00	0.00	-0.17	0.00	-0.17	0.00	-0.17	-0.83	-0.67	-0.33	-0.50	0.00	0.00	0.00	-0.17	-0.17	-0.20

Table 4.2-2. Mackerel eggs first staging.

(A) The numbers of eggs at each modal stage read by each participant. (B) The numbers of eggs allocated to each stage by each participant.
(C) The over / underestimation of stage 1 (1a+1b) by each participant. (D) The percentage agreement by modal egg stage by each participant.
(E) The percentage agreement by modal stage 1a and 1b combined, by each participant.
(F) The bias is indicated by the percentage over or under estimation of each egg stage, as estimated by each participant, in relation to the modal stage.
For each table the combined result is also given.

A

NUMBER OF EGG STAGE READINGS BY MODAL EGG STAGE

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Stage 1a ==>	0	41	29	21	19	21	11	14	16	17	15	19	29	49	25	10	16	352
Stage 1b ==>	1	11	6	6	8	6	6	5	5	5	6	7	8	10	7	3	9	108
Stage 2 ==>	2	10	2	15	7	12	13	5	3	15	7	4	16	19	14	11	20	173
Stage 3 ==>	3	100	69	114	90	106	95	93	44	104	104	78	111	129	103	117	125	1582
Stage 4 ==>	4	9	13	16	7	16	18	15	-	14	16	12	18	17	16	16	17	220
Stage 5 ==>	5	6	6	6	6	6	7	6	2	7	6	6	7	7	6	6	7	97
Total	0-5	177	125	178	137	167	150	138	70	162	154	126	189	231	171	163	194	2532

B

EGG STAGE COMPOSITION

	Stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Stage 1a ==>	0	26	23	10	18	27	10	16	20	8	22	24	28	53	19	7	14	325
Stage 1b ==>	1	27	12	15	8	0	0	3	2	14	1	3	9	5	15	5	7	126
Stage 2 ==>	2	21	4	18	14	10	15	12	5	21	6	1	17	13	10	11	31	209
Stage 3 ==>	3	91	66	114	84	107	102	93	33	101	101	85	111	136	98	116	130	1568
Stage 4 ==>	4	6	11	13	3	18	17	7	8	15	20	9	16	12	20	19	6	200
Stage 5 ==>	5	6	9	8	10	5	6	7	2	3	4	4	8	12	9	5	6	104
Total	0-5	177	125	178	137	167	150	138	70	162	154	126	189	231	171	163	154	2532

C

OVER- / UNDERESTIMATION OF STAGE 1 (=1A+1B)

MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
1a+1b	2%	0%	-7%	-4%	0%	-41%	0%	5%	0%	10%	4%	0%	-2%	6%	-8%	-16%	-2%

D

PERCENTAGE AGREEMENT BY EGG STAGE

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Stage 1a ==>	0	59%	66%	48%	79%	100%	45%	93%	94%	47%	100%	95%	90%	90%	60%	70%	75%	76%
Stage 1b ==>	1	91%	67%	83%	50%	0%	0%	40%	20%	100%	17%	43%	88%	20%	57%	100%	56%	52%
Stage 2 ==>	2	80%	50%	100%	100%	83%	54%	100%	33%	87%	86%	25%	100%	63%	64%	82%	90%	80%
Stage 3 ==>	3	86%	94%	98%	93%	98%	94%	92%	75%	91%	96%	99%	97%	96%	90%	96%	92%	94%
Stage 4 ==>	4	67%	69%	75%	29%	94%	61%	40%	-	71%	94%	42%	78%	53%	75%	88%	18%	65%
Stage 5 ==>	5	100%	100%	83%	100%	83%	86%	83%	100%	43%	67%	50%	100%	100%	100%	83%	71%	84%
Weighted mean	0-5	79.1%	83.2%	89.3%	86.1%	92.8%	78.7%	84.8%	74.3%	82.7%	91.6%	84.9%	94.2%	87.0%	81.3%	92.0%	81.4%	85.7%
RANKING		14	10	5	7	2	15	9	16	11	4	8	1	6	13	3	12	

E

PERCENTAGE AGREEMENT STAGE 1A and 1B combined

MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
1a+1b	97%	89%	86%	93%	97%	44%	100%	100%	96%	96%	93%	95%	96%	97%	86%	78%	92%
RANKING	5	12	13	11	4	16	1	1	6	6	10	9	6	3	14	15	

F

BIAS

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Stage 1a ==>	0	0.49	0.52	0.67	0.21	0.00	1.45	0.07	0.06	0.53	0.00	0.16	0.14	0.16	0.40	0.40	0.50	0.33
Stage 1b ==>	1	-0.09	0.00	0.17	0.00	-1.00	0.50	-0.60	-0.80	0.00	-0.83	-0.57	-0.13	-0.50	-0.43	0.00	0.00	-0.26
Stage 2 ==>	2	-0.10	1.00	0.00	0.00	0.17	0.15	0.00	0.00	0.13	0.14	0.75	0.00	0.32	0.14	0.18	0.00	0.12
Stage 3 ==>	3	-0.16	-0.14	-0.03	-0.07	0.02	-0.02	-0.06	0.07	-0.07	0.00	0.01	-0.01	-0.02	0.05	0.03	-0.05	-0.03
Stage 4 ==>	4	-0.33	0.08	0.13	0.29	-0.06	-0.44	-0.33	-	-0.29	-0.25	-0.92	-0.11	0.12	0.13	-0.13	-0.82	-0.20
Stage 5 ==>	5	0.00	0.00	-0.17	0.00	-0.17	-0.14	-0.17	0.00	-0.57	-0.33	-0.50	0.00	0.00	0.00	-0.17	-0.29	-0.16

Table 4.2-3. Horse Mackerel eggs first staging.

(A) The numbers of eggs at each modal stage read by each participant. (B) The numbers of eggs allocated to each stage by each participant.
(C) The over / underestimation of stage 1 (1a+1b) by each participant. (D) The percentage agreement by modal egg stage by each participant.
(E) The percentage agreement by modal stage 1a and 1b combined, by each participant.
(F) The bias is indicated by the percentage over or under estimation of each egg stage, as estimated by each participant, in relation to the modal stage.
For each table the combined result is also given.

A

NUMBER OF EGG STAGE READINGS BY MODAL EGG STAGE

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Stage 1a ==>	0	51	71	70	65	68	69	64	67	70	71	65	32	58	71	64	64	1020
Stage 1b ==>	1	22	29	26	22	18	23	22	25	32	28	15	9	9	26	27	28	361
Stage 2 ==>	2	38	66	48	52	45	37	53	52	46	55	44	9	31	33	43	41	693
Stage 3 ==>	3	27	39	10	21	11	23	31	68	5	11	58	5	5	11	6	7	338
Stage 4 ==>	4	5	8	6	8	6	2	6	11	3	6	8	1	2	6	6	1	85
Stage 5 ==>	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	0-5	143	213	160	168	148	154	176	223	156	171	190	56	105	147	146	141	2497

B

EGG STAGE COMPOSITION

	Stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Stage 1a ==>	0	40	34	55	55	83	92	53	43	43	102	74	29	60	61	48	33	905
Stage 1b ==>	1	36	64	40	29	4	2	33	40	61	1	10	9	16	35	5	60	445
Stage 2 ==>	2	33	68	39	54	22	34	56	52	41	42	28	13	14	32	79	40	647
Stage 3 ==>	3	31	40	23	19	30	25	31	73	8	16	69	4	12	9	5	8	403
Stage 4 ==>	4	3	7	3	11	9	1	3	15	3	9	8	1	3	10	9	-	95
Stage 5 ==>	5	-	-	-	-	-	-	-	-	-	1	1	-	-	-	-	-	2
Total	0-5	143	213	160	168	148	154	176	223	156	171	190	56	105	147	146	141	2497

C

OVER- / UNDERESTIMATION OF STAGE 1 (=1A+1B)

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
	1a+1b	4%	-2%	-1%	-3%	1%	2%	0%	-10%	2%	4%	5%	-7%	13%	-1%	-42%	1%	-2%

D

PERCENTAGE AGREEMENT BY EGG STAGE

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Stage 1a ==>	0	47%	44%	67%	60%	90%	94%	75%	54%	56%	99%	97%	75%	79%	66%	69%	50%	70%
Stage 1b ==>	1	36%	86%	81%	32%	11%	0%	86%	76%	94%	4%	53%	44%	44%	50%	4%	89%	52%
Stage 2 ==>	2	82%	79%	71%	77%	42%	86%	91%	69%	78%	73%	61%	100%	42%	61%	91%	83%	74%
Stage 3 ==>	3	100%	85%	90%	57%	82%	96%	87%	94%	100%	64%	98%	80%	100%	64%	17%	100%	88%
Stage 4 ==>	4	40%	63%	33%	63%	100%	50%	50%	91%	100%	83%	75%	100%	100%	100%	100%	0%	74%
Stage 5 ==>	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Weighted mean	0-5	64.3%	68.5%	70.6%	61.3%	65.5%	77.9%	82.4%	74.0%	72.4%	71.9%	84.7%	75.0%	66.7%	63.3%	62.3%	69.5%	71.0%
	RANKING	13	10	8	16	12	3	2	5	6	7	1	4	11	14	15	9	

E

PERCENTAGE AGREEMENT STAGE 1A and 1B combined

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
	1a+1b	55%	89%	94%	89%	92%	98%	93%	79%	53%	98%	98%	93%	96%	88%	56%	93%	90%
	RANKING	5	12	6	13	11	2	8	15	7	1	3	9	4	14	16	10	

F

BIAS

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Stage 1a ==>	0	0.55	0.66	0.41	0.52	0.21	0.10	0.28	0.73	0.49	0.03	0.03	0.34	0.22	0.42	0.59	0.55	0.38
Stage 1b ==>	1	-0.55	0.14	-0.04	-0.32	-0.89	-1.00	0.05	0.00	0.06	-0.89	-0.13	-0.56	-0.11	-0.04	0.93	0.11	-0.16
Stage 2 ==>	2	-0.13	-0.09	0.00	-0.12	0.20	-0.19	-0.13	-0.12	-0.17	-0.05	0.00	0.00	-0.45	-0.48	-0.12	-0.20	-0.12
Stage 3 ==>	3	0.00	-0.10	-0.10	-0.05	0.18	-0.04	-0.13	0.06	0.00	0.36	0.02	-0.20	0.00	0.36	0.17	0.00	0.01
Stage 4 ==>	4	-0.60	-0.38	-0.67	-0.38	0.00	-0.50	-0.50	-0.09	0.00	0.17	0.00	0.00	0.00	0.00	0.00	-1.00	-0.21
Stage 5 ==>	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4.2-4. All eggs second staging.

(A) The numbers of eggs at each modal stage read by each participant. (B) The numbers of eggs allocated to each stage by each participant.
(C) The over / underestimation of stage 1 (1a+1b) by each participant. (D) The percentage agreement by modal egg stage by each participant.
(E) The percentage agreement by modal stage 1a and 1b combined, by each participant.
(F) The bias is indicated by the percentage over or under estimation of each egg stage, as estimated by each participant, in relation to the modal stage.
For each table the combined result is also given.

A

NUMBER OF EGG STAGE READINGS BY MODAL EGG STAGE

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Stage 1a ==>	0	114	117	117	113	117	116	116	107	117	116	117	114	105	117	117	110	1830
Stage 1b ==>	1	44	45	45	45	45	44	45	42	45	45	45	44	41	45	44	44	708
Stage 2 ==>	2	107	110	110	108	110	109	107	100	110	107	110	108	109	109	110	106	1730
Stage 3 ==>	3	99	99	99	99	99	99	99	99	99	99	99	96	91	99	101	99	1577
Stage 4 ==>	4	21	21	21	21	23	23	23	21	21	23	21	21	21	21	23	21	346
Stage 5 ==>	5	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	64
Total	0-5	389	396	396	390	398	395	394	373	396	394	396	389	371	395	399	384	6255

B

EGG STAGE COMPOSITION

	Stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Stage 1a ==>	0	71	80	84	117	139	131	115	93	117	142	151	94	84	152	109	132	1811
Stage 1b ==>	1	88	73	71	46	22	29	48	52	45	20	22	57	69	26	30	33	731
Stage 2 ==>	2	114	118	110	113	78	113	108	99	105	107	49	109	79	67	137	91	1597
Stage 3 ==>	3	92	102	105	90	130	91	94	105	105	100	138	93	112	105	73	86	1621
Stage 4 ==>	4	19	19	22	20	26	28	25	20	20	21	32	32	23	40	46	38	431
Stage 5 ==>	5	5	4	4	4	3	3	4	4	4	4	4	4	4	5	4	4	64
Total	0-5	389	396	396	390	398	395	394	373	396	394	396	389	371	395	399	384	6255

C

OVER- / UNDERESTIMATION OF STAGE 1 (=1A+1B)

MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
1a+1b	1%	-6%	-4%	3%	-1%	0%	1%	-3%	0%	1%	7%	-4%	5%	10%	-14%	7%	0%

D

PERCENTAGE AGREEMENT BY EGG STAGE

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Stage 1a ==>	0	58%	68%	72%	80%	99%	97%	89%	81%	91%	98%	96%	79%	57%	92%	90%	96%	84%
Stage 1b ==>	1	77%	93%	87%	44%	47%	57%	76%	76%	80%	40%	47%	82%	63%	36%	48%	50%	63%
Stage 2 ==>	2	94%	95%	92%	91%	68%	97%	94%	90%	89%	96%	41%	90%	67%	61%	97%	84%	84%
Stage 3 ==>	3	89%	96%	97%	88%	95%	90%	90%	95%	98%	97%	90%	87%	91%	81%	69%	82%	90%
Stage 4 ==>	4	67%	86%	90%	95%	96%	96%	91%	86%	95%	91%	95%	90%	86%	90%	96%	100%	91%
Stage 5 ==>	5	100%	100%	100%	100%	75%	75%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	97%
Weighted mean	0-5	78.1%	86.4%	86.6%	81.8%	83.2%	90.4%	89.3%	87.1%	91.4%	90.4%	73.5%	85.1%	71.2%	74.4%	82.5%	84.1%	83.5%
RANKING		13	7	6	12	10	2	4	5	1	3	15	8	16	14	11	9	

E

PERCENTAGE AGREEMENT STAGE 1A and 1B combined

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
	1a+1b	96%	94%	94%	97%	99%	98%	99%	96%	96%	99%	97%	92%	97%	99%	85%	99%	96%
RANKING		12	14	13	7	4	6	2	10	11	5	8	15	9	1	16	3	

F

BIAS

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Stage 1a ==>	0	0.49	0.39	0.30	0.23	0.01	0.03	0.11	0.20	0.09	0.02	0.09	0.26	0.49	0.09	0.13	0.04	0.18
Stage 1b ==>	1	-0.05	0.02	0.13	-0.47	-0.49	-0.34	-0.20	0.00	0.02	-0.56	-0.49	0.05	-0.32	-0.64	0.39	-0.45	-0.21
Stage 2 ==>	2	-0.04	0.05	0.07	-0.11	0.32	-0.03	-0.02	0.05	-0.03	-0.02	0.20	0.04	0.02	-0.05	0.00	-0.11	0.02
Stage 3 ==>	3	-0.03	-0.04	0.01	-0.12	0.01	0.00	-0.02	-0.01	-0.04	-0.03	0.10	0.13	0.02	0.19	0.17	0.16	0.03
Stage 4 ==>	4	-0.62	-0.14	-0.10	-0.05	-0.04	-0.04	-0.09	-0.14	-0.05	-0.09	-0.19	-0.24	-0.14	0.00	-0.04	0.00	-0.12
Stage 5 ==>	5	0.00	0.00	0.00	0.00	-0.25	-0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-0.03

Table 4.2-5. Mackerel eggs second staging.

(A) The numbers of eggs at each modal stage read by each participant. (B) The numbers of eggs allocated to each stage by each participant.
(C) The over / underestimation of stage 1 (1a+1b) by each participant. (D) The percentage agreement by modal egg stage by each participant.
(E) The percentage agreement by modal stage 1a and 1b combined, by each participant.
(F) The bias is indicated by the percentage over or under estimation of each egg stage, as estimated by each participant, in relation to the modal stage.
For each table the combined result is also given.

A

NUMBER OF EGG STAGE READINGS BY MODAL EGG STAGE

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Stage 1a ==>	0	50	52	57	54	67	67	64	38	67	58	49	59	58	65	59	63	927
Stage 1b ==>	1	6	12	12	12	13	14	14	8	12	13	8	13	13	13	11	12	186
Stage 2 ==>	2	40	45	51	50	62	62	62	45	62	57	38	59	55	61	51	61	861
Stage 3 ==>	3	67	68	68	68	68	68	67	68	68	62	66	61	60	68	68	68	1063
Stage 4 ==>	4	11	14	15	14	20	20	20	13	19	9	11	18	17	18	17	18	254
Stage 5 ==>	5	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	64
Total	0-5	178	195	207	202	234	235	231	176	232	203	176	214	207	229	210	226	3355

B

EGG STAGE COMPOSITION

	Stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Stage 1a ==>	0	41	27	31	41	72	75	57	35	56	65	62	44	40	79	49	68	842
Stage 1b ==>	1	18	31	37	27	8	7	22	10	21	7	3	26	30	13	17	14	291
Stage 2 ==>	2	38	52	51	50	62	62	62	46	63	57	24	62	58	47	57	54	845
Stage 3 ==>	3	67	67	69	66	68	66	67	68	69	61	70	61	61	66	63	65	1054
Stage 4 ==>	4	9	14	15	14	21	22	19	13	19	9	13	17	14	19	20	21	259
Stage 5 ==>	5	5	4	4	4	3	3	4	4	4	4	4	4	4	5	4	4	64
Total	0-5	178	195	207	202	234	235	231	176	232	203	176	214	207	229	210	226	3355

C

OVER- / UNDERESTIMATION OF STAGE 1 (=1A+1B)

MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
1a+1b	5%	-9%	-1%	3%	0%	1%	1%	-2%	-3%	1%	14%	-3%	-1%	18%	-6%	9%	2%

D

PERCENTAGE AGREEMENT BY EGG STAGE

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Stage 1a ==>	0	76%	50%	54%	65%	99%	96%	86%	84%	79%	98%	100%	75%	60%	92%	83%	95%	81%
Stage 1b ==>	1	50%	83%	92%	50%	54%	29%	93%	63%	75%	46%	38%	85%	62%	54%	82%	58%	64%
Stage 2 ==>	2	93%	98%	96%	88%	98%	97%	97%	96%	98%	98%	63%	95%	100%	77%	100%	89%	93%
Stage 3 ==>	3	97%	97%	100%	94%	97%	94%	97%	99%	100%	98%	97%	98%	97%	96%	91%	96%	97%
Stage 4 ==>	4	73%	93%	93%	100%	95%	95%	95%	100%	100%	100%	100%	89%	82%	89%	94%	100%	94%
Stage 5 ==>	5	100%	100%	100%	100%	75%	75%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	97%
Weighted mean	0-5	87.1%	83.6%	85.5%	82.7%	94.9%	91.1%	93.5%	93.2%	92.2%	95.1%	88.1%	89.3%	84.1%	86.9%	91.0%	92.0%	89.5%
	RANKING	11	15	13	16	2	7	3	4	5	1	10	9	14	12	8	6	

E

PERCENTAGE AGREEMENT STAGE 1A and 1B combined

MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
1a+1b	100%	91%	97%	96%	100%	100%	100%	96%	97%	100%	100%	93%	99%	100%	94%	100%	98%
RANKING	1	16	11	13	1	1	1	12	10	1	1	15	9	1	14	1	

F

BIAS

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Stage 1a ==>	0	0.24	0.60	0.47	0.41	0.01	0.04	0.14	0.16	0.24	0.02	0.00	0.31	0.41	0.08	0.20	0.05	0.21
Stage 1b ==>	1	-0.50	0.00	0.08	-0.33	-0.46	-0.71	-0.07	-0.13	-0.25	-0.54	-0.63	0.15	-0.38	-0.46	0.16	-0.42	-0.27
Stage 2 ==>	2	-0.02	0.04	0.02	-0.10	0.02	-0.02	-0.02	-0.02	0.02	-0.04	-0.26	-0.02	0.00	-0.44	0.00	-0.16	-0.06
Stage 3 ==>	3	0.00	-0.03	0.00	-0.06	0.00	0.00	-0.03	-0.01	0.00	-0.02	0.03	0.02	-0.03	0.04	0.03	0.04	-0.00
Stage 4 ==>	4	-0.27	-0.07	-0.07	0.00	-0.05	-0.05	-0.05	0.00	0.00	0.00	0.00	-0.28	-0.18	0.00	-0.05	0.00	-0.07
Stage 5 ==>	5	0.00	0.00	0.00	0.00	-0.25	-0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-0.03

Table 4.2-6. Horse Mackerel eggs second staging.

(A) The numbers of eggs at each modal stage read by each participant. (B) The numbers of eggs allocated to each stage by each participant.
(C) The over / underestimation of stage 1 (1a+1b) by each participant. (D) The percentage agreement by modal egg stage by each participant.
(E) The percentage agreement by modal stage 1a and 1b combined, by each participant.
(F) The bias is indicated by the percentage over or under estimation of each egg stage, as estimated by each participant, in relation to the modal stage.
For each table the combined result is also given.

A

NUMBER OF EGG STAGE READINGS BY MODAL EGG STAGE

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Stage 1a ==>	0	52	54	53	50	49	46	49	52	49	49	55	36	34	48	52	46	774
Stage 1b ==>	1	35	34	34	33	28	27	28	34	28	28	35	23	25	26	32	28	478
Stage 2 ==>	2	63	60	59	58	47	46	44	53	47	44	63	30	38	45	59	44	800
Stage 3 ==>	3	32	32	32	32	32	32	32	30	32	32	33	29	31	29	34	32	506
Stage 4 ==>	4	6	6	6	6	3	3	3	6	2	3	7	3	3	2	6	3	70
Stage 5 ==>	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	0-5	190	186	184	179	159	154	156	175	158	156	193	121	131	150	183	153	2628

B

EGG STAGE COMPOSITION

	Stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Stage 1a ==>	0	25	46	48	68	62	51	52	48	56	64	74	32	30	63	55	59	833
Stage 1b ==>	1	61	41	34	19	14	21	26	36	23	13	19	24	36	13	13	19	412
Stage 2 ==>	2	71	60	59	63	16	51	46	51	42	45	22	26	13	19	79	37	700
Stage 3 ==>	3	25	34	36	24	62	25	26	35	36	32	63	24	44	36	10	21	533
Stage 4 ==>	4	6	5	7	5	5	6	6	5	1	2	15	15	8	19	26	17	150
Stage 5 ==>	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	0-5	190	186	184	179	159	154	156	175	158	156	193	121	131	150	183	153	2628

C

OVER- / UNDERESTIMATION OF STAGE 1 (=1A+1B)

MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
1a+1b	-1%	-1%	-6%	5%	-1%	-1%	1%	-2%	3%	0%	3%	-5%	12%	3%	-19%	5%	-1%

D

PERCENTAGE AGREEMENT BY EGG STAGE

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Stage 1a ==>	0	44%	85%	91%	96%	98%	93%	88%	83%	100%	98%	96%	83%	53%	92%	98%	98%	88%
Stage 1b ==>	1	83%	94%	85%	45%	46%	67%	68%	76%	82%	43%	51%	87%	72%	35%	38%	54%	64%
Stage 2 ==>	2	94%	93%	90%	91%	30%	98%	91%	85%	77%	93%	32%	77%	29%	42%	97%	77%	76%
Stage 3 ==>	3	72%	94%	91%	72%	91%	78%	72%	90%	91%	91%	79%	59%	81%	48%	26%	50%	74%
Stage 4 ==>	4	75%	83%	83%	83%	100%	100%	67%	67%	50%	67%	100%	100%	100%	100%	100%	100%	86%
Stage 5 ==>	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Weighted mean	0-5	73.7%	90.9%	89.1%	80.4%	67.3%	87.0%	81.4%	82.9%	87.3%	84.6%	64.2%	76.9%	57.3%	58.7%	73.8%	73.9%	77.2%
RANKING		12	1	2	8	13	4	7	6	3	5	14	9	16	15	11	10	

E

PERCENTAGE AGREEMENT STAGE 1A and 1B combined

MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
1a+1b	95%	97%	92%	89%	97%	96%	99%	95%	94%	97%	98%	93%	98%	99%	78%	93%	95%
RANKING	12	9	15	1	7	10	2	11	13	7	6	14	5	3	16	3	

F

BIAS

	MODAL stage	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Soo IG Reader 5	Soo FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Stage 1a ==>	0	0.58	0.15	0.09	0.04	0.02	0.07	0.12	0.17	0.00	0.02	0.05	0.22	0.47	0.13	0.02	0.02	0.13
Stage 1b ==>	1	0.06	0.06	0.15	-0.48	-0.46	-0.19	-0.25	0.00	0.11	-0.50	-0.43	-0.04	-0.20	-0.65	0.44	-0.39	-0.16
Stage 2 ==>	2	-0.06	0.03	0.10	-0.14	0.70	-0.04	-0.02	0.06	-0.09	0.00	0.46	0.23	0.13	0.44	-0.02	-0.05	0.10
Stage 3 ==>	3	-0.16	-0.06	0.03	-0.28	0.03	-0.03	-0.03	-0.03	-0.16	-0.09	0.21	0.41	0.13	0.52	0.44	0.38	0.08
Stage 4 ==>	4	-0.25	-0.17	-0.17	-0.17	0.00	0.00	-0.33	-0.33	-0.50	-0.33	0.00	0.00	0.00	0.00	0.00	0.00	-0.14
Stage 5 ==>	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 4.2-1 All eggs first staging

In the egg stage bias plots below the mean egg stage recorded +/- 2stdev of each stage reader and all stage readers combined are plotted against the MODAL egg stage. The estimated mean egg stage corresponds to MODAL egg stage, if the estimated mean egg stage is on the 1:1 equilibrium line (solid line). Bias is the egg stage difference between estimated mean egg stage and MODAL egg stage.

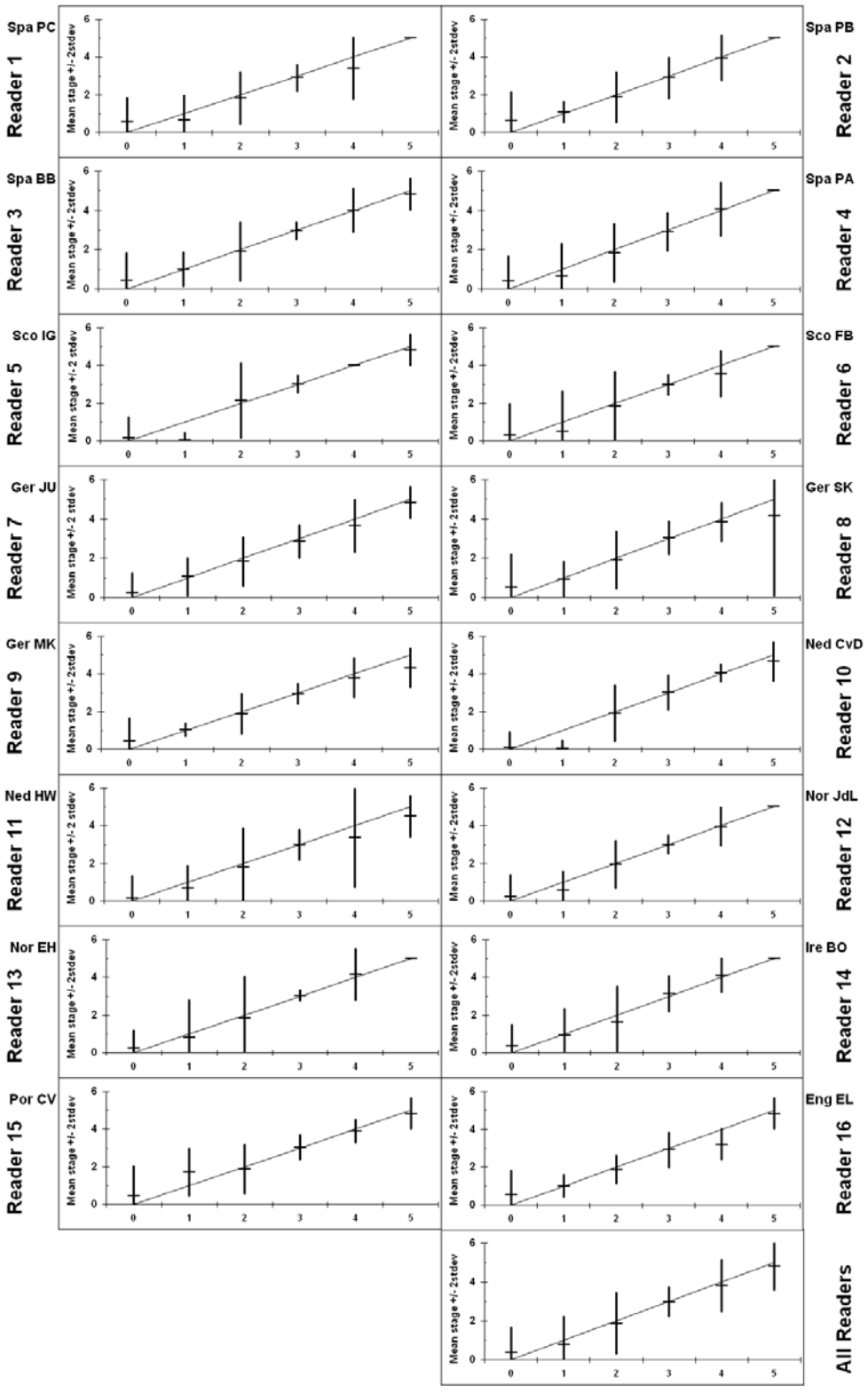


Figure 4.2-2 Mackerel eggs first staging

In the egg stage bias plots below the mean egg stage recorded +/- 2stdev of each stage reader and all stage readers combined are plotted against the MODAL egg stage. The estimated mean egg stage corresponds to MODAL egg stage, if the estimated mean egg stage is on the 1:1 equilibrium line (solid line). Bias is the egg stage difference between estimated mean egg stage and MODAL egg stage.

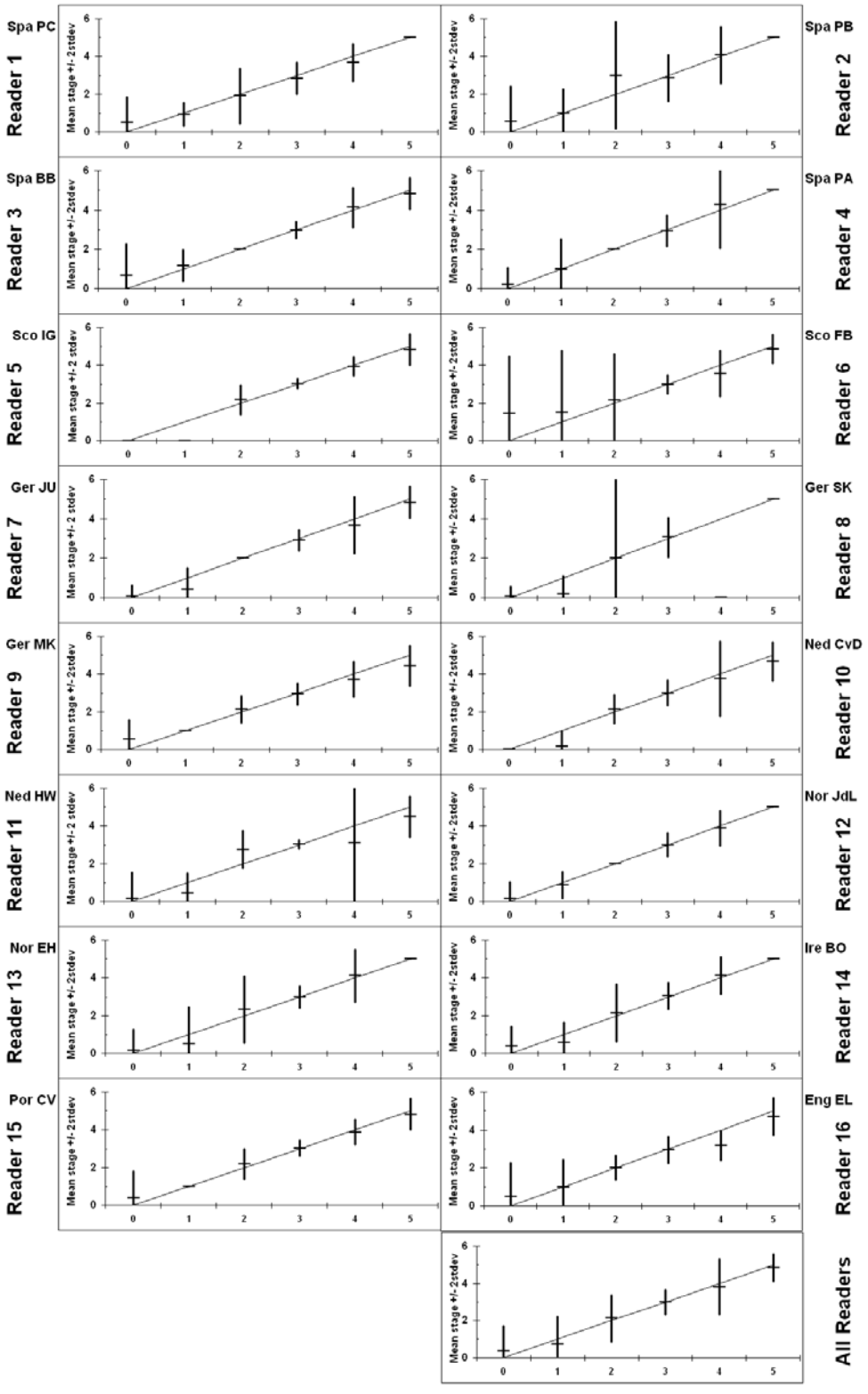


Figure 4.2-3 Horse mackerel eggs first staging

In the egg stage bias plots below the mean egg stage recorded +/- 2stdev of each stage reader and all stage readers combined are plotted against the MODAL egg stage. The estimated mean egg stage corresponds to MODAL egg stage, if the estimated mean egg stage is on the 1:1 equilibrium line (solid line). Bias is the egg stage difference between estimated mean egg stage and MODAL egg stage.

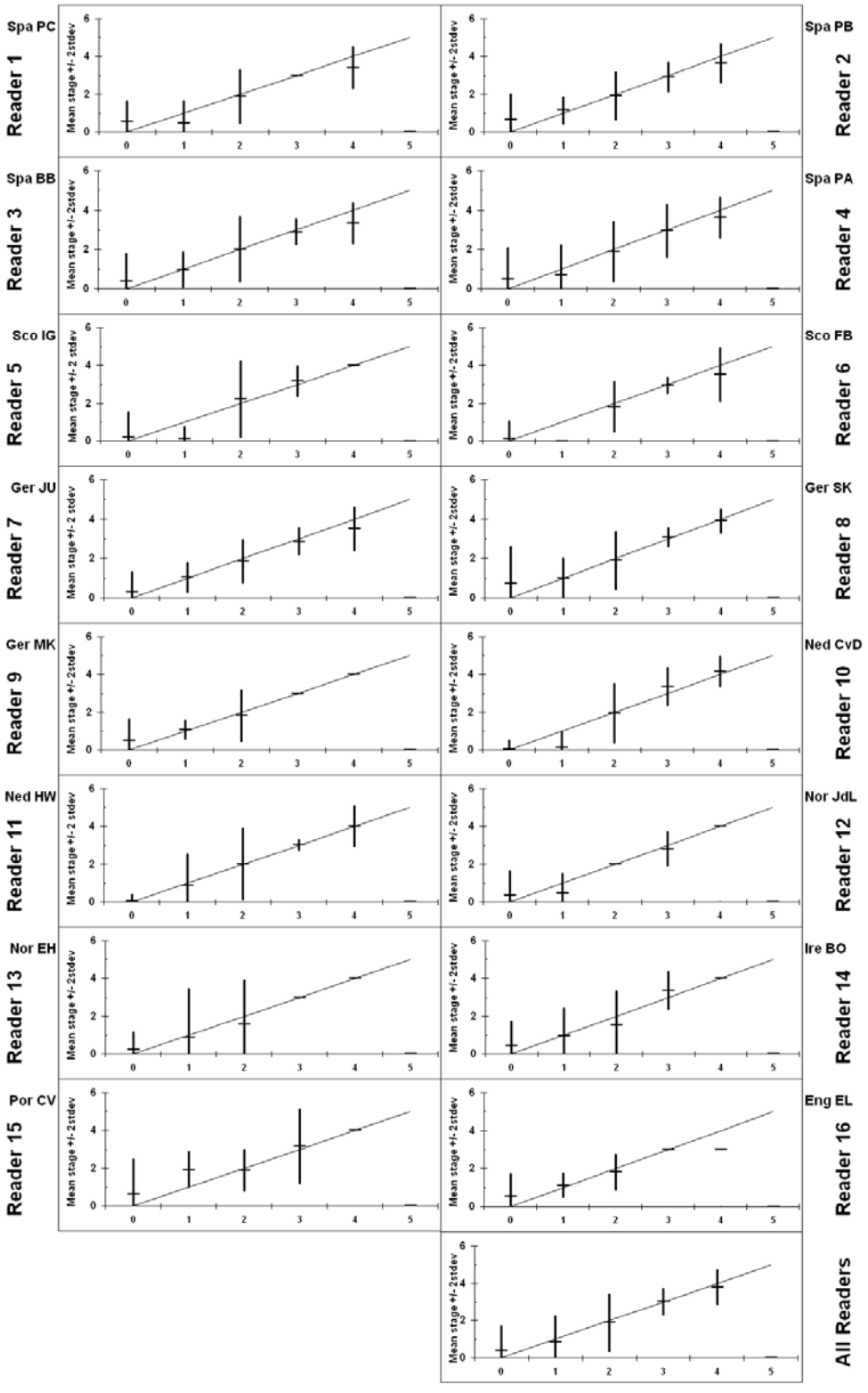


Figure 4.2-4 All eggs second staging

In the egg stage bias plots below the mean egg stage recorded +/- 2stdev of each stage reader and all stage readers combined are plotted against the MODAL egg stage. The estimated mean egg stage corresponds to MODAL egg stage, if the estimated mean egg stage is on the 1:1 equilibrium line (solid line). Bias is the egg stage difference between estimated mean egg stage and MODAL egg stage.

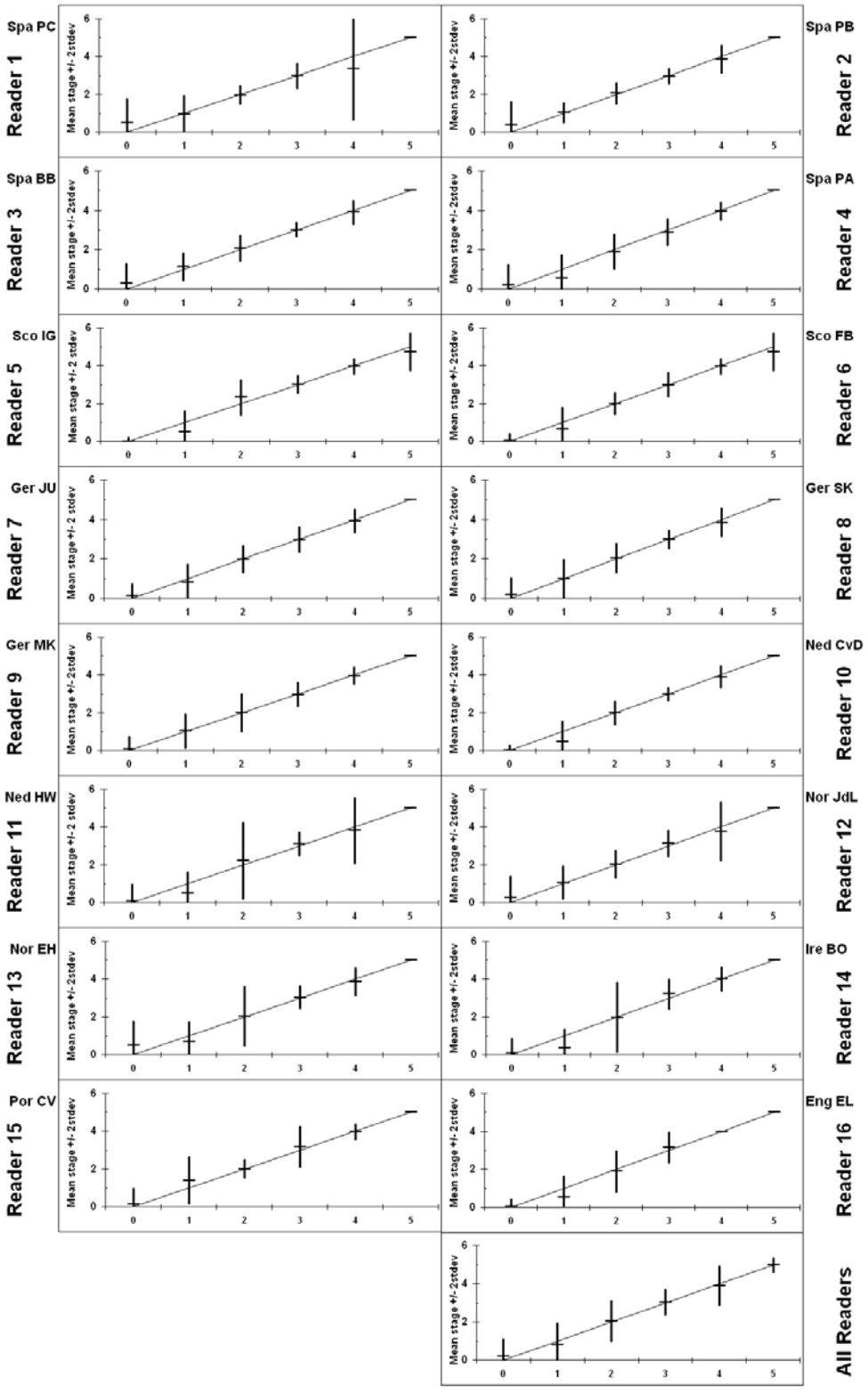


Figure 4.2-5 Mackerel eggs second staging

In the egg stage bias plots below the mean egg stage recorded +/- 2stdev of each stage reader and all stage readers combined are plotted against the MODAL egg stage. The estimated mean egg stage corresponds to MODAL egg stage, if the estimated mean egg stage is on the 1:1 equilibrium line (solid line). Bias is the egg stage difference between estimated mean egg stage and MODAL egg stage.

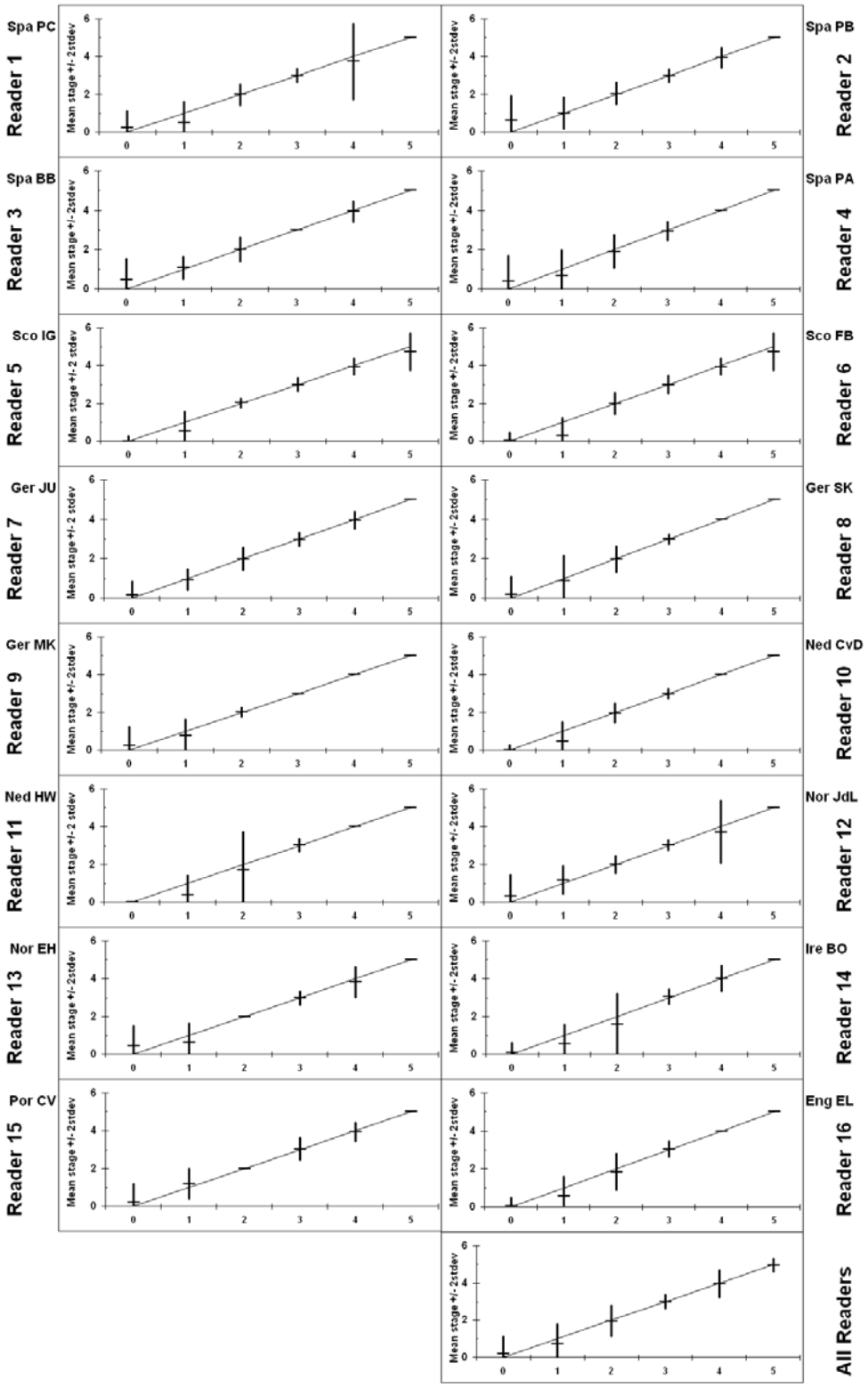
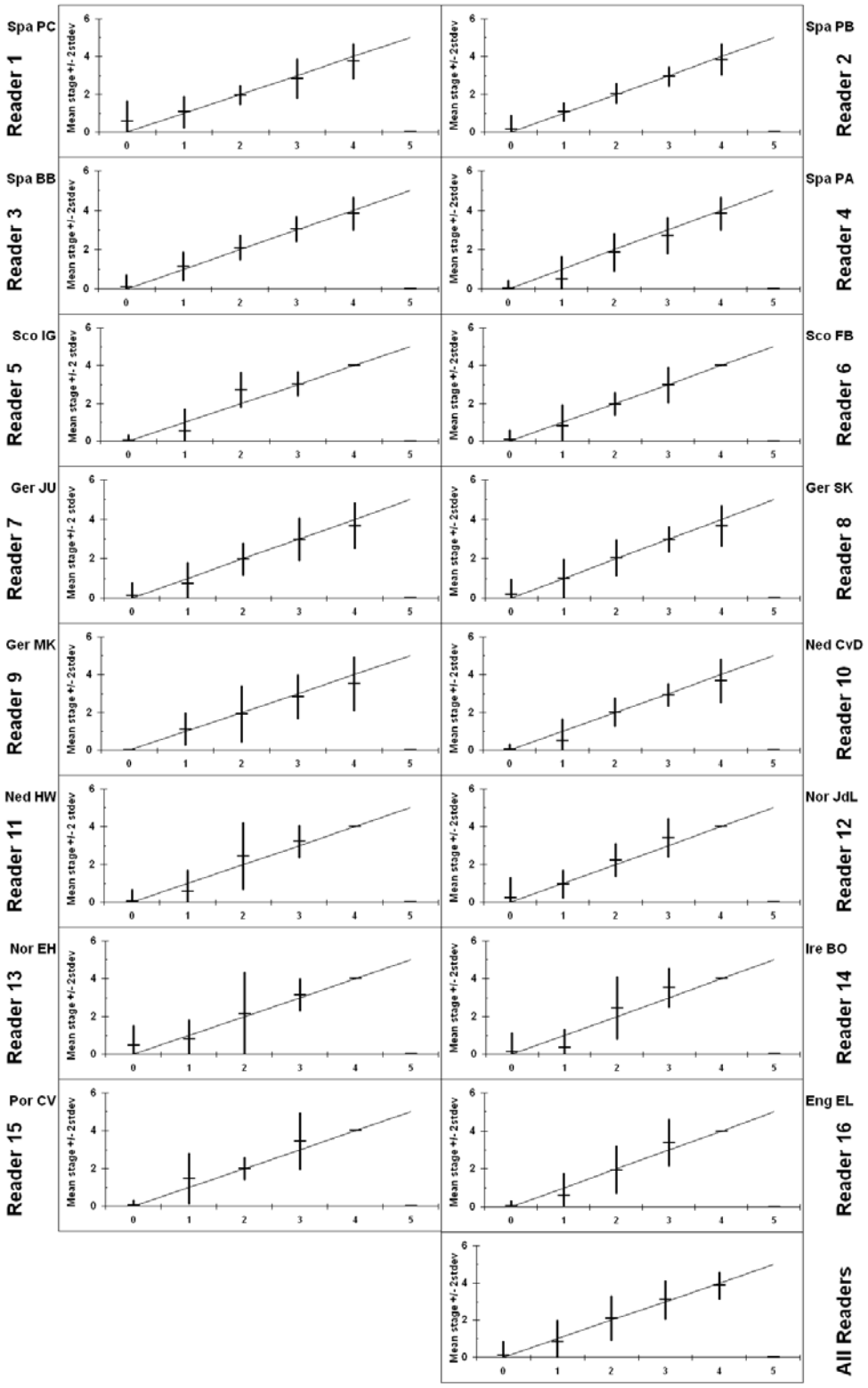


Figure 4.2-6 Horse mackerel eggs second staging

In the egg stage bias plots below the mean egg stage recorded +/- 2stdev of each stage reader and all stage readers combined are plotted against the MODAL egg stage. The estimated mean egg stage corresponds to MODAL egg stage, if the estimated mean egg stage is on the 1:1 equilibrium line (solid line). Bias is the egg stage difference between estimated mean egg stage and MODAL egg stage.



4.3 Results of the egg species identification exercise

The same trays of eggs, which were used for egg staging, were also used for the egg identification exercise. For the first time at these workshops, all the eggs used were from artificial fertilisations and so the species of each egg was definitely known. It was hoped that by using eggs of known species any problems associated with identification would be highlighted clearly and better descriptions of each species could be prepared.

The original assessment of species identification for each egg, by each participant, was input into a primary result table (not presented here). Once the results were available from every participant two methods of analysis were conducted. The results were initially compared with the actual species of egg, which should have been present in the wells of each tray. However, due to concerns about unintentional movement of eggs between wells, a modal species determination was also calculated for each egg. It would have been useful to judge each participant against eggs of known species but it was obvious in some cases that eggs had been unintentionally moved from their original wells. Both sets of results from the first round of analysis are presented below. It is possible that most of the differences between these tables can be accounted for by movement of eggs from one well to another.

Summaries of the results from the two rounds of egg species determination are presented in Tables 4.3-1 to 4.3-3. Each of these tables is divided into four sub-tables labelled A-D, where the performance of each participant is judged against the actual species and modal species determination.

Sub-tables A show the number of eggs at each actual or modal species that were assessed by each participant. The numbers at each modal species will therefore be the same for all participants that read all the eggs.

Sub-tables B show the numbers of eggs of each species as assessed by each participant.

Sub-tables C show the percentage under or over-estimation by each participant for each species.

Sub-tables D show the percentage agreement in species identification between the assessment of each participant and the actual or modal species.

Tables 4.3-1 and 4.3-2 show differences in the results from the first round of analysis, where ‘actual’ species of eggs were used (Table 4.3-1) and where modal determinations (Table 4.3-2) were used to compare with participants’ assessment of species. The differences between these tables probably reflect the extent to which some eggs were unintentionally moved between cells during the first round of analysis. This is apparent when comparing the results in sub-tables C and D (Tables 4.3-1 and 4.3-2) and is particularly highlighted by the difference between ‘actual’ and ‘modal’ species determinations for ‘other species’. If participants are judged against ‘actual’ species they appear to have underestimated ‘other species’ by 13% but if comparisons are made with modal species they appear to have overestimated ‘other species’ by 92%.

The results of the second round of analysis show no difference between the use of ‘actual’ or ‘modal’ species determination and hence only one table (Tables 4.3-3) is presented here. These results show that the modal value produced was always the same as the ‘actual’ species i.e. the modal species determined from the analysis was 100% correct. This is very re-assuring and demonstrates that participants, in general, were able to correctly identify the eggs provided. It also shows that they took more care when manipulating the eggs during the second round of analysis, to prevent movement between wells in the trays.

The results show significant improvements in the allocation of eggs to the various species, from the first to the second round of analysis. However, they also highlight the difficulties in

being able to positively identify eggs where there are few distinguishing features other than the size of egg and oil globule diameters. After the first round of analysis there was some discussion on the features which aid fish egg identification. Some references and criteria were produced (see Section 3.3.2) to help with the identification of eggs which are similar to those of mackerel and horse mackerel. This helped with the identification of horse mackerel eggs during the second round of analysis where the percentage over-estimation decreased from 15% to 7%.

These discussions and criteria also helped to improve the mean percentage agreement between participants' identification of eggs to species (Tables 4.3-1D and 4.3-2D compared with Table 4.3-3D). For mackerel eggs the percentage agreement increased from 79% to 90% and for horse mackerel the improvement rose from 79–80% to 96%. Overall, the percentage agreement rose from 72% ('actual' spp.) and 79% (modal spp.) in the first round to 93% in the second round of analysis. These results were very re-assuring particularly as most of the microscopes were not fitted with eyepiece graticules to enable measurement of egg or oil globule diameters.

Table 4.3-1 Species identification by participants compared with ACTUAL species. First determination.

The species compositions based on actual species reflecting the best estimates based on only those eggs that were used for species identification by the participant (A), the species compositions as obtained per participant (B), the percentages over- and underestimation (C) and the percentages agreement with modal species or actual species (D) are shown per species by participant and for the whole group that took part in the species identification excercise on fish eggs. A weighted mean percent agreement is given by person and all persons combined.

A **Species compositions using actual species (second last column input table)**

	Modal or actual species	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Mackerel	1	174	176	170	156	168	170	174	184	185	180	187	186	187	186	186	180	2849
Horse Mackerel	2	139	147	141	135	135	147	129	129	141	145	146	142	147	140	146	145	2254
Other species	3	54	57	57	42	53	53	55	61	62	55	63	61	64	62	64	53	916
Total	1-3	367	380	368	333	356	370	358	374	388	380	396	389	398	388	396	378	6019

B **Species compositions as estimated per participant and whole group**

	Species	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Mackerel	1	200	126	179	142	173	160	139	76	162	155	131	191	256	185	164	202	2641
Horse Mackerel	2	143	229	160	169	148	159	179	258	157	171	198	56	105	153	146	150	2581
Other species	3	24	25	29	22	35	51	40	40	69	54	67	142	37	50	86	26	797
Total	1-3	367	380	368	333	356	370	358	374	388	380	396	389	398	388	396	378	6019

C **Percentage overestimation / underestimation**

	Modal or actual species	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Mackerel	1	15%	-28%	5%	-9%	3%	-6%	-20%	-59%	-12%	-14%	-30%	3%	37%	-1%	-12%	12%	-7%
Horse Mackerel	2	3%	56%	13%	25%	10%	8%	39%	100%	11%	18%	36%	-61%	-29%	9%	0%	3%	15%
Other species	3	-56%	-56%	-49%	-48%	-34%	-4%	-27%	-34%	11%	-2%	6%	133%	-42%	-19%	34%	-51%	-13%

D **Percentage agreement in species identification per species**

	Modal or actual species	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Mackerel	1	74%	59%	98%	81%	96%	75%	78%	36%	79%	85%	62%	85%	95%	82%	84%	95%	79%
Horse Mackerel	2	73%	99%	95%	100%	90%	65%	94%	87%	79%	95%	77%	20%	58%	78%	75%	77%	79%
Other species	3	9%	21%	44%	29%	32%	34%	49%	38%	40%	47%	62%	30%	11%	27%	34%	34%	34%
Weighted mean	1-3	64.3%	68.7%	88.3%	82.3%	84.3%	65.1%	79.3%	53.7%	72.9%	83.4%	67.4%	52.7%	67.6%	71.9%	72.7%	79.6%	72.0%
	RANKING	14	10	1	4	2	13	6	15	7	3	12	16	11	9	8	5	

Table 4.3-2 Species identification by participants compared with MODAL species. First determination.

The species compositions based on modal species reflecting the best estimates based on only those eggs that were used for species identification by the participant (A), the species compositions as obtained per participant (B), the percentages over- and underestimation (C) and the percentages agreement with modal species or actual species (D) are shown per species by participant and for the whole group that took part in the species identification excercise on fish eggs. A weighted mean percent agreement is given by person and all persons combined.

A **Species compositions using modal species (second last column input table)**

Modal or actual species		Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Mackerel	1	178	182	176	162	172	176	178	188	189	186	192	189	193	192	191	186	2930
Horse Mackerel	2	161	170	164	152	165	175	153	159	171	167	176	172	177	169	177	165	2673
Other species	3	28	28	28	19	19	19	27	27	28	27	28	28	28	27	28	27	416
Total	1-3	367	380	368	333	356	370	358	374	388	380	396	389	398	388	396	378	6019

B **Species compositions as estimated per participant and whole group**

	Species	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Mackerel	1	200	126	179	142	173	160	139	76	162	155	131	191	256	185	164	202	2641
Horse Mackerel	2	143	229	160	169	148	159	179	258	157	171	198	56	105	153	146	150	2581
Other species	3	24	25	29	22	35	51	40	40	69	54	67	142	37	50	86	26	797
Total	1-3	367	380	368	333	356	370	358	374	388	380	396	389	398	388	396	378	6019

C **Percentage overestimation / underestimation**

Modal or actual species		Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Mackerel	1	12%	-31%	2%	-12%	1%	-9%	-22%	-60%	-14%	-17%	-32%	1%	33%	-4%	-14%	9%	-10%
Horse Mackerel	2	-11%	35%	-2%	11%	-10%	-9%	17%	62%	-8%	2%	13%	-67%	-41%	-9%	-18%	-9%	-3%
Other species	3	-14%	-11%	4%	16%	84%	168%	48%	48%	146%	100%	139%	407%	32%	85%	207%	-4%	92%

D **Percentage agreement in species identification per species**

Modal or actual species		Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Mackerel	1	75%	59%	99%	80%	94%	77%	78%	37%	80%	83%	60%	87%	95%	81%	84%	95%	79%
Horse Mackerel	2	73%	96%	97%	94%	85%	71%	94%	86%	87%	97%	71%	27%	59%	85%	82%	84%	80%
Other species	3	0%	36%	86%	68%	89%	100%	100%	67%	100%	100%	89%	32%	7%	59%	93%	63%	67%
Weighted mean	1-3	68.1%	74.2%	97.0%	85.6%	89.9%	75.4%	86.3%	60.2%	84.8%	90.3%	66.9%	56.8%	73.1%	81.2%	83.8%	88.1%	78.7%
RANKING		13	11	1	6	3	10	5	15	7	2	14	16	12	9	8	4	

Table 4.3-3 Species identification by participants compared with ACTUAL/MODAL species. Second determination.

The species compositions based on actual/modal species reflecting the best estimates based on only those eggs that were used for species identification by the participant (A), the species compositions as obtained per participant (B), the percentages over- and underestimation (C) and the percentages agreement with modal species or actual species (D) are shown per species by participant and for the whole group that took part in the species identification excercise on fish eggs. A weighted mean percent agreement is given by person and all persons combined.

A **Species compositions using actual/modal species (second last column input table)**

	Modal or actual species	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Mackerel	1	229	232	232	230	234	233	233	218	232	233	232	228	218	232	236	226	3678
Horse Mackerel	2	155	159	159	155	159	157	156	150	159	156	159	156	148	158	158	153	2497
Other species	3	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	80
Total	1-3	389	396	396	390	398	395	394	373	396	394	396	389	371	395	399	384	6255

B **Species compositions as estimated per participant and whole group**

	Species	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	TOTAL
Mackerel	1	178	195	207	203	234	236	231	176	232	203	181	220	219	235	211	226	3387
Horse Mackerel	2	206	196	184	179	159	154	156	176	158	156	210	121	131	151	183	153	2673
Other species	3	5	5	5	8	5	5	7	21	6	35	5	48	21	9	5	5	195
Total	1-3	389	396	396	390	398	395	394	373	396	394	396	389	371	395	399	384	6255

C **Percentage overestimation / underestimation**

	Modal or actual species	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Mackerel	1	-22%	-16%	-11%	-12%	0%	1%	-1%	-19%	0%	-13%	-22%	-4%	0%	1%	-11%	0%	-8%
Horse Mackerel	2	33%	23%	16%	15%	0%	-2%	0%	17%	-1%	0%	32%	-22%	-11%	-4%	16%	0%	7%
Other species	3	0%	0%	0%	60%	0%	0%	40%	320%	20%	600%	0%	860%	320%	80%	0%	0%	144%

D **Percentage agreement in species identification per species**

	Modal or actual species	Spa PC Reader 1	Spa PB Reader 2	Spa BB Reader 3	Spa PA Reader 4	Sco IG Reader 5	Sco FB Reader 6	Ger JU Reader 7	Ger SK Reader 8	Ger MK Reader 9	Ned CvD Reader 10	Ned HW Reader 11	Nor JdL Reader 12	Nor EH Reader 13	Ire BO Reader 14	Por CV Reader 15	Eng EL Reader 16	ALL
Mackerel	1	76%	84%	89%	87%	99%	100%	99%	80%	98%	86%	76%	94%	94%	97%	87%	99%	90%
Horse Mackerel	2	97%	100%	100%	98%	98%	98%	100%	97%	97%	100%	97%	78%	89%	93%	97%	98%	96%
Other species	3	100%	100%	100%	100%	100%	100%	100%	100%	80%	40%	100%	100%	0%	100%	100%	100%	89%
Weighted mean	1-3	84.8%	90.7%	93.7%	91.5%	98.5%	99.2%	99.5%	87.1%	97.5%	90.9%	84.6%	87.4%	90.3%	95.2%	91.2%	98.4%	92.6%
	RANKING	15	11	7	8	3	2	1	14	5	10	16	13	12	6	9	4	

4.4 Results of the fecundity and atresia determination exercise

4.4.1 Results of the potential fecundity analysis

The overall cv counting both normal and atretic follicles before and after discussion of the interpretation was 3.8 and and 2.3% respectively (Table 4.4.1). Comparing the scores before and after discussion for either unstained or stained images the reduction in cv was greatest for the unstained sample. However, after discussion the cv of the counts for each preparation was very similar with or without staining at 2.6, 1.9 and 2.3 for PAS, Rose Bengal and unstained respectively. Atretic follicles were considered to be more easily detected before staining because the stain masked the contents of the follicle making it less easy to see the fragmented chorion. The Workshop considered that the extra time required to carry out the staining protocol was not reflected in easier identification of follicle classes. Post ovulatory follilces (POF) counting also improved following discussion and it was agreed that the sample should be rejected from the potential fecundity data set if 5 or more similar POF structures were found during the first stage of whole mount examination.

Table 4.4.1. Results of the fecundity counts comparing analysts working with 3 images prepared from follicle samples stained with either Periodic acid Schiffs (PAS), Rose Bengal or unstained. Follicles were scored as normal vitellogenic (VF) atretic (atre) and post ovulatory (pof). The columns to the left of the centre line refer to results before discussion whilst those to the right were scored after discussion.

	PAS				PAS			
Participant	VF	pof	atre	Total	Participant	VF	pof	atre	Total
anders	227	1	0	227	anders	207	2	0	207
merete	225	1	0	225	merete	215	8	0	215
lorraine	222	8	1	223	lorraine	222	8	1	223
Mairead	222	7	2	224	Mairead	223	6	1	
peter	226	7	0	226	peter	224	6	0	224
cindy	232	5	0	232	cindy	218	8	0	218
Hanz	225	2	0	225	Hanz	224	8	0	224
Marai S	221	0	0	221	Marai S	216	2	1	217
Maria k	222	0	0	222	Maria k	221	2	1	222
mean	225	3	0	225	mean	219	6	0	219
stdev	3	4	0	3	stdev	6	6	1	6

	Rose Bengal				Rose Bengal			
Participant	VF	pof	atre	Total	Participant	VF	pof	atre	Total
anders	183	1	0	183	anders	169	1	1	170
merete	190	1	2	192	merete	178	1	1	179
lorraine	177	3	1	178	lorraine	181	1	0	181
Mairead	179	2	1	180	Mairead	178	2	1	
peter	176	2	0	176	peter	177	6	0	177
cindy	197	4	0	197	cindy	175	2	1	176
Hanz	182	2	2	184	Hanz	176	4	0	176
Marai S	180	0	0	180	Marai S	177	3	4	181
Maria k	184	0	0	184	Maria k	177	3	3	180
mean	183	2	1	184	mean	176	3	1	178
stdev	7			7	stdev	3	2	1	4

	Unstained				Unstained			
Participant	VF	pof	atre	Total	Participant	VF	pof	atre	Total
anders	238	8	2	240	anders	203	4	7	210
merete	224	5	8	232	merete	211	5	4	215
lorraine	215	4	2	217	lorraine	219	4	2	221
Mairead	207	3	1	208	Mairead	211	5	6	
peter	209	2	3	212	peter	207	6	6	213
cindy	236	2	0	236	cindy	205	5	6	211
Hanz	233	0	0	233	Hanz	208	4	5	213
Maria S	213	0	0	213	Maria S	211	9	10	221
Maria k	214	0	0	214	Maria k	213	7	11	224
mean	221	3	2	223	mean	210	5	6	216
stdev	12	2	2	12	stdev	5	6	6	5

4.4.2 Results of the mackerel atresia assessment exercise

A comparison of the alpha atresia counts, after combining the values for each stage, showed that staining method (Table 4.4.2) was not a significant factor in the results produced by the most experienced analysts (CEFAS and IMR, Table 4.4.3). Although there were differences in the allocation of scores to the three follicle classes this was always between adjacent categories and the likely consequence when applied to the stereometric method under these circumstances is therefore low. Counts made on H&E stained sections were also not significantly different for AZTI compared with Cefas, IMARES or IMR whilst IMARES was different to both Cefas and IMR. The other scores from PM and TB stained images were very different comparing either AZTI or IMARES with Cefas or IMR and further work is required to identify the source of variation. In summary the analysis suggests that either stain can be used for the assessment of atresia because AZTI will use H&E whilst Cefas and IMR will use PM and TB respectively. The differences with IMARES are not likely to be important because they are not participating in the processing of atresia samples from the 2007 surveys.

Table 4.4.2. Results from four Institutes scoring images for alpha atresia in three follicle classes, from six sections (between five and seven images per section) each stained with either Heamotoxylin and Eosin (H&E) PAS Mallory (PM) and Toluidine blue (TB). In each case the data is presented for total of all three classes combined (all) and each class separately yolk vesicle (YV) yolk vesicle / yolk granule (YV-YG) and yolk granule (YG). The data are presented as the mean count with the standard deviation in brackets.

	Institute				Overall
Class	AZTI	CEFAS	IMARES	IMR	mean
H&E all	3.56 (3.23)	3.92 92.11)	2.46 (1.80)	3.68 (3.14)	3.40 (2.70)
H&E YV	1.27 (2.33)	0.31 (1.34)	0.05 (0.32)	0.24 (0.76)	0.48 (1.48)
H&E YV-YG	1.51 (1.95)	2.17 (2.46)	0.85 (1.77)	2.61 (3.52)	1.78 (2.59)
H&E YG	0.78 (1.29)	1.44 (1.87)	1.56 (1.42)	0.83 (1.30)	1.14 (1.50)
PM all	3.64 (2.09)	4.17 (2.27)	2.17 (1.40)	4.14 (2.26)	3.53 (2.18)
PM YV	1.53 (2.85)	0.56 (1.55)	0.00	1.44 (2.64)	0.88 (2.16)
PM YV-YG	0.81 (1.34)	2.19 (2.95)	0.42 (0.73)	1.33 (1.67)	1.19 (1.96)
PM YG	1.31 (1.45)	1.42 (1.72)	1.75 (1.40)	1.36 (1.89)	1.46 (1.62)
TB all	3.17 (2.74)	3.44 (2.88)	1.72 (1.59)	3.83 (4.18)	3.04 (3.06)
TB YV	0.64 (1.19)	0.36 (0.79)	0.03 (0.16)	1.86 (3.93)	0.72 (2.18)
TB YV-YG	1.58 (2.40)	2.00 (3.19)	0.64 (1.17)	1.17 (1.29)	1.35 (2.21)
TB YG	0.94 (1.32)	1.08 (1.46)	1.06 (1.52)	0.81 (1.45)	0.97 (1.43)

Table 4.4.3. P values from Paired T tests comparing counts (n=36–41) for each stain type made by each Institute.

	Toluidine			H&E			PAS		
Institute	AZTI	CEFAS	IMARES	AZTI	CEFAS	IMARES	AZTI	CEFAS	IMARES
AZTI		0.00274			0.41002			0.00000	
IMARES	0.00274	0.00071		0.13336	0.00446		0.00000	0.00000	
IMR	0.07643	0.38148	0.00368	0.67465	0.94796	0.07988	0.00045	0.89301	0.00000

5 Discussion

5.1 Discussion of the egg sorting exercise

The evaluation of the 'Spray technique' for the removal of fish eggs from plankton samples proved to be very valuable. The results (Table 4.1-1) indicate that on average 83% of the eggs were removed during the first spraying and that after three sprayings 91% of the eggs had been removed. However, these are probably conservative estimates as each individual result assumes that there were 500 eggs available in the sample to be removed. This was probably only the case for the first participant to use each sample, as eggs were lost during the spraying and in the reconstitution of the samples.

The problems experienced during this evaluation of the 'Spray technique' were mainly generated by the fact that only a few samples were prepared with exactly 500 eggs in them. This meant that each sample had to be used on several occasions and that each time they were used, eggs were lost. This would not happen when using this technique on actual survey samples.

However, the problem of egg loss did highlight the fact that great care should be taken at all times when using the spray and all participants were made aware of this before the second sample was used. The first sample used appears to have been spilt or the eggs sustained some considerable damage quite early in the exercise, as only 416 eggs were removed (from an inoculation of 500 eggs) during the first spraying and subsequent sorting. Further fish eggs were lost during each subsequent spraying of this sample. The third participant (a novice) found large numbers of eggs but it is likely that many of these were eggs of crustacean species which should not have been counted.

The second sample produced better results but it too was spilt after two participants had used it. These experiences helped to emphasise the importance of exercising great care when using the spray technique and all participants, particularly those with less experience, learnt a good lesson from this exercise.

A third sample was used by three sets of participants. The results were much better, with a mean 93% of eggs being removed by the first spraying and between 94% and 100% of eggs being removed after several sprayings.

The results are encouraging as long as participants exercise care in the handling and spraying of samples. The technique has been fully documented in a draft paper (Eltink, 2006) which is currently awaiting submission to a reputable journal. It is hoped that it will be published in the near future.

5.2 Discussion of the egg staging exercise

The criteria for staging mackerel eggs (Lockwood *et al.,* 1977) and horse mackerel eggs (Pipe and Walker, 1987) have been used by WGMEGS participants since the instigation of the tri-ennial surveys. Following discussions at previous egg-staging workshops in 2000 and 2003 (ICES, 2001 and ICES, 2004), and further consultations at this workshop, these egg staging criteria have been enhanced by the addition of some secondary characteristics (Section 3.2.2). These characteristics are the result of many years of personal experience (from various participants) in staging preserved fish eggs from plankton samples. These characteristics proved invaluable to less experienced participants during this workshop, particularly during the second round of analysis when much greater levels of agreement on egg stages were obtained (Section 4.2.1).

A weakness of the analytical method previously used for assessing the results is that the modal stage is not necessarily the true stage. In some difficult cases with a low percentage of

agreement the majority of the group could be incorrect in its judgement and only a minority of participants (often the most experienced) could be correct in their assessment of egg stage. This would lead to the modal stage being 'incorrect', and therefore the assessment made by the more experienced readers would appear to be wrong. This problem is difficult to overcome unless eggs of validated stages are available for these exercises.

One of the main problems encountered during the workshop was, once again, the distinction between the stages Ia and Ib. However, this appeared to be less contentious than at the previous workshops because the eggs (from artificial fertilisations) were generally in better condition, as they had not suffered the trauma of being towed in a plankton net before preservation. Discussions after the first round of analysis provided further clarification of egg stage criteria (Section 3.2.2). This led to improvements in the percentage agreement of stage 1 (1a+1b) eggs of both target species, with agreement for mackerel increasing from 92% to 98% and agreement for horse mackerel increasing from 90% to 95%.

Unfortunately, there were not many late stage eggs (stages 4 and 5) available for the analysis whilst there were larger numbers of early stage eggs. This could have affected the results, as a participant who arbitrarily assigned all the eggs to stage 1 would have achieved a high level of agreement for that stage. It is important for future workshops that all egg stages are fully represented perhaps in the proportions expected from an average survey sample.

These results (Tables 4.2-1 to 4.2-6) certainly highlight the need to conduct regular quality assurance workshops and the very valuable benefit, which can be gained by bringing practitioners together to discuss problems and clarify procedures.

5.3 Discussion of the egg identification exercise

The eggs used for species identification were the same as those used for the egg staging exercise. They were all 'validated' eggs (of known species) from artificial fertilisations.

The exercise proved to be extremely valuable, not least in the production of some egg identification criteria (Section 3.3.2) from both published sources and from the experience gained by several participants over many years. The benefits are highlighted by the increase in the mean percentage agreement in the identification of each species (Tables 4.3-1 to 4.3-3). For mackerel, the percentage agreement for species identification increased from 79% to 90%. For horse mackerel the agreement increased from 79% ('actual' species.) or 80% (modal species) to 96% and for other eggs the agreement increased from 34% ('actual' species.) or 67% (modal species) to 89%. These results are very comparable to those obtained at the 2003 workshop where the percentage agreement in species identification after the second round of analysis were 95% for mackerel, 88% for horse mackerel and 78% for 'other eggs'. This is very encouraging, particularly given the number of inexperienced participants at this workshop

The mean agreement for horse mackerel eggs improved by 16% or 17% mainly because the validated horse mackerel eggs were sometimes unusual in their appearance. Many of the participants, particularly those not familiar with samples from the southern area, found these eggs extremely difficult to identify in the first round of analysis. The oil globules were sometimes fragmented and there was little sign of segmentation in the yolk. As a result of discussions, participants were made aware of these features, which seem to be more common for horse mackerel eggs from the southern area. They were then able to correctly identify these eggs in the second round of analysis.

The levels of agreement seen in these results (both for stage and species) are probably lower than in the analysis of real survey samples. There were a number of inexperienced participants at this workshop who were identifying and staging fish eggs for the first time. These analysts benefited greatly from participating in the workshop and from the knowledge gained from

other, more experienced, participants. They will be able to utilise this knowledge when they begin to process plankton samples collected on the 2007 surveys. The accidental movement of eggs from one well to another, also caused problems. This led to low levels of agreement (both in staging and identification) between participants as they were sometimes analysing different eggs, which had been moved between wells. The eggs also became more and more damaged during the course of each round of analysis as sixteen participants manipulated each egg to look for the salient features. Because of the movement of eggs and the damage incurred to some eggs it was decided to replace all the eggs prior to the second round. It is unlikely that this would have significantly affected the results as all the eggs used were from the same artificial fertilisations, although there were fewer 'other' eggs available for the second round.

Discussion amongst participants was difficult to prevent whilst the eggs were being analysed. Independent assessment of the eggs is critical to prevent the introduction of bias or incorrect assignment of modal stages/species. All discussions should be reserved for the plenary sessions to enable every participant to comment fully on the features observed.

The participants were unfamiliar with the microscopes used for the analysis. This did lead to some problems at the beginning of the analysis where the lighting on some microscopes was not adjusted to its optimum settings. In addition, few of the microscopes were fitted with eye-piece graticules which would have made the speciation of mackerel and horse mackerel eggs easier, as horse mackerel eggs are generally slightly smaller.

6 Working documents

6.1 An image processing method for the identification of mackerel and horse mackerel eggs

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This is a short description of a PC based method developed at the BFAFi to measure egg and oil globule diameters from a mixed mackerel and horse mackerel egg sample.

Material and methods

The image analysis is based on the ImageJ package which has been developed by Wayne Rasband at the American National Institute of Health as a public domain software which can be downloaded from the web. The program is widely used by scientists around the world. As a java program it is compatible with all platforms and operating systems. The program is freeware and the source code is available for free as well. An extensive documentation and a lot of plugins are available for download at http://rsb.info.nih.gov/ij.

Working with the "Egg Finder" Plugin

You need a digital image of an egg. With certain hardware equipment it is possible to use ImageJ for acquiring images; please refer to the Plugins → Acquisition section on the ImageJ website for latest information.

The EggFinder-Plugin tries to recognize the egg and globules on the image and then shows their outlines which can be manually adjusted if necessary (Fig.1). The diameters of the outlines are measured and the results are shown in a separate window, as well as the ratio between egg and globules diameter; this can be an aid in the staging process. Immediately after measuring a dialog window will open which gives you the possibility to choose the species from a menu as well as the stage, and to enter a comment if desired (Fig.2). The information is added to the results table as well. When you have finished all your measurements, you can transform the results table to a tab delimited text file (Fig.3).

The plugin is free for distribution to all workshop members. If interested please send a mail to juergen.schlickeisen@ish.bfa-fisch.de or jens.ulleweit@ish.bfa-fisch.de

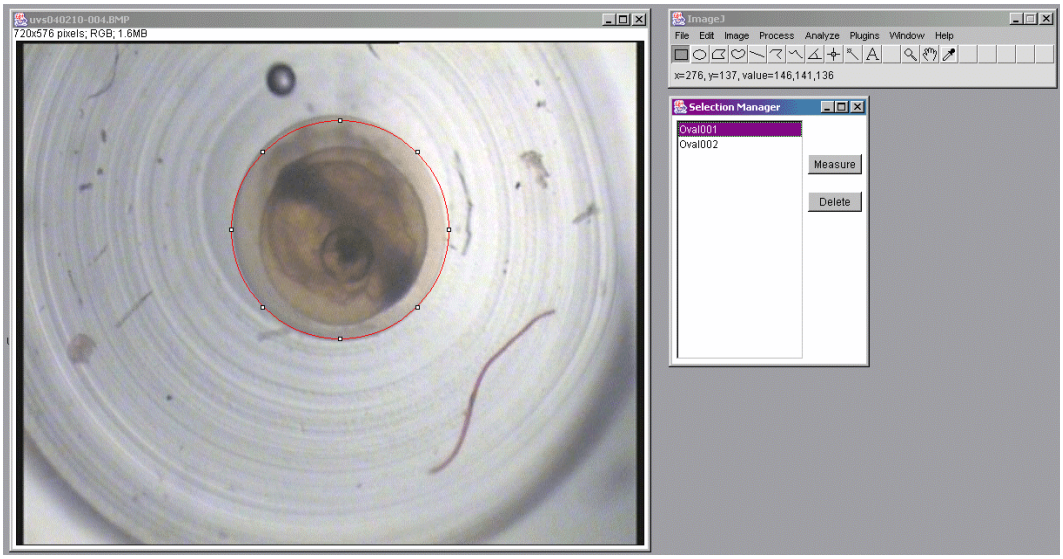

Figure 1: Image with recognized and correctable egg outline.

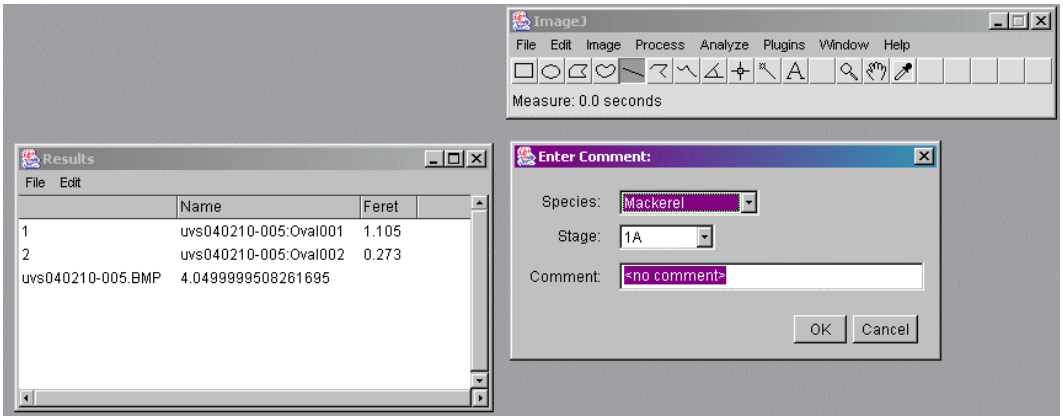

Figure 2: Entering Species, Stage and Comment.

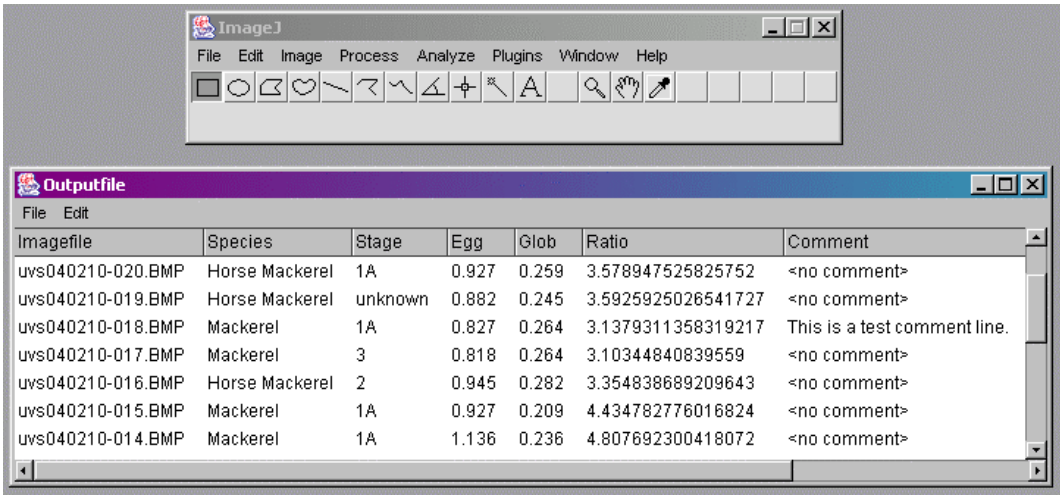

Figure 3: Results in a tab delimited text file.

6.2 The occurrence of Snake pipefish on the German WGMEGS survey 2004

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Abstract

A major side outcome of the German 2004 MEGS was the occurrence of snake pipefish in a large number of plankton samples. In the surveyed area between 46°00' and 58°30' N west of the British Isles snake pipefish occurred almost exclusively and regularly in samples off the shelf over deep waters. The occurrence of snake pipefish in the samples presumably was the first notice of a mass occurrence of pelagic snake pipefish in the Northeast Atlantic (Kloppmann and Ulleweit 2006). In the following years pelagic snake pipefish appeared to become even more numerous in the Northeast Atlantic as well as in the North Sea with a northward extension of its occurrence up to Spitsbergen (Harris *et al.,* 2006). Because of the strong increase in abundance, snake pipefish were regularly observed to be utilised as food by fish and dolphins (van Damme and Couperus 2006) as well as by seabirds (Harris *et al*., 2006). Because of the poor digestibility of snake pipefish particularly for the chicks this change in seabird feeding habit subsequently caused serious failures in breeding success in populations of some species such as puffin and terns (Harris 2006; Harris *et al*., 2006).

Because increase of snake pipefish abundance is an ongoing process, probably linked to phenomenon associated to climate change, participants of the 2007 MEGS were encouraged to note any occurrences of snake pipefish in the plankton samples, and to measure and sex the specimens. Results should be sent to Cindy van Damme (IMARES) and Jens Ulleweit (BFA Fisch).

References

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6.3 Mass occurrence of snake pipefish: result of a change in climate?

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Abstract

In 2004 a sudden mass occurrence of snake pipefish *Entelurus aequoreus* appeared in the North-eastern Atlantic and has been increasing since. Before 2004 snake pipefish was mainly found in coastal areas and occasionally in oceanic waters.

Indices (numbers of fish caught per hour) from inshore surveys remain at the same level, while the indices from surveys conducted in deeper offshore areas show a very strong increase since 2004. The length distributions of all surveys differ significantly from each other. Coastal snake pipefish are larger compared to pelagic specimens. Although the outward appearance of the coastal pipefish seems different from the pelagic specimens, no differences were found

when comparing taxonomic features. The mean numbers of rings and fin rays are well within the ranges mentioned for snake pipefish. Apart from appearance the habitat is different for the two types of snake pipefish. The oceanic form lives free in the water column while the coastal form is found among seaweeds or in sea grass beds.

Although food is available in high quantities, the oceanic specimens of snake pipefish are much leaner than the coastal specimens. While the snout length would make the species more suitable for preying on less mobile prey, the stomach contents of the oceanic snake pipefish revealed remains of relatively small calanoids (mean length 2.4 mm). The calanoid population has recently changed and is nowadays dominated by the smaller *Calanus helgolandicus*. Here we put forward the hypothesis that the sudden appearance of the snake pipefish in the deeper waters is a result of the change in the average lengths of calanoids which in turn is caused by changes in the hydroclimatic environment.

The mass occurrence of the snake pipefish is affecting the whole ecosystem. Seabirds are feeding their chicks with them and they are also found in stomachs of fish and sea mammals.

6.4 Comparison between Bongo designs used by IEO, IPIMAR and AZTI for triennial Mackerel and Horse Mackerel egg surveys

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Abstract

A primary design criterion for any quantitative plankton sampling net calls for sustained efficient filtration. Changes in filtration efficiency during a tow affect the representation of the water column. The most important design features in regard to sustained filtration efficiency are mesh size and filtering area (Smith, *et al*., 1968).

Bongo 40 sampler is the standard sampler used by IEO, IPIMAR and AZTI during triennial Mackerel and Horse Mackerel egg surveys in the Southern area. However subtle differences in the design of the nets used by the different labs have been detected. The net shapes used are cone for IEO and IPIMAR Bongo nets and cylinder-cone for AZTI Bongo net, with a 0.4 m diameter mouth opening, 2.48 m long for IEO Bongo, 2.40 m long for IPIMAR Bongo and 2.94 m long for AZTI Bongo. All nets are made of nylon gauze at 46% porosity. However IEO and IPIMAR nets present a mesh size of 250 μm whereas AZTI net presents a mesh size of 335 μm. The filtering area of IEO Bongo net is about 1.69 m^2 (R= 6.21), IPIMAR Bongo net is 1.70 m^2 (R= 6.23) and for AZTI Bongo net is 2.48 m^2 (R= 9.06).

The generic equations to estimate the relation of filtering area to the mouth area (R) (Sameoto *et al*., 2000) was applied to the mean volume of water filtered per sample during the 2004 triennial egg survey by Bongo sampler (120 m^3) and the resulting estimated were R = 9.17 in coastal or green water and R = 4.10 in oceanic or blue water.

A suggested modification could be to increase filtering area in IPIMAR and IEO nets (with a R less than 9) by attaching an anterior cylinder net to the conical net with a mesh area of at least of 0.80 m^2 (length of 0.65 m).

On the other hand differences in mesh size between IEO and IPIMAR net (250 μm) and AZTI net (335 μm) will have a significant effect on plankton selection since mesh width is the most important measurement affecting the size selection of plankton (Tranter and Smith, 1968). In order to unify the gears the suggestion is that AZTI should change the mesh size to a 250–280 μm as has been recommended by the WGMEGS (ICES, 2003).

6.5 Genetic identification of fish by species-specific DNA markers for use in stock biomass assessments and detection of commercial fraud (MARINEGGS)

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Abstract

Marineggs project (QLK5-CT 1999-01157; 2000–2003) was focused on the identification of fish eggs by means of species-specific DNA markers for use in stock biomass assessments. The project provides specific method to extract DNA from fish eggs preserved in formaldehyde 4%. Two specific methods have been applied to extract and amplify the DNA using mitochondrial gene like target sequences: i) when previous knowledge of the DNA sequences is required: PCR amplification followed by fragment size identification in agarose or acrylamide gels and ii) when No previous knowledge of the DNA sequences is required: SSCP (single-stranded conformation polymorphism). These methods were successfully determined from 85% (CV = 8.4%) and 95% of formaldehyde-fixed eggs and larvae recorded from the plankton samples. Comparing costs, first one is more expansive because it requires sophisticated equipment. However, in order to propose these methods for routine genetic analyses in plankton surveys additional samples should be analysed.

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6.6 Effects of temperature on development and mortality of Atlantic mackerel fish eggs

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Abstract

The development of Northeast Atlantic mackerel (*Scomber scombrus*) eggs, obtained from artificial fertilisation during the spawning season in the Biscay Bay area, was monitored at five temperatures (ranging from 8.6 to 17.8◦C). The times to intermediate stages (III–V) and total hatching, obtained in this study, agree with the results of previous studies undertaken some years ago. However, the times over stages IA, IB and 50% hatching indicate that development rates differed significantly between the studies. According to these results, the mackerel eggs production could be under-estimated. The percentage is temperature dependent and varied between 3.5 to 14%.

However, these differences could be related to an effect of the previous thermal history of the eggs, or to experimental biases. These possible effects will be tested by mean of a new artificial experimentation which will be carried out by Marine Institute of Galway (Ireland).

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Annex 2: Agenda

1) Introduction and welcome. S. Milligan (CEFAS).
2) Presentation of working documents.
3) Discussion on egg identification and staging to be used during the workshop.
4) Discussion on plankton sorting techniques, including the 'spray technique' developed by RIVO and AZTI.
5) Visual identification and staging of prepared samples of mackerel, horse mackerel, and morphologically similar fish eggs.
6) Applying the 'spray technique' to remove eggs from prepared plankton samples.
7) Statistical analysis of the results from the comparative egg identification and staging exercise.
8) Discussion and evaluation of results including group analysis of those egg stages which are highlighted as causing the greatest difficulties.
9) Repeat of the egg identification and staging exercise.
10) Statistical analysis and interpretation of the second set of results.
11) Discussion and evaluation of the performance of individual participants.
12) Discussion and evaluation of the effectiveness of the 'spray technique'.
13) Production of the report on the statistical evaluation of individual performance.
14) Discussion on the interpretation of egg stages and species recognition.
15) Discussion on preservation of fish eggs.
16) Production of a technical manual to include sorting, identification and staging of fish eggs for the use of all participants in the WGMEGS 2007 surveys.
17) Recommendations.
18) Any other business.

Annex 3: WKMHMES Terms of Reference for the next meeting

The **Workshop on Mackerel and Horse mackerel egg staging and identification** [WKMHMES] (Chair: C. van Damme*, Netherlands) will meet in IJmuiden, Netherlands in the Autumn of 2009 to:

a) carry out comparative plankton sorting trials on typical survey samples. This should follow the pattern of trial – analysis – retrial – identification of problem areas;

b) carry out a comparative egg staging trial for mackerel and horse mackerel eggs following the pattern used in the 2006 egg staging workshop;

c) update a set of standard pictures and descriptions for species identification and egg staging;

d) provide a review of any available documentation on identifying eggs to species and define standard protocols;

e) provide a review of any information available on other egg identification procedures – particularly DNA probes.

f) carry out inter-calibration work on fecundity determination and harmonise the analysis and interpretation of fecundity samples;

WKMHMES will report by January 2010 for the attention of the Living Resources Committee as well as WGMHSA.

Supporting Information

PRIORITY:	Information quality, used to provide fisheries advice through WGMHSA, will be impaired if this workshop is not conducted.
SCIENTIFIC JUSTIFICATION AND RELATION TO ACTION PLAN:	Sorting eggs from plankton samples, Identification of eggs to species and the staging of those eggs remains one of the key areas in the execution of the mackerel and horse mackerel egg surveys. As this process is carried out by a number of different operators in many different countries, and then the data combined, it is vital that the process be standardised. WGMHSA and WGMEGS strongly feel that this is best done through the mechanism of sample exchange programmes and regular workshops to compare results. In the context of the triennial egg surveys it would seem appropriate to hold a workshop prior to every survey to standardise approaches and methodologies in the run-up to the surveys. This will have the advantage of training new operators as well as harmonising the approach of experienced operators. Egg staging workshops were held in 2000, 2003and 2006 and were very successful in achieving these aims. It is proposed that these be used as a model for the proposed workshop in 2009. It is expected that the workshop will use the proven method of carrying out a set of sorting trials, analysing the results and identifying problems, and then repeating the trials on the basis of the new understanding. The workshop will also be tasked to update a standard manual of descriptions and photographs to assist in the plankton sample handling procedure. This material was assembled into an agreed standard manual at previous workshops. Currently identification to species depends on visual examination. A number of other approaches to egg identification are available, notably the use of DNA probes. The workshop is asked to examine the state-of-the-art in species identification and make appropriate recommendations. In the context of these surveys, fecundity estimation is very important for conversion of egg production to biomass. Fecundity estimation is carried out using histological methods and the analysis and interpretation of this material also requires standardization across participating institutes. Standardization of this aspect of the work will be included in the workshop. Goal 1. Understand the physical, chemical, and biological functioning of marine ecosystems Modernise technologies and sampling designs for collecting, measuring, and enumerating marine organisms, and improve the precision and accuracy of resource surveys. Goal 4. Advise on the sustainable use of living marine resources and protection of the marine environment

	Develop quality assurance protocols to enhance confidence in scientific advice.
RESOURCE REQUIREMENTS:	None
PARTICIPANTS:	Mainly scientists (approximately 20) involved in the surveys.
SECRETARIAT FACILITIES:	None.
FINANCIAL:	No financial implications.
LINKAGES TO ADVISORY COMMITTEES:	ACFM.
LINKAGES TO OTHER COMMITTEES OR GROUPS:	WGMEGS and WGMHSA.
LINKAGES TO OTHER ORGANIZATIONS:	None.

Annex 4: Recommendations

RECOMMENDATION	ACTION
1. WKMHMES participants propose that Cindy van Damme, IMARES, Netherlands, becomes the new Chair of WKMHMES from January 2008.	WKMHMES recommend to LRC that Cindy van Damme is chosen as the new Chair of this workshop.
2. It is almost impossible to organise and run workshops such as this without some financial assistance. Standardisation of procedures and techniques is a requirement of all ICES working groups and is recognised as being vitally important. However, without access to central financial resources, each participant is wholly reliant on funding from their own institute for travel and subsistence. It is recommended that each institute include workshops such as this in their bid for EU Data Regulations funding.	All participants.
3. It is recommended that all microscopes at the next workshop are fitted with eyepiece graticules. These graticules should be calibrated to the same standard i.e. that one eyepiece unit (epu) should be equivalent to the same number of millimetres, regardless of microscope used.	New chairperson to consider before next workshop in 2009.
4. It is recommended that all participants carry out artificial fertilisations of any species, which have eggs similar to those of mackerel and horse mackerel. It would be useful if egg and oil globule diameters are measured and that photographs are taken of as many stages as possible. It would also be beneficial if the eggs were preserved at various stages of development and any morphological changes noted following fixation. These eggs should be made available for analysis during the next workshop (scheduled for 2009).	All participants to consider providing eggs for analysis at the next workshop. IMR, Norway encouraged to provide mackerel and horse mackerel eggs from tank experiments for the 2009 workshop.
5. The Spray technique should be used as the primary method for sorting eggs from the rest of the plankton during the 2007 tri-ennial surveys. Following the use of the 'Spray Technique' to remove the eggs, each sample should subsequently be resorted by hand to remove any remaining eggs.	All participants.
6. All participants are reminded that the procedures described in the WGMEGS survey manual should be followed during the 2007 surveys. Particularly that 4% formaldehyde, buffered with sodium acetate tri-hydrate, is the standard survey fixative and that plankton samples should never come into contact with formaldehyde of a concentration greater than 4%. All participants are encouraged to check the pH of their fixative on a regular basis.	All participants.
7. Based on the experiences at the workshop a recommended binocular microscope should have the following features: • Options for a black or white stage plate for use with incident (top) light. • A transparent stage plate for transmitted (bottom) light. • Dark field illumination for contrast. • Adjustable brightness. • Magnification with click stops. • A choice of 10x and 20x eyepieces. • Adjustable binocular head and ergonomic design to allow flexibility of movement. • Adjustable focus on all eyepieces.	New chairperson to consider before next workshop in 2009.

• Calibrated eyepiece graticules. • Double (fibre optic) cold light source, with adjustable focus, to avoid shadows. • Mechanical stages to position samples easily in the field of view and to hold the samples firmly. • Filters and polarisation.	
8. Comparisons between the performance of Bongo and Gulf type samplers should be carried out during the 2007 survey.	Participants from IEO, AZTI and IPIMAR to carry our these comparisons on their surveys in 2007.
9. To consider changing the design of the well plates used during the workshop to try to limit the amount of movement of eggs between wells.	Chairperson and participants to consider new designs before the next workshop.
10. A manual should be written during the next workshop to describe the procedures involved in handling the data collected and collated during these workshops.	Chairperson to delegate responsibility for this to suitable participants.
11. All analysts who are engaged in the analysis of fecundity and atresia mackerel and horse mackerel samples must complete the intercalibration exercise before starting the analysis of the 2007 Triennial survey samples.	Members of WGMEGS participating in the 2007 Triennial survey (AZTI, Cefas, IEO, IMARES and IMR).

Annex 5: WGMEGS Survey manual

[All changes to the last version of the survey manual (ICES, 2003) and recommendations are highlighted in bold text].

A manual for the conduct of egg surveys, targeted at the AEPM, is given in Section 8 of the Report of the Mackerel/Horse Mackerel Egg Production Workshop (ICES, 1994). Those instructions are repeated in ICES 1997 (Sections 6.4.1 to 6.4.8) and incorporate changes, additions or clarifications, which are underlined. Additional changes and recommendations for further standardisation between participants are given in Section 3.3 of ICES, 2003.

This annex now incorporates the current protocols (together with recent changes) for the collection and analysis of adult fish parameters required for the AEPM method. It is recommended that this annex is updated on a regular basis and is distributed for use by all participants on the 2007 and future tri-ennial surveys.

1. Sampling areas and sampling effort

The spatial and temporal distribution of sampling is designed to ensure an adequate coverage of both mackerel (*Scomber scombrus* L.) and horse mackerel (*Trachurus trachurus* L.) spawning. Sampling effort is targeted at producing estimates of stage 1 egg production for both species.

The north-east Atlantic shelf area is sub-divided (by WGMEGS) into 'western' and 'southern' areas for the purposes of estimating spawning stock biomass (SSB) of mackerel and horse mackerel. The 'southern' area is regarded as being from 36º N to 45º N. It includes southern Biscay, the Cantabrian Sea and from the Portuguese coast to 11º W. Sampling usually begins in January in this area and continues until June in the Cantabrian Sea.

The 'western' area is from 44º N to 60º N. It includes Biscay, the Celtic Sea and the shelf edge to the northwest of Scotland. Sampling is focussed along the shelf edge (200m isobath) but also occurs from the French and Irish coasts out to 16º W. Sampling in this area usually begins in March and continues into early July.

In the western area plankton samplers are deployed at the centre of half standard ICES rectangles, which are 0.5º latitude, by 0.5º longitude. To the north of Spain (Cantabrian Sea) and to the south of Portugal (south of 37ºN) the sampling positions are separated by 10' latitude and 20' longitude because of the proximity of the shelf edge to the coast. To the west of Portugal (from 37ºN to 43º 10'N) the station positions are separated by 20' latitude by 10' longitude to provide greater spatial resolution across the shelf break.

Since the surveys began in 1977 considerable changes have been made to the 'standard' sampling area and some of these were described in Section 8.4 (ICES, 1994). Based on the expansion of the "standard area" since 1977, it was agreed (ICES, 2002a) to reconsider its use. It was agreed that the existing "standard area" should be retained only as a guide to the core survey area for cruise leaders, and that the extent of coverage should be decided based on finding the edges of the egg distribution only i.e. boundaries should be set based on the adaptive sampling guidelines given below (Section 2.). The core areas for the western and southern surveys together, are presented in Figure 1. The sampling area in the south has been modified from the design used in 2001 and previously (Figure 2). Figures 1 and 2 are provided as a planning guide only. The limits of the survey in both areas should be established on the basis of two consecutive zero samples, and not by the boundaries on these maps.

2. Sampling strategy

The sampling strategy in the western and southern areas will be targeted at the AEPM only. However, Portugal will collect both plankton and adult fish samples to produce a DEPM estimate for horse mackerel in their waters, in 2007.

Two important factors needed to be considered when planning the survey strategy. Firstly, a set of rules must be established in order to decide when to stop sampling along a given transect, in order to ensure that the whole area of egg distribution is sampled with no effort wasted outside the spawning area. Secondly, some guide-lines need to be provided to cruise leaders on the number and spacing of transects which may be omitted in order to best match available effort to the size of the area to be surveyed. As a first guide to planning the distribution of sampling effort, historic egg distributions should be reviewed with particular reference to the latest WGMEGS reports. The main areas of egg abundance, identified for each of the different sampling periods, should always be sampled to the north/south and east/west limits although individual transects may be omitted. When sampling along transects, shipboard enumeration of results should be undertaken several rectangles before the limit of the core area is reached. The introduction of the 'Spray technique' (WD, Eltink) should allow a rapid assessment of the numbers of eggs present in each station. Sampling will be completed along a transect when two consecutive stations contain no mackerel or horse mackerel eggs. In some cases it may be necessary to sample beyond the core area limits (Figure 1).

The amount of ship time available and the size of the area to be covered will determine the spacing and omission of sampling transects. During periods when several ships are available it should be possible to sample all transects, while at other times it may be necessary to omit several, at least during the first pass over the designated sampling area. No more than one consecutive transect should ever be omitted. Given that the area to be covered is more or less known, as is ship time, cruise leaders should be able to estimate fairly accurately the number of the full transects they will be able to make. **It is strongly recommended that, where practical, and even where total coverage is expected, a first pass over the area be made on alternate transects. The intervening transect should be sampled on the return leg**. If time is limited on the return leg, sampling should concentrate in areas where high densities were observed in the first pass. The cruise leader should be aware of edge definition problems where the contours run east-west. In this way, weather problems, equipment failure and vessel breakdown need not seriously prejudice results. Such a strategy, furthermore, enables better evaluation of distributional change with time, which is likely to be important in modelling the results. An example of an appropriate sampling strategy where one in two transects is fully sampled is given in Fig. 6.16 in ICES (1994).

Where possible, additional sampling should be carried out in areas where high densities of either mackerel or horse mackerel eggs are encountered. This will enable an estimate of sampling error to be calculated.

3. Standardisation of survey gears

The standard plankton samplers for use on these surveys are national variants of 'Gulf type' or Bongo 'high-speed' samplers (Nash *et al*., 1998). These samplers generally incorporate conductivity, temperature and depth probes (CTD's) and are fitted with either mechanical or electronic flowmeters to enable the volume of water filtered on each deployment to be calculated. These sensors either relay 'real-time' environmental data back to a shipboard computer or log the information, ready for downloading once the station has been completed.

It would be preferable to use a standard survey sampler for the tri-ennial surveys. **As a first step, it is therefore recommended that each participating nation should review the design of their sampling equipment (including flowmeters) against published sampler designs.**

This information will be collated by C. van Damme (for Gulf type samplers) and G. Costas (Bongo samplers). It will be presented at the next meeting of WGMEGS in 2008, and included in an updated version of this annex. Nash *et al*., 1998, provides a comprehensive description for a Gulf type sampler, which they call a Gulf VII. A useful review of Bongo designs and a suggested standard is given by Coombs et al (1996) in an annex to the final report of EU AIR project AIR3 CT94 1911. **Each participant is requested to compare their samplers against these suggested designs, report the differences at the next WGMEGS meeting and attempt to modify their sampler designs to make them more similar to the published standard.**

The estimation of volume of water filtered by each sampler is critical in the calculation of egg abundance. Again, the suggestions provided by Nash et al (1998), and Coombs et al (1996) provide an acceptable standard. **It is recommended that participants follow these standards as closely as possible.** It is also critical that participants understand the importance of calibrating flowmeters and changes in flowmeter performance when they are mounted in the apertures of plankton samplers (EU AIR3 CT94 1911). **It is recommended that all participants review the performance of their flowmeters and regularly their check their calibration in-situ (i.e. within the sampling device).** The current flowmeters used in the survey are largely considered as state-of-the-art; however, new developments are being made in non-intrusive flow meters. **It is recommended that participants investigate the utility and cost-benefits of these and report back to WGMEGS as appropriate.**

Although a mesh size of 500 micron aperture is adequate for sampling mackerel and horse mackerel eggs, a nylon mesh with an aperture between 250 and 280 microns is the recommended size for these surveys. This allows the plankton samples to be more widely used for investigations on other species and taxa. In the North Sea surveys, where clogging is a problem, a 500 micron aperture mesh is used by both the Netherlands and Norway. Norway is the only participant to use 500 micron aperture mesh in the western (or southern) area.

The aperture on the Gulf type sampler should be 20 cm in diameter in order to ensure that an adequate volume of water is filtered. The aperture of the Bongo samplers should be either 40 cm or 60 cm diameter. It is recommended that no ad hoc changes take place.

Different mouth openings for Bongos do not seem to make a difference in sampling efficiency or performance, although 60 cm nets (vs. 40 cm) are apparently more prone to clogging. **Portugal used a 60 cm Bongo until the 2004 survey, but in 2007 they will use a 40 cm diameter Bongo, similar to that used by both AZTI and IEO, Spain for all their tri-ennial surveys.**

4. Plankton sampler deployment

It is recommended that the Gulf type samplers are deployed on a double oblique tow, at **4 knots**, **(note change from 5 knots),** from the surface to maximum sampling depth (see below) and return. The Bongo samplers are deployed at 2–3 knots on similar, double oblique tows. The aim is for an even (not stepped) 'V' shaped dive profile, filtering the same volume of water from each depth band. The aim is to shoot and haul at the same rate with the sampler spending 10 seconds in each 1 metre depth band (ICES, 2001). At shallow stations, multiple double-oblique dives may be necessary to enable a sufficient volume of water to be filtered. **A minimum sampler deployment time of 15 minutes is recommended.**

Norway uses the Gulf type samplers in the western area but deployed a Bongo in the North Sea until the 2005 survey when a Gulf VII sampler was used. **Both Norway and the Netherlands now use Gulf VII samplers on the North Sea surveys and this is now the recommended sampling device for this survey. Norway has also changed from a stepped tow profile (used with the Bongo) to the recommended double oblique tow used by all**

other nations. This is a welcome standardisation both in terms of gear design and in deployment method, and is to be encouraged.

Recommended maximum sampling depth is to 200 m, or to within 5 m of the bottom where the bottom is less than 200 m. In the presence of a thermocline greater than 2.5°C in 10m depth, sampling can be confined to a maximum depth of 20 m below the base of the thermocline.

Vessels can only achieve the high frequency of samples taken at exactly the recommended maximum depth if they have automatic devices controlling the sampling depth, or by samplers fitted with real-time pressure sensors. **As a result, and because depth is an important parameter when calculating egg densities, the working group recommends that depth measurements are recorded carefully, with the use of real-time depth, flowmeter and temperature monitoring systems.**

5. Plankton sample collection and fixation

It is recommended that the standard plankton samples collected for the SSB estimates will be handled carefully and preserved as soon as practicable. The recommended procedure will be as follows:-

a) Remove the end bag used on the station before washing down the net.

b) Attach a clean end bag and **gently** wash down the net from both ends of the sampler, taking care to wash the lower surface of the net just in front of the end bucket.

c) Always wash down from the nosecone end last.

d) Make sure the net is clean, using more than one end bag if necessary.

e) Make doubly sure that a clean end bag is left on the sampler ready for the next station.

f) Wash the plankton from the end bags into a jar with the 4% formaldehyde solution in a wash bottle.

g) Top up the jar with 4% formaldehyde, making sure that the volume of plankton does not exceed 50% of the volume of the jar.

h) Any excess sample should be fixed separately in additional jars.

i) Put labels containing station details in pencil into all jars.

The standard fixative for use on these surveys will be a 4% solution of buffered (pH 7 – 8) formaldehyde in either distilled or fresh water. (420g of sodium acetate trihydrate is dissolved in 10 litres of 4% formaldehyde, ICES, 2001). This solution is approximately iso-osmotic with seawater and will minimise damage and distortion of the eggs. The sample should be directly fixed with the addition of the 4% formaldehyde solution and should not come into contact with formaldehyde strength in excess of 4%.

There was some discussion at WGMEGS, 2006 about the suitability of the sodium acetate buffer for the preservation of fish eggs. It is recommended that all participants review their preservation methods and present any results for discussion at WKMHMES, 2006. Any conclusions will be included in the WKMHMES report and will be available for the next meeting of WGMEGS in 2008.

The volume of plankton in the sample jar must never exceed 50% of the volume of the jar. Excess sample should be fixed separately in additional jars. Details of an alternative fixative, giving better definition of egg development stage, for a more precise estimate of elapsed time since spawning, were given in ICES (1988). That fixative is 9.5 parts ethanol (95%); 1 part formalin (10%); 0.5 part glacial acetic acid.

6. Plankton sample sorting

Following practical demonstrations and trials with a 'spray technique' for the removal of fish eggs from plankton samples at WKMHMES (ICES, 2004b), it was recommended that this technique was used on samples collected during the 2004 tri-ennial survey. Since then, enhancements have been made to the equipment and methods (WD, Eltink), which will again be evaluated at WKMHMES in 2006. **It is recommended, that where suitable, the spray technique be used at sea to quickly remove the majority of fish eggs from plankton samples. This will allow a rapid decision to be made on whether to continue sampling along a transect or to move to the next transect line.**

The eggs removed by the 'spray technique' can be stored in separate vials within the plankton sample jar. **It is recommended that every sample is subjected to a manual sorting and removal of any remaining eggs, to ensure that all eggs are removed from each sample.** The use of the spray technique will remove the need for any sub-sampling of the plankton samples collected.

Immediately before the manual sorting, it is recommended that the 4% formalin is drained from the sample and the sample washed gently with seawater. The sample can then be placed in a sorting/observation fluid (Steedman, 1976), which also acts as a preservative. The observation fluid stock solution is made with 50ml of propylene phenoxetol mixed with 450 ml of propylene glycol (propane-1,2-diol). Before use, 5ml of the stock solution is diluted with 95 ml of distilled water to produce a sorting fluid which is non-toxic and pleasant to use (odourless).

All sorted eggs should be kept in tubes, in, 4% buffered formaldehyde, inside the sample container for future reference and use. Usually only the eggs of mackerel and horse mackerel need be identified to species and staged.

7. Egg identification and staging

This is a key area for standardization and has been the subject of considerable attention by the working group. Egg staging was the subject of a detailed workshop held at Cefas, Lowestoft in 2000 (WKMHMES, ICES, 2001). This workshop produced a detailed manual on plankton sample handling and analysis, which was used by all survey participants during the 2001 surveys. A subsequent exchange programme on plankton sorting, species identification and staging revealed some deficiencies, mainly in the species identification (see section 9.3). It should be noted that this was a small-scale exercise, and was mainly intended to highlight areas for further work rather than as an analysis exercise in itself. Based on these findings a further WKMHMES (ICES, 2004b) was held in 2003, which included, sample sorting, species identification and egg staging. The results of this workshop were very re-assuring and a further WKMHMES is planned in 2006, to train and evaluate the performance of the plankton analysts involved with the 2007 survey. The results of this workshop will be presented to ICES by the end of 2006.

The eggs and larvae of most of the species found in the area are well described by Russell, 1976. This book is well known and used by all the participants of the ICES tri-ennial surveys. It is generally regarded as the definitive work on the subject in this area.

Some difficulties do occur, particularly with the identification of fish eggs, which do not show great differences in their morphological features. In some instances it is even difficult to recognise differences between mackerel and horse mackerel eggs when the segmentation of the yolk is not distinct in the latter.

Some difficulties can occur with the identification of hake eggs, which are similar in size and appearance to several other species including mackerel, ling and megrim. The 'surface

adhesion test' (SAT) described by Porebski (1975) and Coombs (1994) does help to separate hake eggs from those of other species, although it does not always produce consistent results.

Within WGMEGS the eggs of mackerel are classified into one of five morphological stages (I, II, III, IV and V) (Lockwood *et al.*, 1981) (Figure 3), following the development criteria described for plaice (Simpson, 1959). For horse mackerel the description of stages is the same with the exception of stage V, which does not exist. Horse mackerel larvae hatch at the end of egg stage IV (Pipe and Walker, 1987).

For the estimation of daily egg production for both mackerel and horse mackerel, only the counts of stage I eggs are used. This is recognised as a conservative estimate of the total eggs spawned because of mortality which occurs during development. However until there is consistency in the identification of the other stages, between all countries, the other stages cannot be used for the estimation of mortality rates and backtracking to total eggs spawned.

8. Calculation of daily egg production

To convert abundance of eggs into daily egg production, data on the rate of development is required. For mackerel the relationship between egg development rate and temperature was described by Lockwood *et al*. (1977, 1981). This has been used as the basis for calculating daily egg production of stage I eggs on all the surveys from 1977. For horse mackerel similar egg development data are given by Pipe and Walker (1987) and have also been used for the calculation of stage I egg production since 1977. The formula for calculating the duration of stage I mackerel eggs from the sea temperature (T°C) is:

$$\text{Log}_e \text{ time (hours)} = -1.61 \log_e (T°C) + 7.76$$

For calculating the duration of stage I horse mackerel eggs the formula is:

$$\text{Log}_e \text{ time (hours)} = -1.608 \log_e (T°C) + 7.713$$

Work aimed at reviewing the existing calculation to estimate the rate of development is taking place (see Section 11). The temperature at 20 m depth (5m for the North Sea) should be used for the calculation of egg stage duration. If that is not available then the sub-surface temperature (ca. 3m) should be used.

9. Standardisation of plankton data analysis

Detailed procedures for the post analysis of egg abundance data to produce daily and, finally, annual egg production estimates are given below. This analysis has previously been carried out by two data coordinators (one for the western and one for the southern area), using data submitted in a standard format. **However, F. Burns, FRS, Aberdeen will manage the results for the entire 2007 survey.** This analysis is subject to examination and approval by the full working group and will ensure a standard approach and methodology. **It is recommended that participants will supply their plankton data either in a standard MS Excel spreadsheet or Paradox database input form, to be distributed by the data co-ordinator.**

To convert the number of eggs in each sample (or sub-sample) to the number of eggs per m^2, the following calculations are made. Firstly the volume of sea water filtered by the sampler during the haul is calculated.

$$\text{Volume filtered } (m^3) = \frac{\text{Flowmeter-revs x Aperture}}{\text{Flowmeter calibration}} \times \text{Efficiency Factor}$$

The number of egg m^{-2} is calculated from the formula:

$$\text{Eggs/m}^2 = \frac{\text{Eggs counted x Factor}}{\text{Volume Filtered } (m^3)} \times \text{Depth Sampled}$$

Where:

Flowmeter-revs.	=	Number of revolutions of the flow meter during tow
Aperture	=	The area of the mouth opening of the sampler in m^2
Flowmeter calibration	=	The number of flow meter revolutions per metre towed, obtained from the flume or sea calibration in free flow.
Eggs counted	=	Number of eggs in sub-sample
Factor	=	Raising factor from the sub-sample to the whole sample
Depth Sampled	=	The maximum depth of the sampler during the tow in metres
Efficiency Factor	=	The sampler efficiency from flume or towing tank calibration

Numbers of eggs per m^2 are raised to number per m^2 per day using development equation for both species in the following way:

For stage I **mackerel** eggs:

$$\text{Eggs/m}^2\text{/day} = 24 \times \text{Eggs/m}^2 \;/\; \exp\left[-1.61 \log_e (T°C) + 7.76\right]$$

For stage I **horse mackerel** eggs:

$$\text{Eggs/m}^2\text{/day} = 24 \times \text{Eggs/m}^2 \;/\; \exp\left[-1.608 \log_e (T°C) + 7.713\right]$$

Eggs/m^2/day are then raised to the area of the rectangle they represent. The rectangle values are summed to give numbers of stage 1 eggs per day over the survey area for each sampling period. Rectangle areas are calculated by each ½° row of latitude using the formula:

$$\text{Area (m}^2\text{)} = (\cos(\text{latitude}) \times 30 \times 1853.2) \times (30 \times 1853.2)$$

The next stages in the estimation of annual egg production are:

- Estimating the daily egg production for each survey period in turn
- Integrating the daily egg production histogram, to give annual egg production
- Calculating the variance of the estimate of annual egg production

The method was modified for use in the analysis of the 1995 survey data. It is fully described in Section 5.3.3 of the report of those surveys (ICES, 1996b). The same methods will be used for the analysis of the 2007 survey data. **It is recommended that the flowmeters and sampling devices deployed in the survey should be calibrated in terms of the volume of water filtered**. There are two aspects to calibration: The first requirement is to know and understand the relationship between flowmeter revolutions and distance travelled through the water. The second is to relate flowmeter revolutions, (whilst mounted *in-situ* in the aperture of a plankton sampler), to volume filtered by the sampler. The only way in which the second aspect can be accurately determined is to calibrate the flowmeter and sampler under controlled conditions in a circulating water channel or in a large towing tank. These facilities provide independent measures of water or towing speed and also enable water velocity to be measured extremely accurately at numerous positions across the sampler aperture (EU AIR CT94 1911). Such facilities are extremely expensive and alternative methods to calibrate flowmeters *in-situ* have been employed by various participants. This usually involves calibration at sea using a reference flowmeter mounted on the outside of the sampler and two tows in opposite directions to overcome the effects of tides or currents on ship and sampler speed through the water. Such calibrations will provide a crude estimate of volume filtered (under non-clogged

net conditions) but it must be remembered that there are differences in water velocity across the aperture of any sampler and that this water velocity profile may change as clogging of the net progresses. **However, it is recommended that participants conduct calibrations of their flowmeters *in-situ* over a range of towing speeds at least at the beginning and end of each survey.**

There is also a well defined protocol to interpolate egg densities for some unsampled rectangles which fulfil the following criteria. In order to qualify for an interpolated value an unsampled rectangle must have a minimum of two sampled rectangles immediately adjacent to it. Once qualified the sample values of all surrounding rectangles, both immediately adjacent and diagonally adjacent are used to calculate the interpolated value. The interpolated value is the arithmetic mean of all those surrounding rectangles. Once calculated, interpolated values are not used in order to calculate values for other unsampled rectangles, or to qualify those rectangles for interpolation. No values are to be extrapolated outside the sampled area. As a general recommendation, the cruise leader should try to avoid situations where interpolation is going to be problematic.

On some occasions and in particular where multiple observations are made within a rectangle sampling positions may fall on a dividing line between rectangles. When this occurs the sample is allocated to the rectangle to the north of the line of latitude and to the west of the line of longitude. **However, it must be remembered that sampling should be attempted at the centre of the designated rectangles wherever possible**.

10. Standardization of adult sampling – data collection and analysis

The working group prepared an updated protocol for the collection and analysis of adult parameters; fecundity, atresia, and parameters for condition and feeding in the case of horse mackerel. These are detailed in Sections 3.4 to 3.6 (ICES, 2003). The analysis of these samples, particularly with reference to fecundity estimation, the use of the Auto-diametric approach and oocyte diameter determination, were standardised at WKMHMES (ICES, 2004). This fecundity and atresia manual will again be updated at the next meeting of WKMHMES to be held at Cefas, Lowestoft, in October 2006.

10.1 Sampling for mackerel potential fecundity and atresia in the Western and Southern areas

Following WGMEGS decision to use only formaldehyde fixative (ICES, 2003) it will be possible to provide a unified sampling scheme for fecundity and atresia for use on the 2007 survey. **Following the experience of the 2004 survey the Auto-diametric method, (although useful where the fecundity sub-sample weight is not known) produces more variable fecundity data compared to the Gravimetric method (Hunter *et al.*, 1989). The Working Group recommends that the latter technique is used for the 2007 survey.** All changes in the sampling protocol and methods between the 2004 and 2007 surveys are given in table 10.1.1

Table 10.1.1. Changes for 2007 compared to 2004.

2004	2007
Auto-diametric method (Thorsen and Kjesbu 2001)to estimate fecundity was more variable than Gravimetric results	Gravimetric fecundity (F) method (Hunter *et al.* 1989). F = O * C*S where O= ovary weight ± 0.1g, C=count of vitellogenic follicles in the sub-sample weight S (± 0.0001g)
Fecundity sub-sample weight assumed equivalent to pipette displacement (0.026mg)	Tubes + fixative weighed prior to survey and after filling with sample. 4 replicates should be taken
No instruction to add sample into the tube	Ensure sub sample is covered by fixative
Non standardized staining of slides for mackerel atresia	Staining of slides stained by agreed protocol following October 2006 workshop.
No exchange of atresia samples for mackerel in the Southern area	Fecundity and atresia samples from Southern and Western spawning components shared between all Institutes participating in the analysis

Samples for estimation of mackerel potential fecundity and atresia will be mostly taken on vessels participating in the egg survey or from commercial fishing vessels. Recognising the constraints of the egg survey, cruise leaders should try to distribute trawl stations across the whole survey area. Details on the numbers, timing and spatial coverage of the samples required will be provided by participants of each relevant WGMEGS, planning working group (e.g. Tables 3.1.2 a-b, this report).

If the size range of fish is restricted in the catch the remaining sample quota should be taken from the more abundant classes to fill the weight classes in Table 10.1.2 below. In order not to concentrate the sampling on spawning fish it is preferable that trawling is not concentrated on the 200 metre depth contour but is adapted to fit in conveniently with the egg survey along the transects over the continental shelf. In 2007 Cefas will not be contributing towards the collection and analysis of mackerel fecundity and atresia so the samples will be redistributed to Norway, Scotland, and Spain. Ireland has been requested to take over allocation of samples that were previously processed by Cefas. Details of preparation for fecundity sampling at sea is shown in Table 10.1.3.

Table 10.1.2 Weight classes for sampling females of maturity stages 2–6 (Walsh *et al.,* 1990)for Potential fecundity and atresia

WEIGHT CATEGORY [G]	<250	251 – 400	401–550	>551	Total
NUMBER OF FISH	5	5	5	5	20

Table 10.1.3.

Protocol for processing and distribution of mackerel ovary sub-samples for either fecundity or atresia analysis, 2007.

Prior to cruise departure:

Norway (Maria Kruger Johnsen) will coordinate the analysis of mackerel fecundity samples and assign tube reference numbers to cruise leaders for labelling the Eppendorf tubes used on their cruises.

Coordinators to assign unique codes to each participating cruise.

Procure Eppendorf type tubes and place in suitable racks.

Attach a spot label to the Eppendorf lid and add 1.2 ml of 3.6% formaldehyde buffered with 0.1M sodium phosphate (referred to below as 'fixative') to each tube using a dispenser. The label should contain 3 alpha or numeric characters for a primary key in the fecundity database. Prepare 4 replicates for each tube label and colour the replicate white, red, blue and green respectively. Measure and record the weight of each tube including fixative (±0.0001 g) using the tube label code and colour for reference.

Procure sample bottles for the remaining ovary tissue should have parallel walls and without a restricted neck opening (otherwise we cannot extract the ovary without cutting of the jar top). The largest ovaries will require 250 ml sample bottles but in many cases a 100 ml or smaller capacity jar will be adequate. Label the bottle with the Eppendorf code and cruise.

Procure 25–50 µl capillary pipettes and test performance of the pipette by taking 25 µl water samples and weighing the dispensed fluid.

Procedures to follow at sea to collect samples and for sample analysis in the laboratory are shown in Tables 10.1.4 and 10.1.5 respectively. In order to compare estimates of fecundity made by each country 100 samples should be analyzed by all participants but, for the remainder, at least 2 of the quadruplicate samples should be analyzed. Overall targets for estimating realized fecundity are shown in Table 10.1.6. Provisional reporting of estimates for potential fecundity and atresia are required for the 2007 Mackerel Horse Mackerel Working group in September and final results for WGMEGS in the spring of 2008. If the participants or fecundity coordinator are not certain of the data quality the concern should be passed on to the Working Group Coordinator (Findlay Burns).

Table 10.1.4. Adult mackerel sampling programme Flow diagram.

Mackerel and Horse Mackerel Egg Survey 2007

MACKEREL SAMPLING

Estimation of potential fecundity in pre-spawning fish and the estimation of atresia for realised fecundity

Sampling at Sea (for details on cruises see Table 2.2)

Area	Sampling by	Period/samples 1	2	3	4	5	total no. of samples	
Southern	POR/IPIMAR	40					40	
	ESP/IEO	80	20				100	
	ESP/AZTI			40			40	**180**
Western	ESP/AZTI	60		40			100	
	GER/BFA Fi	80	40				120	
	IRL/MI	120				100	220	
	ESP/IEO	20	60				80	
	SCO/FRS		100		60		160	
	NED/IMARES			60	60		120	
	NOR/IMR			40	20		60	**860**

(a) obtain length, weight and maturity distribution of larger sample (>100 fish) ⇢ length, maturity, weight distribution

(b) measure, weigh (total), gut (***carefully!***), select mature, late pre-spawning, spawning or spent (stages 3-6) females (20 per station in 4 weight classes [<250/-400/-550/>550 g]) ⇢ lenght, weight (total), sex, maturity

(c) remove ovaries ***undamaged!***, weigh ovaries take 4 parallel pipette samples of one ovary **(25 µl)** in pre-filled Eppendorf tubes and put the other ovary in formalin jars, weigh liver, guts (including contents) and carcass ⇢ ovary, liver, guts, carcass weight

(d) send on one of every parallel sample to Vigo, Aberdeen, Bergen, Galway

→ histological analysis at IEO, FRS, IMR, MI ⇢ potential fecundity, atresia, realised fecundity

For the desired temporal and spatial distribution of the fecundity samples please refer to Table 3.1.2; for further instructions please refer to paragraphs 3.1.

Collection of samples for genetic population analysis (refering to 3.4)

(a) Collect a larger sample of mackerel

(b) Cut a tissue sample (about 10*5*5mm) of the muscle, put each tissue sample in a 1.5ml Eppendorf tube in absolute alcohole

(c) Freeze 50 fish individually in plastic bags

(d) Send the Eppendorf tubes and the frozen fish to IMR (Bergen)

Sample analysis targets for Ireland, Norway, Scotland and Spain participating in estimation of mackerel fecundity and atresia 2007. Each country carrying out the various is responsible for distributing their sample collection alternately to the countries carrying out the fecundity analysis. Norway will coordinate mackerel fecundity sample analysis in 2007.

Table 10.1.5.

Processing ovary and pipette samples on return from sea

After a minimum of 1 week fixation, cut cross-sections 4 mm thick from the ovary not previously sampled and place them in labelled histological cassette. The cassettes should be engraved with an indelible label corresponding to each replicate set of Eppendorf tubes. Cefas can provide engraved cassettes under contract but procurement locally would be more convenient.

Cover the cassettes with fixative or 70% ethanol and pack them in a leak proof bottle. Pack the consignments for each country with a maximum volume of 1000 ml solution in each package. On the outer cover of the package indicate the volume of fixative and that it is within the limits for unclassified transport. Retain the remaining ovary until analysis of data is completed at the 2008 WGMEGS.

Record weight of the Eppendorf tubes, fixative and added tissue 1 week and 4 weeks after return to estimate quantity of tissue taken by the pipette.

Table 10.1.6.

PROTOCOL FOR LABORATORY ANALYSIS OF MACKEREL FECUNDITY SAMPLES		
TASKS	COUNTRIES	TIMING FOR WORK COMPLETION
Training coordinated by Cefas	England, Ireland, Norway, Scotland and Spain	October Workshop
Examine Eppendorf samples to identify and select pre-spawning fish based on the absence of spawning markers such as hydrated follicles or <5 POF type structures in the sample. Apply image analysis protocol based on the fecundity manual to determine fecundity (number of follicles >0.185mm) using the gravimetric method ((Hunter *et al.,* 1989). The outputs from the image analysis macro should be configured to fill all the fields in the Gravimetric sampling table of the fecundity database. The fecundity manual will be revised during the 2006 Workshop based on procedures developed during the 2004 survey. Ensure that at least 100 tube samples are analysed by all institutes for quality control and that each fish has at least 2 replicate fecundity estimates. Ovaries that have either commenced the annual spawning or are recently spent should be processed to estimate atresia below.	Ireland? Norway Scotland and Spain	Provisional results completed for 2007 Assessment Working in September. Completed results for WGMEGS 2008
Prepare resin sections from all mature fish identified as either in spawning or spent to determine the intensity and prevalence of atresia. Each Institute will process ¼ of the atresia samples.		
Determine atresia in mature fish identified as either spawning or spent above by Stereometric analysis using the protocol in the fecundity manual. Configure the macro used to process the atresia analysis results to complete all the columns in the histology table of the fecundity database.	All participating countries	

10.2 Sampling for horse mackerel fecundity in the Western area

Following the experience of the 2004 survey and discussion at the Vigo planning meeting, 2006 the following changes have been recommended for the 2007 survey. **In this context the Auto-diametric method, although useful where the fecundity sub-sample weight is not known, produces more variable fecundity data especially in the case of horse mackerel compared to the Gravimetric method (Hunter *et al.*, 1989). The Working Group recommends that the latter technique is used for the 2007 survey.** All changes in the sampling protocol and methods between the 2004 and 2007 surveys are given in table 10.2.1

Table 10.2.1. Changes for 2007 compared to 2004.

2004	2007
Auto-diametric method (Thorsen and Kjesbu, 2001)to estimate fecundity was unreliable for horse mackerel	Gravimetric fecundity (F) method (Hunter *et al.*, 1989). F = O * C*S where O= ovary weight ± 0.1g, C=count of vitellogenic follicles in the sub-sample weight S (± 0.0001g)
Fecundity sub-sample weight assumed equivalent to pipette displacement (0.026mg)	Tubes + fixative weighed prior to survey and after filling with sample. 4 replicates should be taken
No instruction to add sample into the tube	Ensure sub sample is covered by fixative
Lipid content determined on whole body homogenate after solvent extraction and gravimetric determination of extracted fat carried out by all countries collecting horse mackerel	Fat content determined using a fat meter at IMARES. Fish sampled for fecundity (table 3.2.2) to be frozen and sent to IMARES (after consultation) for lipid analysis.
Lipid levels determined in the Southern and Western spawning components	Lipid levels determined in early maturing fish collected from commercial sources in October and November 2006 and from mature fish caught in the Western area surveys from March to July.
Standing stock of fecundity determined in fish selected as pre-spawning from collections made in the Southern and Western spawning areas	Standing stock of fecundity determined in mature fish collections made in the Southern and Western spawning areas Table 3.2.2 a-b by Ireland, Netherlands Norway and IEO Spain. This data will provide information on trends in ovary weight, batch fecundity, spawning fraction and residual standing stock of fecundity.

In 2007 horse mackerel will be collected from the Southern and Western spawning components selecting fish in maturity stages 3–6 fish > 25 cm collected on trawl hauls spread both temporally and spatially throughout the survey.

Protocols for the 2007 horse mackerel sampling preparations, sampling at sea and analysis in the laboratory and analysis are shown in Tables 10.2.2–10.2.4 respectively. **Cindy Van Damme from the Netherlands** will coordinate the analysis of horse mackerel fecundity samples. 50 samples will be analysed by all 4 countries for quality assurance but at least two sub-samples should be analysed for all the remaining fish.

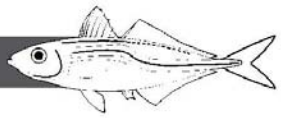
Table 10.2.2.

Protocol for processing and distribution of mackerel ovary sub-samples for either fecundity or atresia analysis.
Prior to cruise departure. **Cindy Van Damme (Netherlands)** will coordinate the analysis of horse mackerel fecundity sample and assign tube reference numbers to cruise leaders for labelling the Eppendorf tubes used on their cruises. Procure Eppendorf type tubes and place in suitable racks. Attach a spot label to the Eppendorf lid and add 1.2 ml of 3.6% formaldehyde buffered with 0.1M sodium phosphate (referred to below as 'fixative') to each tube using a dispenser. The label should contain 3 alpha or numeric characters for a primary key in the fecundity database. Prepare 4 replicates for each tube label and colour the replicate red, blue and green respectively. Measure and record the weight of each tube including fixative (±0.0001 g) using the tube label code and colour for reference. Procure 25–50 µl capillary pipettes and test performance of the pipette by taking 25 µl water samples and weighing the dispensed fluid.

Table 10.2.3. Flow chart for selecting and processing horse mackerel samples.

Mackerel and Horse Mackerel Egg Survey 2007

HORSE MACKEREL SAMPLING



① Estimation of lipid content in pre-spawning fish

Market sampling

Area	Sampling by	Month/samples 10	11	12/06	01/07	02	03	total no. of samples
Western	NED	50	50	50	50	50	50	300

a) obtain length distribution of larger sample ---------- ► length distribution

b) (thaw if frozen), weigh (total), gut select mature, pre-spawning females ---------- ► lenght, weight (total), sex, maturity

c) homogenize carcass and organs together, analyze fat content per dry weight ---------- ► fat content, dry weight

or (depending on availability)*

use a fat-meter to analyze the fat content ---------- ► fat content

② Estimation of standing stock fecundity and lipid content in relation to spawning status

Sampling at Sea (for details on cruises see Table 2.2)

Stock Comp.	Sampling by	Period/samples 1	2	3	4	5	total no. of samples	
Southern	POR/IPIMAR	40					40	
Western	ESP/AZTI	30		40			70	
	GER/BFA Fi	40	20				60	
	IRL/MI	60				50	110	
	ESP/IEO	50	90				140	
	SCO/FRS		50		30		80	
	NED/IMARES			30	30		60	
	NOR/IMR			20	10		30	500

a) obtain length distribution of larger sample (>100 fish) ---------- ► length distribution

b) measure, weigh (total), gut, select randomly mature females >= 25cm, weigh (gonad, carcass), determine stomach fullness ---------- ► lenght, weight (total, gonad, carcass, [gut]), sex, maturity (1-6, Walsh scale), stomach fullness (1-4: empty, filled, full, almost bursting)

c) take 4 parallel pipette samples of ovary **(25µl)** in pre-filled Eppendorf tubes

e) send on one of every parallel sample to Vigo, IJmuiden, Bergen, Galway

histological analysis at IEO, IMARES, IMR, MI ---------- ► vitellogenic oocyte frequency presence of POFs/atretic oocytes

* *fish can be frozen (carcass and organs together!) between these two steps for further processing but keep in mind that pipette samples and frozen fish needs the same indication for later identification*

d) (thaw), homogenize carcass and organs together, analyze fat content per dry weight **at sampling lab** to avoid transfers (IPIMAR, IEO, MI, FRS, IMARES, IMR, BFAFi) ----► fat content, dry weight

or (depending on availability)*

send the frozen sample to IMARES for analyzing the samples with a fat-meter ----------► fat content

* will be clarified Oct 2006 (Fecundity Analysis Workshop)

For the desired temporal and spatial distribution of the fecundity samples please refer to Table 3.2.2; for further instructions please refer to paragraph3.2.

Table 10.2.4

PROTOCOL FOR LABORATORY ANALYSIS OF HORSE MACKEREL		
TASKS	COUNTRIES	TIMING FOR WORK COMPLETION
Training coordinated by Cefas	Ireland, Netherlands Norway and IEO Spain	October Workshop
Examine Eppendorf samples to identify and note presence or absence of spawning markers such as hydrated follicles or <5 POF type structures in the sample. Apply image analysis protocol based on the fecundity manual to determine follicle size frequency distribution. The threshold to identify the standing stock of fecundity will be determined for the 2006 Fecundity Workshop. Use the using the gravimetric method ((Hunter *et al.,* 1989). The fecundity manual will be revised during the 2006 Workshop based on procedures developed during the 2004 survey. Ensure that at least 100 tube samples are analysed by all Institutes for quality control and each fish has at least 2 replicate fecundity estimates.	Ireland, Netherlands Norway and IEO Spain All participating countries	Completed results for WGMEGS 2008

10.3 Methodology for taking samples from mackerel and horse mackerel ovaries.

10.3.1 Use of a capillary pipette to take fecundity samples from horse mackerel or mackerel ovaries and associated equipment

Table 10.3.1. Details of equipment and suppliers.

EQUIPMENT	CATALOGUE REFERENCE	SUPPLIER
Transferpettor capillary	307/5502/05	VWR International Dublin Critical Environment Business City west Business Campus Naas Road Dublin 22 Ireland Tel: ++3531 4660111 Fax: ++3531 4660380
Transferpettor capillary	307/5502/15	VMX as above
Eppendorf type tubes	LA-MCT-200-C	Biohit Ltd, Unit 1 Barton Hill Torquay, Devon, TQ2 8JG England Tel. O800 685 4631 email sales@biohit.demon.co.uk
Racks for tubes	LL-9200–0	Biohit above
Laser tough spots, 0.375”	SPOT-1000	Web Scientific Ltd, Business and Technology, Centre Radway Green Venture Park, Radway Green, Crewe, Cheshire CW2 5PR Tel +44 (0) 1270 875172Fax +44 (0) 1270 878186 Website www.webscientific.co.uk

Method

The capillary pipette will remove an ovary sample of standard weight CV 3% from a stage 3 to 5 ovary but not stage 6. In the case of Stage 4 running ovaries squeeze out all the loose eggs before taking the sample. In the case of stage 6 ovaries take a small piece with forceps from the centre of the ovary similar to that removed by the pipette. Repeat for each of the tube replicates.

Operation

- In the case of mackerel take the replicate samples out of the rear half of one of the ovaries leaving the remaining ovary intact for taking histology samples after fixing for 1 week.
- Make a small hole in the ovary tunica
- Depress the piston to the bottom of the capillary
- Push the tool through the hole in the ovary into the centre of ovary
- With the pipette end held within the ovary pull the plunger wire out of the tube until the base of the piston reaches the first blue line on the capillary (see below).
- Push the sample out of the capillary into a 2.5 ml Eppendorf tube containing 1.2 ml 3.6% formaldehyde buffered with 0.1 M sodium phosphate.
- Take 3 more replicate samples as above
- After each station wash the capillary and piston.

Place the other unsampled ovary in a bottle for atresia estimation (mackerel only)

The Piston can be used 300 + times but eventually piston ware causes a drop in suction power and it must cut off and replaced by pushing the plunger wire into a new piston held in the assembly plate. The amount of sample can be controlled by the distance the piston is pulled up the capillary tube. A second blue line indicates the distance to pull out the piston for twice the standard sample volume.

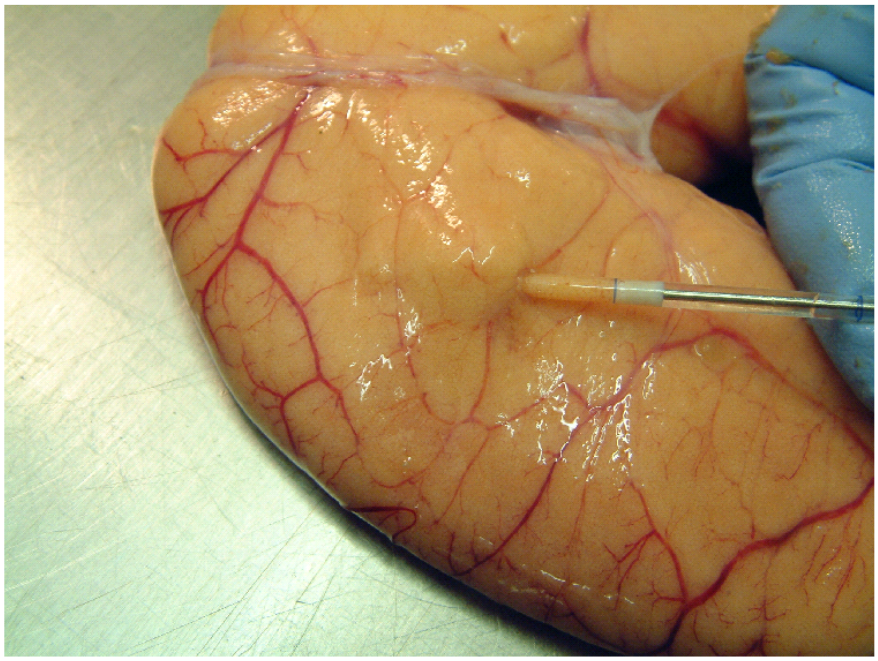


Figure 10.3.1. Method to use a capillary pipette to remove an ovary sample.

Push the plunger to the bottom of the glass tube and then push the tube into the hole previously made in the tunica. Pull up the plunger until the sample reaches the lowest line on the glass pipette (see picture). This will provide a sample of 26 mg of tissue. Ensure there are no air pockets in the sample sucked from the ovary and that it is expelled into the 3.6% formaldehyde solution held in the tube. Ovaries that are nearly spent will not readily provide samples and in these cases use forceps to remove a similar sized sample from the centre of the ovary. Before the cruise ensure operators are familiar with the pipette operation by dispensing water into a container weighed to ±0.0001g

Figure 10.3.2. Picture of a rack holding Eppendorf like tubes for 10 fish with 3 replicates identified by spot labels on the lids. During storage a lid fits on top of the rack to keep the tubes in order during transport.

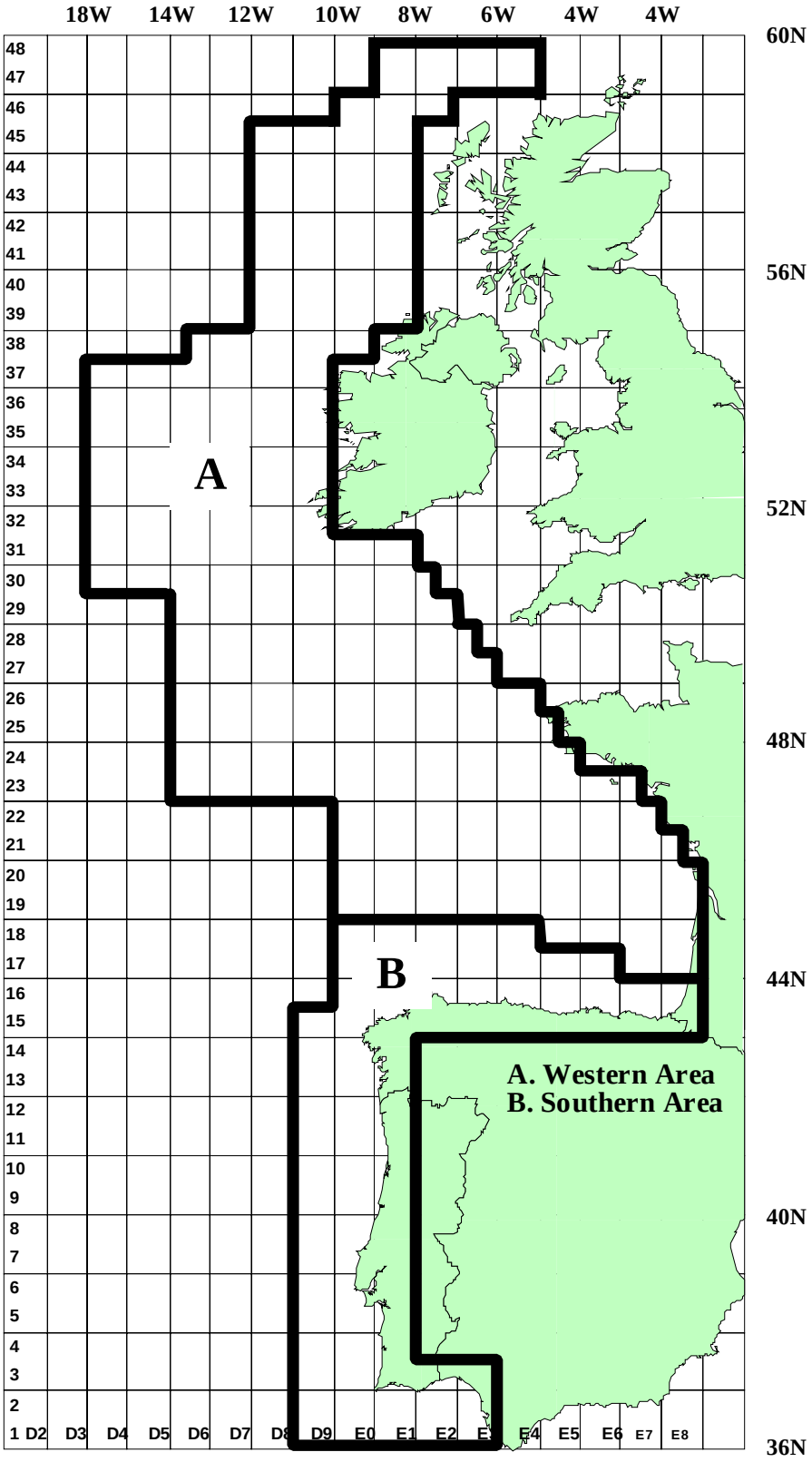


Figure.10.3.3. Core sampling areas for mackerel and horse mackerel eggs in the western and southern areas for 2004. Sampling will be continued outside these limits on surveys based on the adaptive sampling guidelines.

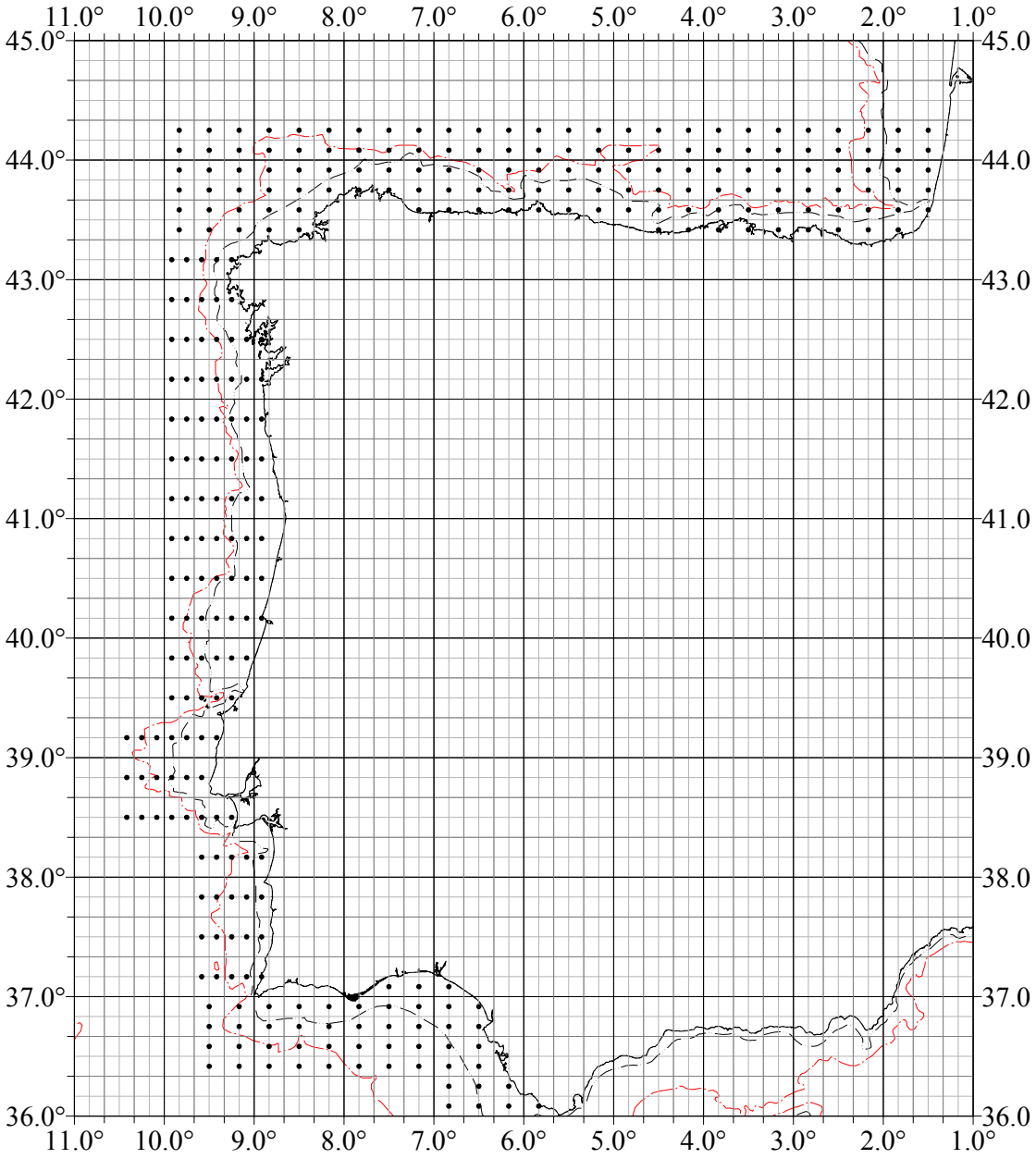


Figure 10.3.4. Provisional station location for mackerel and horse mackerel egg surveys in the southern area in 2004. Offshore boundaries will be based on two consecutive zero rectangles.

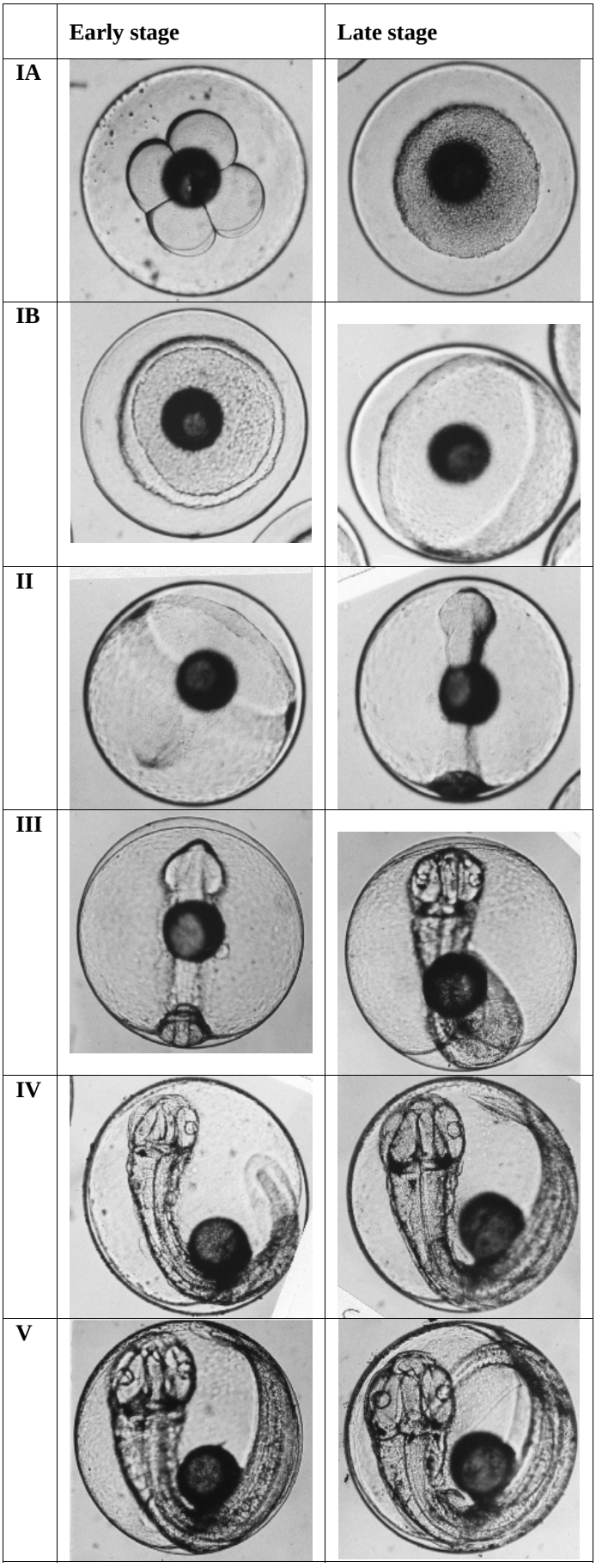
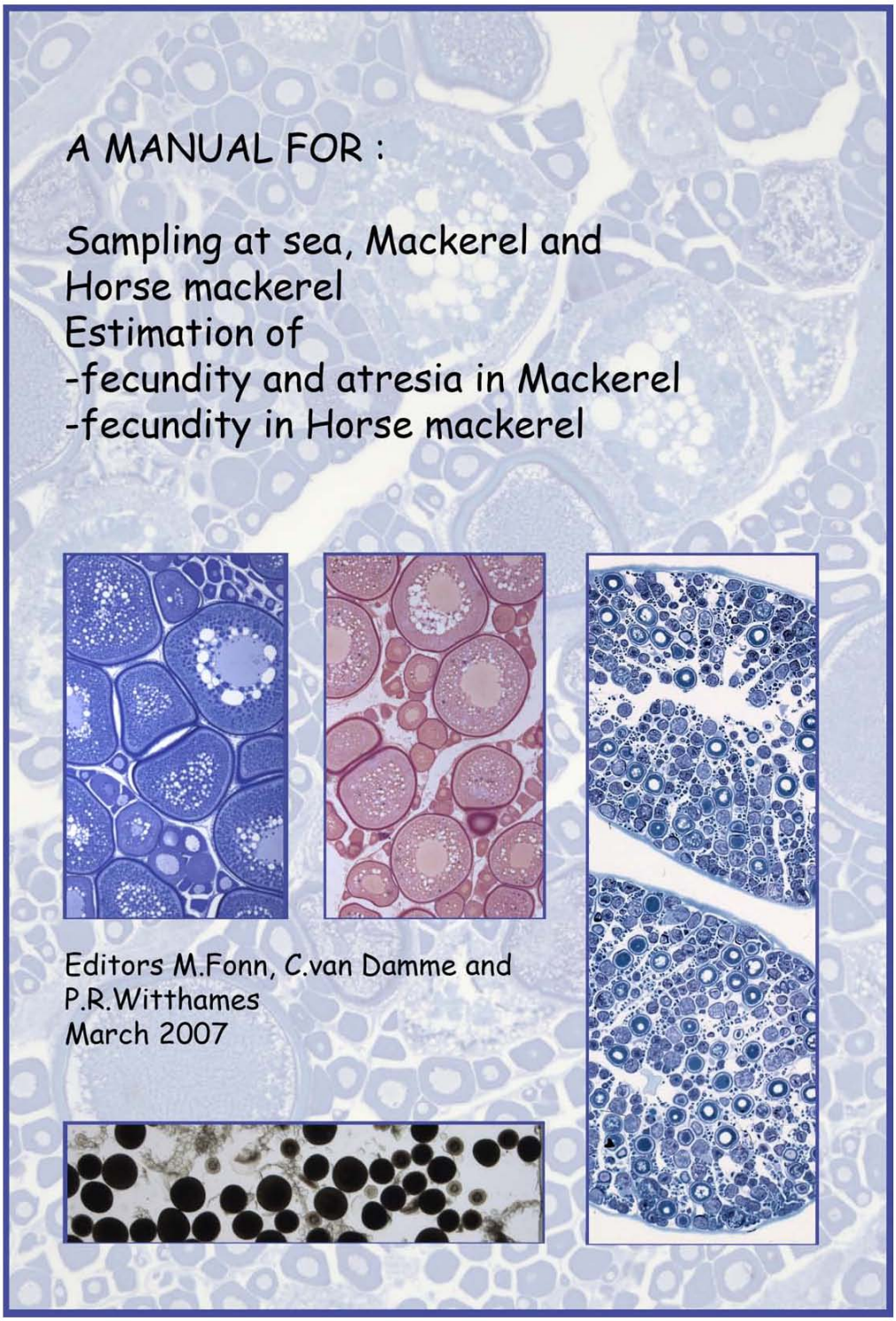


Figure 10.3.5. Mackerel eggs at the beginning and end of the six development stages.

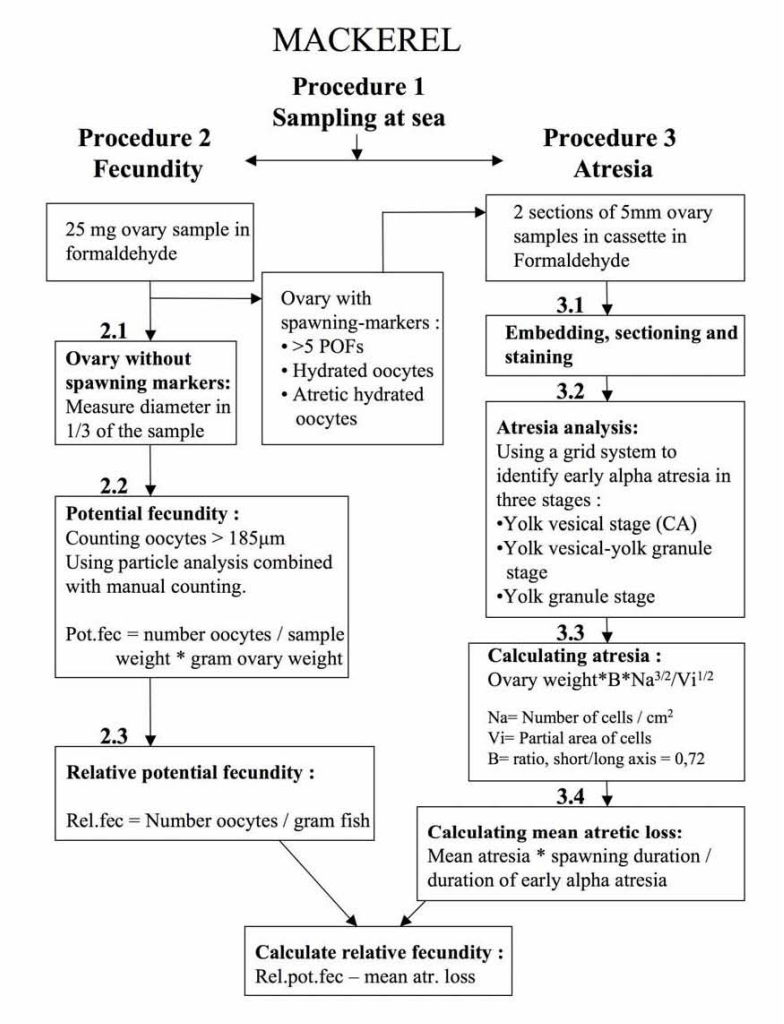
Annex 6: An adult fish sampling and fecundity estimation manual for sampling at sea, Mackerel and Horse mackerel



Contents

Changes in fecundity and atresia estimation methods for Mackerel and Horse mackerel since 2001 (Version 1 of the manual Witthames, 2001).

2000	2006
Mackerel	
On board ovaries were collected whole and fixed in Gilson's fluid (for potential fecundity) and formaldehyde solution (for assessing spawning status and atresia)	On board ovaries are weighed and pipette subsamples of known volume and weight taken and fixed in formaldehyde solution
Potential fecundity Count follicles > 130 µm after Gilson digestion	Gravimetric fecundity estimation Sub samples preserved in 3.6% buffered formaldehyde. F = O * C * S (F = fecundity, O = Ovary weight, C = count follicles > 185 µm in subsample, S = subsample weight) (Hunter *et al.* 1989)
Atresia Stereometric method	Stereometric method
PAS stained sections	H&E -PAS – Toluidine blue
Horse mackerel	
Potential fecundity Stereometric method	Gravimetric fecundity estimation Sub samples preserved in 3.6% buffered formaldehyde. F = O * C * S (F = fecundity, O = Ovary weight, C = count follicles > 175 µm in subsample, S = subsample weight) (Hunter *et al.* 1989)


MACKEREL
Procedure 1
Sampling at sea
Procedure 2
Fecundity
Procedure 3
Atresia
25 mg ovary sample in formaldehyde
2.1
Ovary without spawning markers: Measure diameter in 1/3 of the sample
2.2
Potential fecundity :
Counting oocytes > 185µm
Using particle analysis combined with manual counting.
Pot.fec = number oocytes / sample weight * gram ovary weight
2.3
Relative potential fecundity :
Rel.fec = Number oocytes / gram fish
Ovary with spawning-markers :
• >5 POFs
• Hydrated oocytes
• Atretic hydrated oocytes
2 sections of 5mm ovary samples in cassette in Formaldehyde
3.1
Embedding, sectioning and staining
3.2
Atresia analysis:
Using a grid system to identify early alpha atresia in three stages :
•Yolk vesical stage (CA)
•Yolk vesical-yolk granule stage
•Yolk granule stage
3.3
Calculating atresia :
Ovary weight*B*Na3/2/Vi1/2
Na= Number of cells / cm2
Vi= Partial area of cells
B= ratio, short/long axis = 0,72
3.4
Calculating mean atretic loss:
Mean atresia * spawning duration / duration of early alpha atresia
Calculate relative fecundity :
Rel.pot.fec – mean atr. loss

3

Procedure 1
Mackerel sampling procedure at sea 2007

Before the cruise :
Fill the labelled 2,5 ml eppendorf tubes with 1,2 ml of 3,6% buffered (sodium phosphate) formaldehyde (see excel-file : Buffered formaldehyde) and measure the weight (± 0,0001 g).

During the cruise :
Measure the weight of the whole catch and select a subsample of 100 fish and measure the total weight of the subsample.
Measure total length, weight, maturity (Walsh scale) and sex of each fish in the subsample.

Select females in maturity stages 3-6 (see table 3.1.2 WGMEGS 2006) from the subsample of 100 for DNA, fecundity and atresia analysis. Be sure to divide the females equally into the 4 weight categories : < 250g, 251-400g, 401-550g and >550g.
Measurements :

- Total length
- Total weight
- Maturity
- Otoliths
- Weigth of gut, ovary and liver

DNA sampling:

- Cut a tissue sample (about 10*5*5 mm) of the muscle from the tick muscle behind the head, put each tissue sample in a 1,5 ml eppendorf tube in absolute alcohole.

Fecundity sampling :

- From one half of the ovary take 4 samples of each 25µl with a pipette and immediately put each sample in induvidual coded eppendorf tubes.

Atresia sampling :

- For atresia : Place the other half of the ovary in a bottle filled with 3,6 % buffered (sodium phosphate) formaldehyde.
- Make sure that all the ovary samples are covered with formaldehyde

Parasites sampling :
Select new fish from the catch to freeze individually in plastic bags. The total number of fish should be 50 for each cruise, following table 1.

Table 1.

Area	Sampling by	Period / number of fish				
		1	2	3	4	5
Southern	POR/IPMAR	50				
	ESP/IEO	50	50			
	ESP/AZTI			50		
Western	GER/BFA Fi	50				
	IRL/MI					50
	SCO/FRS		50			
	NED/IMARES			50		
	NOR/IMR				50	

After the cruise :
Measure the weight of the eppendorf tubes containing the sample.
From the fixed half ovary, cut two 5mm thick slices and put them in a labelled cassette. If the ovary is very big you may have to use 2 cassettes. Separate the cassettes into 4 color coded bottles filled with 70% ethanol. Send the cassettes and eppendorf samples for analysis to the different institutes referring to table 2.

Table 2.

Colour code	Country	Institute and address	Responsible person
Blue	Norway	IMR, Nordnesgaten 50,PB 1870, 5817 Bergen-Nordnes, Norway	Merete Fonn
Red	Ireland	MI, Rinville, Oranmore, Co.Galway, Ireland	Brendan O'Hea
Yellow	Scotland	FRS, Marine Laboratory, Victoria Road, Torry, Aberdeen, AB9 8DB, Scotland	Finlay Burns
White- Even numbers	Spain	IEO, Apartado 1552, Cabo Estay, Canido, 36280-VIGO (Pontevedra), Spain	Jose Ramon Perez
White- Uneven numbers	Spain	AZTI, Foundation Herrera Kaia, Portualde z/ g20110 Pasaia, Basque Country, Spain	Maria Santos

Procedure 2
Fecundity whole mount analysis procedure for mackerel

2.1 Spawning markers

Transfer the unstained sample to a tray and try to separate the oocytes.
Under the microscope check for spawning markers, if there are hydrated oocytes, atretic hydrated oocytes or ≥ 5 POFs, the sample should not be analysed for fecundity. For mackerel these sample should be analysed for atresia.

2.2 Potential fecundity

Note the present or absence of atretic oocytes.
Count all the oocytes >185µm in the sample.
Distribute the sample randomly in the tray and measure the diameter for size distribution for all oocytes >185µm in 1/3 of the sample.

Potential fecundity :
Pot.fec. = number of oocytes / weight of the pipette sample * ovary weight

2.3 Relative potential fecundity

Relative potential fecundity :
Rel.pot.fec. = Pot. fec. / total fish weight

Procedure 3
Atresia analysis for mackerel

3.1 Embedding, sectioning and staining

Preparing resin blocks

Use the two 5 mm sections in the cassettes, following these steps :

Step	Infiltration solution	Duration	Process temperature
1	90% ethanol	2 hours	Room temperature
2	Pour out the liquid and add fresh 90% ethanol	1 hour	Room temperature
3	90% ethanol + Technovit 7100 (1:1 ratio) prepared by diluting Technovit 7100 (from used in steps 4).	2 hours or overnight	Store cool (+5°C) after the orbital shaker
4	Replace the liquid with Technovit 7100 (from step 5).	2-3 days	Store cool (+5°C) after the orbital shaker
5	Replace the liquid with freshly prepared Technovit 7100.	1 day	Store cool (+5°C) after the orbital shaker
6	Transfer the sections from the cassettes to the moulds. Store tissue with catalyzed resin in moulds in the freezer.	2-3 hours or overnight	-6°C
7	Polymerise by adding Technovit 7100: hardener (15:1) in the freezer.	2 hours	-6°C
8	Leave overnight	overnight	Room temperature
9	Block up using Technovit 3040.	15 minutes	Room temperature

Store the blocks in a box containing 70% glycerol.

Disposal of waste resin (in the fume cupboard)

After step 3 the 1:1 resin mix should be put in an aluminium tray and left in the fume cupboard over a few days to allow the EMS to evaporate from the resin. Use about 1 g hardener to 100g resin to polymerise and wrap the block in a poly bag for disposal. Caution the reaction is exothermic and potentially hazardous if too much hardener is added.

Sectioning the blocks

Use a microtome to cut 5 µm sections and dry at 100°C.

Staining the sections

Recipe 2 % Toluidine blue

2 % Toluidine blue and 1 % Sodium tetraborat (Borax). The borax is dissolved in the distilled water and then the dye added under constant stirring. Filter the solution before use.

For individual slides: Cover the section with a few drops of 2 % Toluidine blue and pour the excess back in the bottle and rinse the section with hot (60°C) tap water for 20 seconds. Dry on a 60°C hot plate. Cover the section with a cover slip using two drops of mountex.

3.2 Atresia analysis

Classification of atretic oocytes is based mainly on the breakdown of the zona pellucida, but other changes also occur. Subdivision of the alpha stage into early alpha and late alpha atresia is based on the size of breaks and position of the zona pellucida. If any nick or breakdown in the zona pellucida is observed and if the breaks are smaller than twice the width of the zona pellucida thickness, the oocyte is classed as early alpha atretic. If the zona pellucida has breaks more than twice its width and the fragments are displaced inwards from the outer follicle boundary the ooctye is classed as late alpha. After the zona pellucida has disappeared the breakdown progresses from the alpha into the beta stage and the oocyte is now much reduced in size, highly vacuolated and with no yolk contents visible.
For mackerel we score only the early alpha atretic stage.

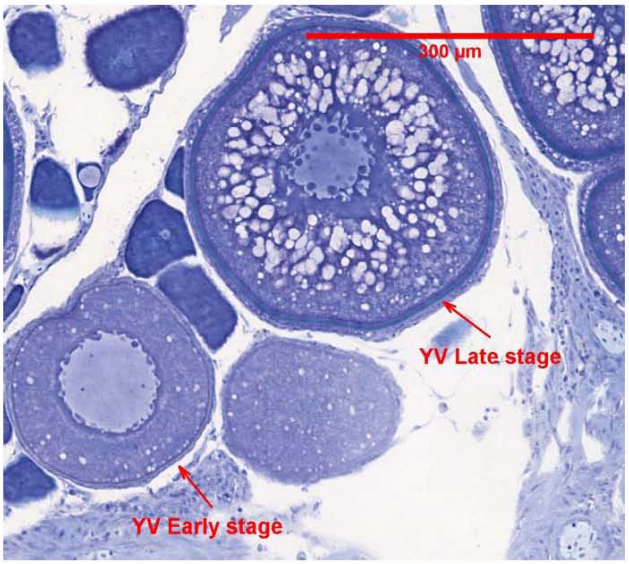
The oocytes are divided into 3 different stages :

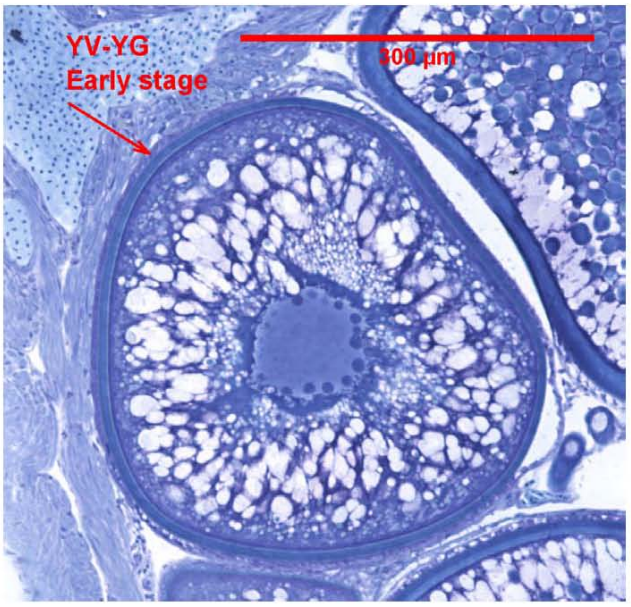
YV (yolk vesical stage) : arises from the smallest vitellogenic oocytes making up the potential fecundity ranging in size from 175 (appearance of corticale alveolie) to 325µm when a complete ring of vacuoles extends throughout the oocyte cytoplasm .

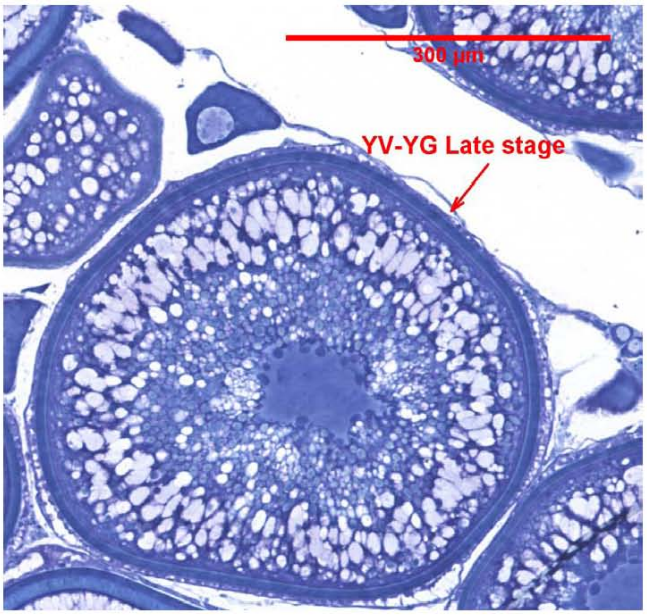
YV-YG (yolk vesical to yolk granule stage) : the oocytes range in size from 325 to 525µm and contain yolk granules that slowly enlarge and start to fill the cytoplasm.

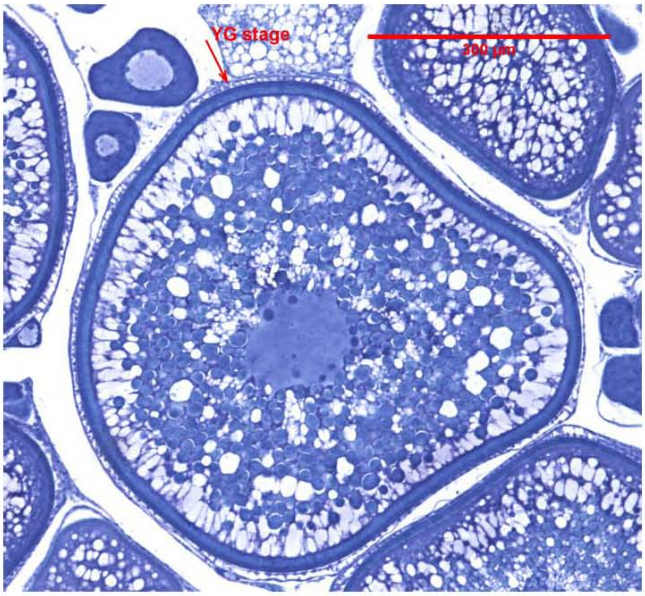
YG (yolk granules) : yolk granules occur throughout the full depth of the cytoplasm. This stage also includes the largest oocytes making up the potential fecundity up to oil droplet formation and the migratory nucleus stage.

Pictures of the 3 different stages in normal oocytes stained with toluidine blue.

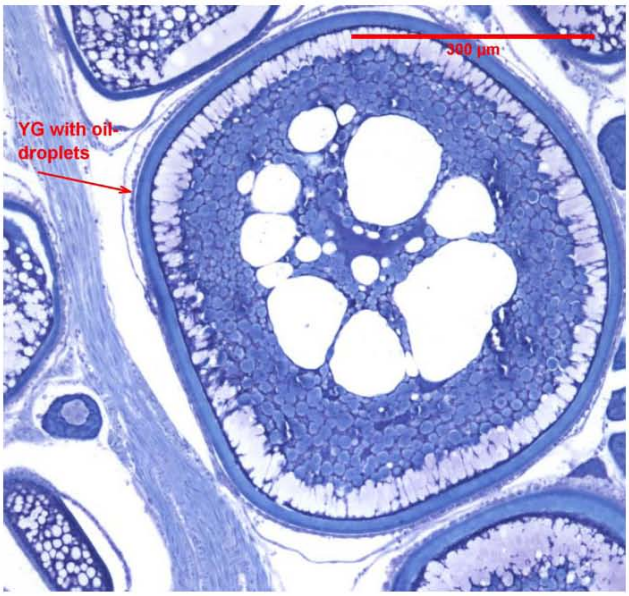


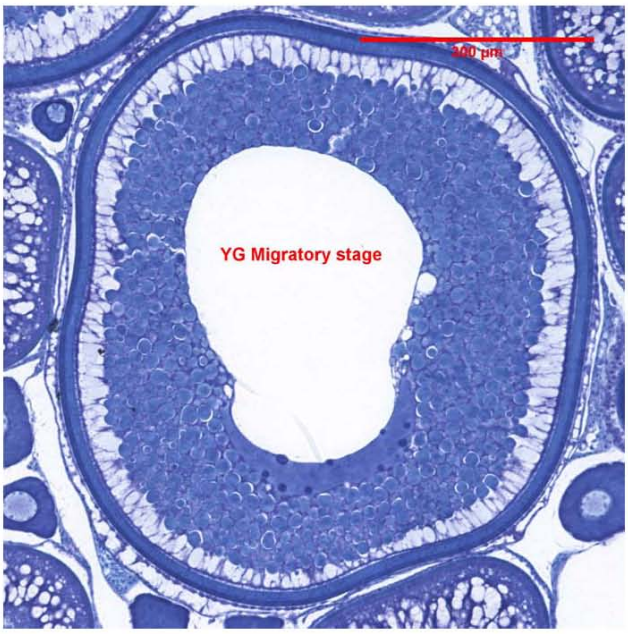




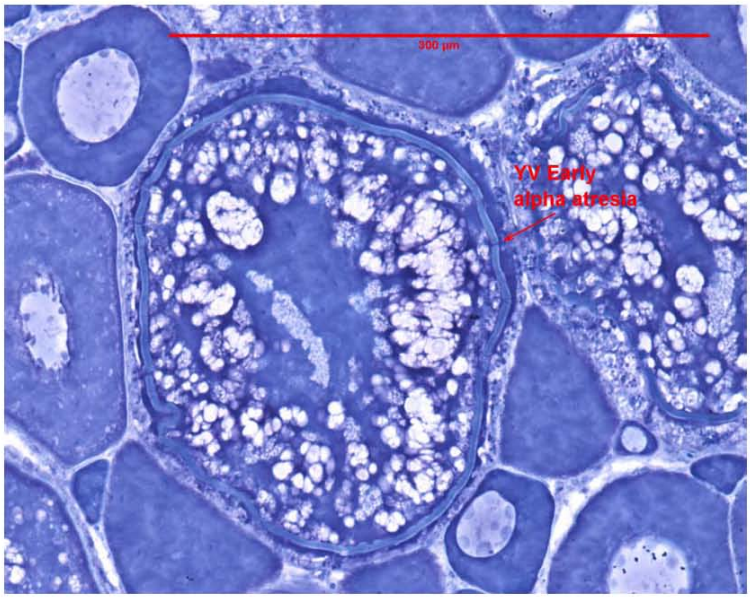


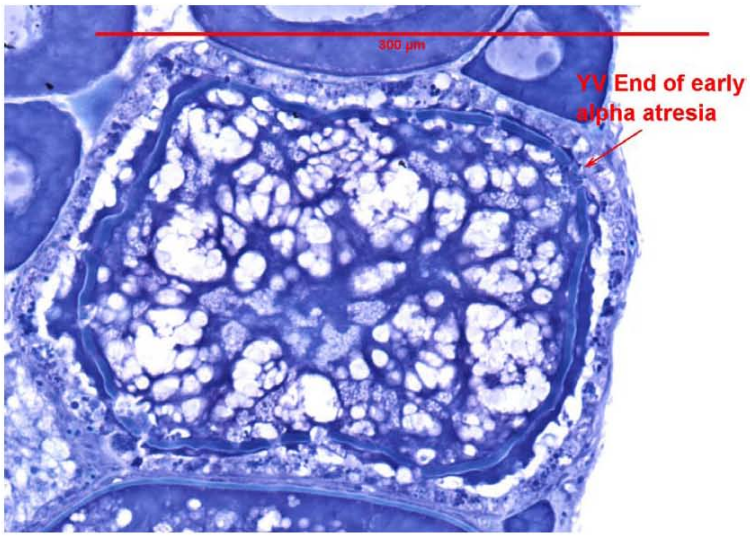
10

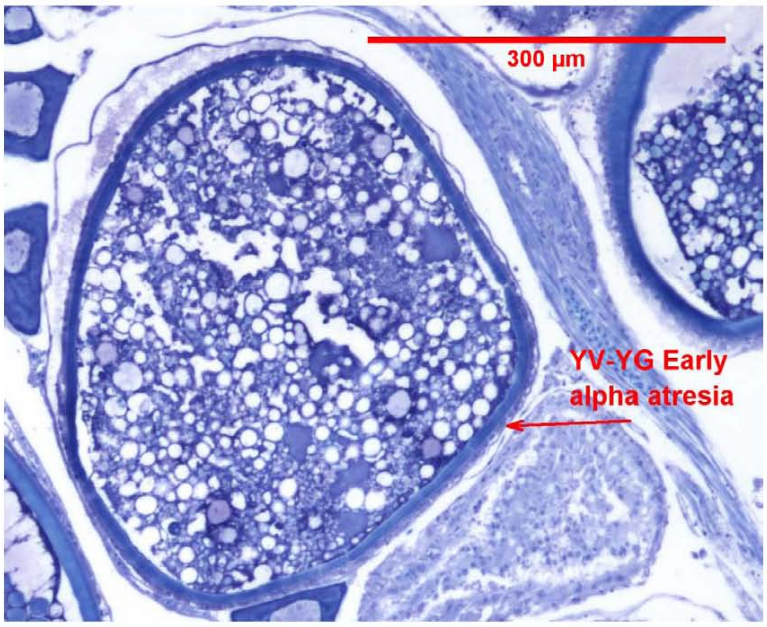

300 µm
YG with oil droplets

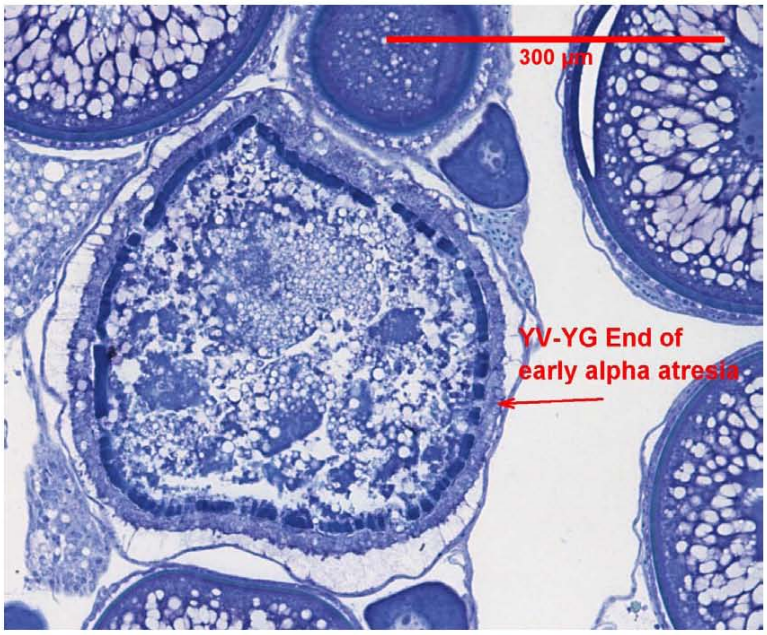

300 µm
YG Migratory stage

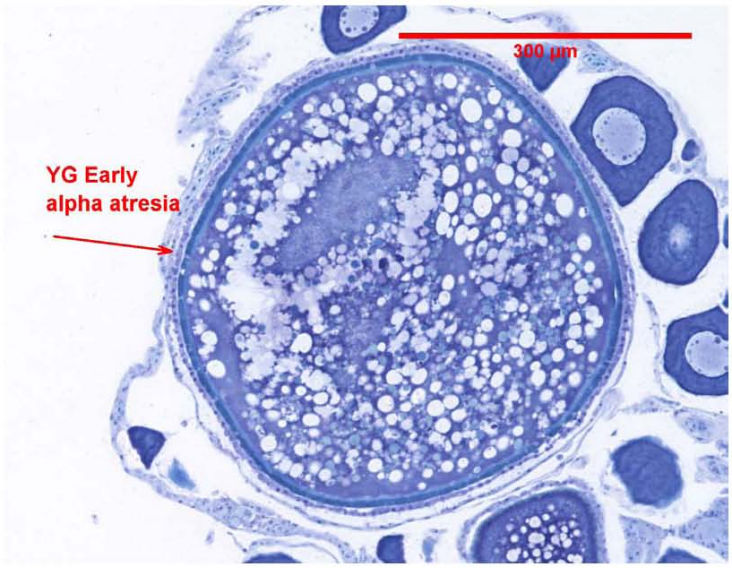
Pictures of the 3 different stages in early alpha atretic oocytes stained with toluidine blue.

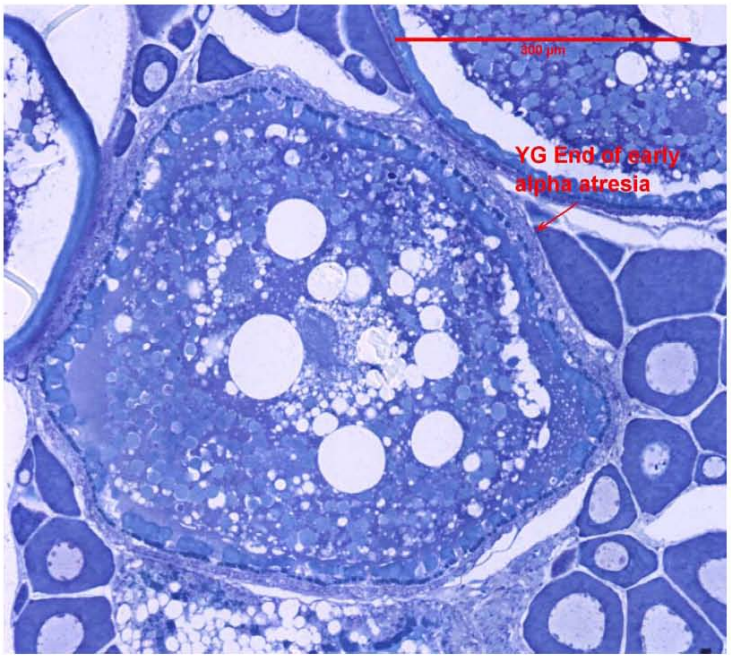





300 µm
YV-YG Early
alpha atresia


300 µm
YV-YG End of
early alpha atresia


300 µm
YG Early
alpha atresia

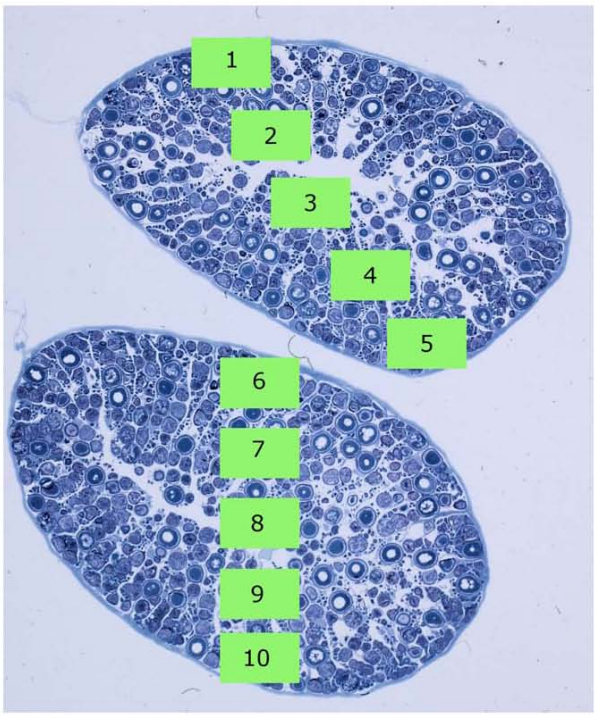

300 µm
YG End of early
alpha atresia

Measurement of Vi :

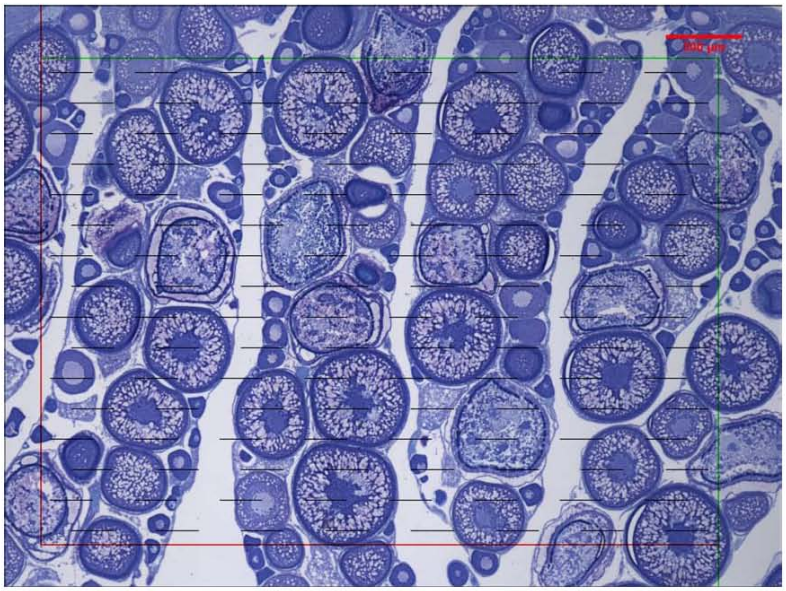
Vi = partial area of vitellogenic oocytes in the histological section.
A number of frames are superimposed across both ovary sections at regular intervals in order to estimate the mean Na and Vi for the fish. The area analysed should be proportional to the ovary weight.
A Weibel grid made up of test points is superimposed on the section in order to estimate the partial area of early alpha atretic oocytes as a proportion of the total surface area in the sample frame. The test points are located at the ends of the lines in a grid.

Ovary weight (g)	**Approximate area to be analysed**	**Number of fields to be analysed if the area is 0,05 cm^2**
2-9	0,3 cm^2	6
10-19	0,4 cm^2	8
20-29	0,6 cm^2	12
>30	0,7 cm^2	14

The outer grids should include area occupied by the ovary tunica and points lying outside the ovary should be discounted (negative grid).



The grid should have about 5000 points per cm^2 to cover the field.
In the example below the area inside the frame is 0,050 cm^2 and there are 256 points, which means that there are 5120 points per cm^2.

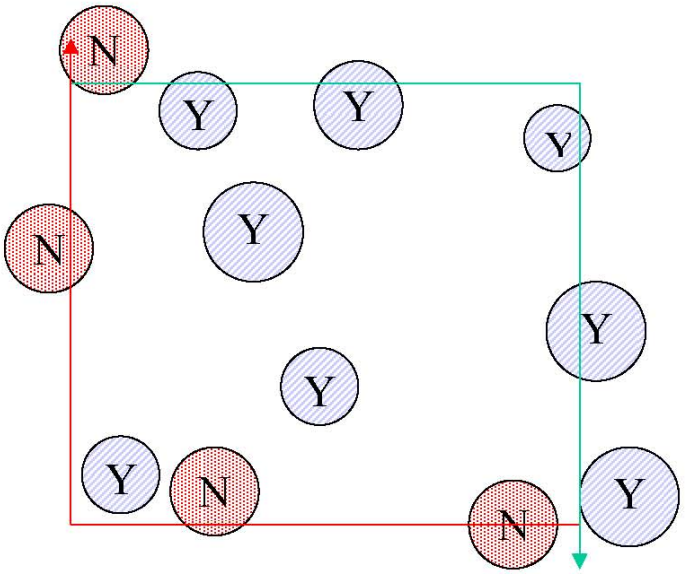


Count the point that hit early alpha atretic oocyte in each of the three stages : YV, YV-YG, YG.

Calculate Vi for each stage using the following equation :
Vi = Number of hits / (total points – negative grid)

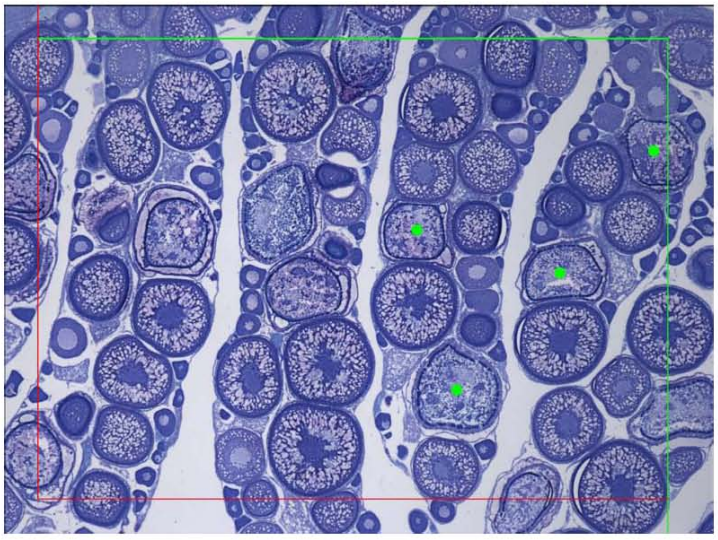
Measurement of Na :

Na = number of vitellogenic oocyte transactions per unit area.
A frame is superimposed over the section and the number of early alpha atretic cells in each class of oocyte counted using the rules shown in the illustration below. Oocytes touching the forbidden line (red) or extended red line will not be counted (N). Oocytes inside the frame or touching only the green line should be counted (Y).



Calculate Na for each stage using the following equation :
Na = Number of profiles / field area

In the example below 4 early alpha atresia cells in the stage (YV-YG) are counted. The area inside the frame is 0,053 cm^2, Na for YV-YG will be 4 / 0,053 = 75,5 profiles / cm^2.



3.3 Calculation of atresia

To estimate the number of atretic oocytes in the the gonade we use the following equation :

$F_{atr} = Ov * B * K * Na^{3/2} / Vi^{1/2} = Ov * 0,72 * Na^{3/2} / Vi^{1/2}$

Ov = ovary weight in gram
B = 0,72 (constant value, ratio between the longest and shortest axis of the oocytes transected)
K = 1 (constant value for atretic oocytes)

Calculate relative atresia :
Rel.atr. = F_{atr} / fish weight (this is the number that should be entered into the database)

Summerize F_{atr} for the 3 stages

Calculate the mean atresia from all the fish examined.

3.4 Calculation of mean atretic loss

To estimate the mean atretic loss we use the following equation :

Mean atr. loss = mean atresia * spawning duration / duration of early alpha atresia

Spawning duration = 60 days
Duration of early alpha atresia = 7,5 days

Procedure 4
Horse mackerel sampling procedure at sea

Before the cruise :
Fill the labelled 2,5 ml eppendorf tubes with 1,2 ml of 3,6% buffered (sodium phosphate) formaldehyde (see excel-file : Buffered formaldehyde) and measure the weight (± 0,0001 g).

During the cruise :
Measure the weight of the whole catch and select a subsample of 100 fish and measure the total weight of the subsample.
Measure total length, weight, maturity (Walsh scale) and sex of each fish in the subsample.

Select females in maturity stages 3-6 (see table 3.1.2 WGMEGS 2006) from the subsample for fecundity analysis. Be sure to divide the females equally into the 4 weight categories : < 150g, 151-250g, 251-350g and >350g.
Measure or take:

- Total length
- Toatal weight
- Maturity
- Otoliths for age reading
- Weigth of gut, ovary and liver
- Stomach fullness (1: empty, 2: filled, 3: full and 4: bursting)

Ovary sampling :

- From the ovary take 4 * 25µl samples with a pipette and immediately put each sample in individual coded eppendorf tubes.
- Make sure that all the ovary samples are covered with formaldehyde.
- Freeze and label the gutted fish separately in plastic bags for lipid measurements. Be sure to use the same code for the eppendorf tubes and frozen fish for each individual

After the cruise :
Measure the weight of the eppendorf tubes containing the sample.
Send all the frozen fish to IMARES, see address in table 1.
Send the eppendorf samples for analysis to the different institutes referring to table 1.

Colour code	Country	Institute and address	Responsible person
Blue	Norway	IMR, Nordnesgaten 50,PB 1870, 5817 Bergen-Nordnes, Norway	Merete Fonn
Pink	Ireland	MI, Rinville, Oranmore, Co.Galway, Ireland	Brendan O'Hea
Green	Netherlands	IMARES, Haringkade 1, 1976 cp Ymuiden, Netherlands	Cindy van Damme
White	Spain	IEO, Cabo Estay-Canido, 36280 - VIGO (Pontevedra) Spain	Jose Ramon Perez

Procedure 5
Fecundity whole mount analysis procedure for Horse mackerel

5.1 Spawning markers

Transfer the unstained sample to a tray and try to separate the oocytes.
Under the microscope check for spawning markers, if there are hydrated oocytes, atretic hydrated oocytes or ≥ 5 POFs, the sample should not be analysed for fecundity.

5.2 Potential fecundity

Note the present or absence of atretic oocytes.
Count all the oocytes >175µm in the sample.
Distribute the sample randomly in the tray and measure the diameter for size distribution for all oocytes >175µm in 1/3 of the sample.

Potential fecundity :
Pot.fec. = number of oocytes / weight of the pipette sample * ovary weight

5.3 Relative potential fecundity

Relative potential fecundity :
Rel.pot.fec. = Pot. fec. / total fish weight