Comparative fecundity estimates in the thumbprint emperor, *Lethrinus harak* (Forsskal, 1775) using volumetirc and stereological methods

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ABSTRACT

Comparative fecundity estimates were made on 10 stage 4 ovaries of the thumbprint emperor, *Lethrinus harak* using volumetric and stereological methods. Results show that the volumetric method tended to under-estimate fecundity by an average of 23% in comparison to the stereological method. The fecundity of 24 other stage 4 ovaries determined volumetrically was therefore standardised by taking this value into consideration. A mean fecundity of 476,000 oocytes per female was established. High positive correlation was found to exist between fecundity and three variables i.e. standard length, total weight, and ovary weight.

Key words: *Lethrinus harak,* fecundity, volume, stereology

INTRODUCTION

The importance of fecundity as a powerful tool in fisheries biology is attested to by its application in a variety of studies. Fecundity, combined with a knowledge of abundance of fish eggs in the plankton, has been used to estimate the size of adult fish stocks (Bannister *et al.*, 1974; Macer, 1974; Lockwood *et al.*, 1981). It has also been used in fish stock assessment studies, in egg and larval studies and in the discrimination of fish stocks (Holden and Raitt, 1974). However, the validity of any fecundity-based application depends largely on the accuracy of the oocyte counting method. Besides solving the problems encountered in estimating fecundity of most commercially important fish species (see Witthames & Walker, 1987), the ideal counting method must also be cheap, simple to use and above all must give accurate fecundity estimates.

Several automated methods for counting fish eggs have been described (Witthames & Walker, 1987). The method described by Davies (1984) and the HIAC Criterion PC 320 Particle Size Analyzer (Witthames & Walker, 1987) have an added advantage in that they also measure egg size distribution. Witthames & Walker, (1987) have further compared HIAC counts with gravimetric and volumetric fecundity estimates and showed that HIAC counts gave better estimates. However, despite this conclusion, they could not solve the problem of counting smaller eggs, i.e. those that are smaller than 200 μm in diameter. As with other methods, the automated egg counting machines have their shortcomings because they are not only expensive but also need constant attention from highly trained technicians (Ntiba, 1989).

Among the laboratory methods used to estimate fish fecundity, the volumetric method is the most widely used. The application of this method poses two problems. Firstly, the Gilson's fluid used to digest the ovary wall tissues in order to allow release of eggs is mercury based fixative and thus unfriendly to the environment and can be poisonous. Secondly, it is very difficult to obtain an even suspension of eggs of different sizes and densities in a sub-sample, and, hence fecundity of a given fish is usually under-estimated (May 1967). In this study we compared fecundity estimates of *L. harak* using volumetric and stereological methods. The aim was to perfect the later method in an endeavour to do away with the method using mercury based fixatives.

MATERIALS AND METHODS

The fish samples used in the study were caught using beach seine nets of variable mesh sizes (bag 28 mm, wings 60 mm) in collaboration with the local fishermen of Gazi and Msambweni areas in the South coast of Kenya. Sampling was carried out on fortnightly basis during spring tides for the period April 1995 - March 1996. Occasionally, additional sampling was done on neap tides when the required sample size of 50 fishes was not materialized. During the sampling period, a total of 827 specimens of *L. harak* were collected of which 386 were males, 426 were females and the sex of the remaining 15 fishes was difficult to determine.

The total and standard lengths were taken on a fish measuring board to the nearest millimeter. The total body weight of the ungutted fish was weighed to the nearest gram using a top loading digital balance. Fish were then dissected, sexed and the gonads were carefully removed and weighed to the nearest milligram. After gross examination, the gonads were assigned to a maturity stage based on their external features such as size, colour, shape and texture following the schemes of Ntiba and Jaccarini (1990). For females this assignment was later validated by histological examination and oocyte diameter measurements.

Oocyte counts were made in different regions (anterior, middle and posterior) of both lobes of the ovary to assess any difference in oocyte numbers and frequency distribution of oocyte diameters between regions of the same lobe and between the two lobes. An analysis of variance showed no significant difference in oocyte counts between the regions of the same ovary and between the two lobes ($p>0.05$). There was also no dissimilarity in frequency distribution of oocyte diameters in all ovarian regions. In subsequent analyses, therefore, portions from the middle region of one lobe per fish were cut and preserved in Gilson's fluid for oocyte size distribution analysis and volumetric oocyte counting while the corresponding region of the other lobe was preserved in Bouin's fixative for histological studies and stereological oocyte counting.

The volume of the ovary (VO) was determined using the formula: $VO = 0.95 \times W$ where, W = weight of ovary (Aherne and Dunnill, 1982).

Gilson's Treatment and Volumetric Counting of Oocytes

As soon as the ovary was removed from the fish and the necessary data recorded, pre-weighed portions from one lobe of stage 4 ovaries were cut longitudinally, turned inside out and preserved in Gilson's fluid in 100 ml, wide mouthed, plastic bottles. The bottles were shaken, at regular periodic intervals, to aid the release of oocytes from the ovarian walls.

Before counting of oocytes was undertaken, the preserved materials were carefully washed into a 0.5 liter capacity beaker, avoiding spillage, and the empty bottles were checked under a magnifying glass to make sure no oocytes were left in the bottles. To help liberate those oocytes, which were still clung onto the ovarian tissues, a hand-held electric Moulinex mixer was used to stir the oocytes, at medium speed, for five minutes. The ovarian debris suspended in the water containing the oocytes were removed by decanting and replacing the water several times until an acceptable level of debris less than 1% was achieved.

After taking several trial sub-samples of 0.5 ml from the 0.5 liter capacity beaker, it was discovered that one sub-sample could give sufficient numbers of large oocytes to yield satisfactory counts and ova diameter distributions. It was also noted that this single sub-sample contained around two thousand small oocytes, which made counting, and measuring rather difficult and time consuming. To solve this problem, it was deemed necessary to adopt, with slight modifications, the method described by Macer (1974) who treated the larger and smaller oocytes separately so that counting of the latter would become easier. In the present study, a dividing size separating the smaller and larger oocytes was adopted at 95 ?m. By increasing or decreasing the sample volume, a 0.5ml subsample was found to contain appreciable numbers of the oocytes for each of the two categories. In the case of sub-sampling of oocytes <95 μm, the water volume was made-up to 400 ml in a one liter capacity beaker after which a subsample of 0.5 ml was taken. The larger oocytes in this sub-sample were counted and measured ignoring the smaller ones. For sub-sampling of oocytes >95 μm, the water volume was diluted to 1600ml in a two liter capacity beaker. In this case, the smaller oocytes were counted and measured leaving out the larger ones. The number of smaller oocytes that would have been contained in the larger oocyte sub-sample, if there were no volume increments, was calculated by multiplying smaller oocyte numbers actually counted by a dilution factor based on the difference between the two sample volumes.

The number so calculated and that for the larger oocytes was then pooled to form a single diameter distribution.

When taking the actual sub-samples for counting, care was always taken to make sure that the oocytes were evenly distributed in the water column. This was achieved by stirring the oocytes using an ordinary plastic ruler with a to and fro motion. A sub-sample was taken after 15 strokes of the ruler, using a 1-ml labsystem finlet pipette. The oocytes in the pipette were washed into a petri-dish with a hand made square grid and their diameters measured along a horizontal axis, irrespective of their shape, under a Wild dissecting microscope with a calibrated eye-piece graticule, at a magnification of 40X. The total number (N) of oocytes in any size class in the ovary was calculated as follows:

$N = (V/V^{o})n \times W/W^{o}$, where,
V = volume of sample,
V^{o} = volume of sub-sample,
n = number of oocytes in the sub-sample counted,
W = weight of ovary, and,
W^{o} = weight of ovarian portions fixed in Gilson's fluid.

Histological Techniques and Stereological Counting of Oocytes

For histology, the middle region of the other lobe was fixed in Bouin's fixative for a maximum of 48 hrs, transferred and rinsed in 50% alcohol and then stored in 70% alcohol. The stored material was dehydrated in graded alcohol, cleared in a mixture of absolute alcohol and xylene and further cleared in xylene. The specimen was infiltrated in two changes of paraplast wax before embedding in paraplast wax. Sections were cut at 8-12 microns thick using a microtome and stained in iron haemotoxylin and eosin. The sections were examined under an ordinary microscope for the general morphology of the ovary.

For stereological counting, the histological sections were viewed under a dissecting microscope using an eyepiece graticule with a lattice grid at a magnification of 40X. The grid had a total of 121 intersecting line points and an area of 2.56 mm^2. The volume fractions representing the proportions of the ovary occupied by the oocytes (grouped into resting, vacuolated, yolked and atretic); the connective tissue and the artifacts were estimated by the point-counting

technique (Aherne & Dunnill, 1982). Superimposing the grid on a randomly selected field within the section and counting the total number of intersecting line points of the grid falling on the different ovarian components did this. Since it was impossible to carry out point-counting and make oocyte diameter measurements simultaneously, the oocyte sizes were determined later by means of a calibrated eyepiece graticule. For oocyte diameter measurement purposes, a representative field containing all oocyte groups were selected from the mid portion of the section. All oocytes cut through the nucleus were measured along a horizontal axis irrespective of their shape. The total number (N_1) of oocytes of any size class in the ovary was calculated using the following formula:

$N_1 = 0.95W/(4/3\ Pi\ R3) \times n^o/n$ where,

W = weight of ovary,

R = mean oocyte radius,

n^o = number of grid intersecting line points falling on oocytes of any size class, and,

n = total number of intersecting line points counted.

RESULTS

Determination of spawnable Oocytes

Figure 1 shows the oocyte diameter frequency distribution of Gilson's treated ovaries for all stages of maturity of *L. harak*. The oocyte diameter distribution in mature ovaries (stages 4 and 5) is bimodal with no distinct size separation between the two modes. In contrast, the oocyte distribution in developing and spent ovaries (stages 1, 2, 3, and 6) is unimodal.

Fecundity estimates are usually done in ovaries at stage 4 because determination at other stage can lead to under-estimation of oocyte counts. For example, at stage 5 some oocytes might have already been released while at stage 3 many oocytes have not fully matured. One difficulty associated with fecundity estimates of fish with asynchronous oocyte distribution in stage 4 ovaries is the identification of the size of the smallest oocyte that could be spawned along

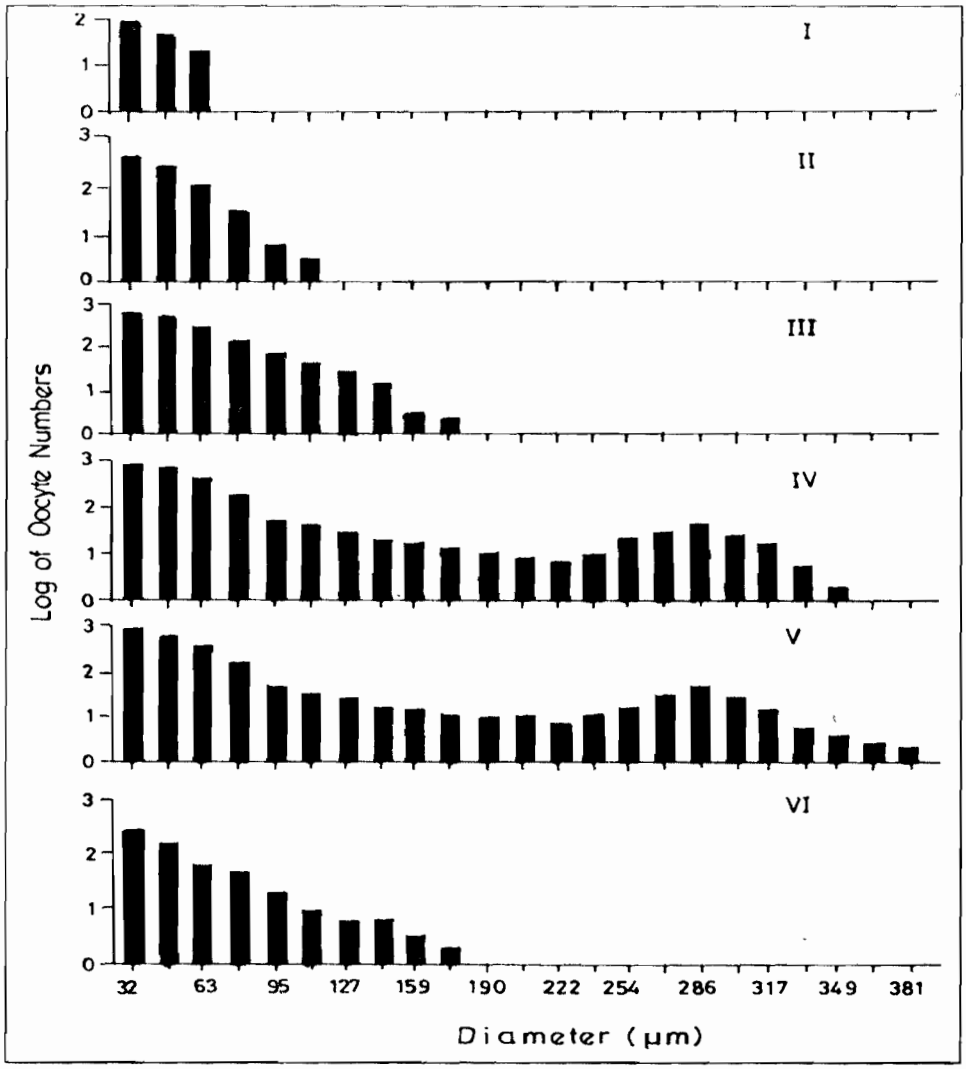


Fig 1. Distribution of oocyte diameters for each of the maturity stages

with the mode of larger oocytes in the coming season. Following Macer, (1974) and Ntiba & Jaccarini, (1992) all oocytes with cytoplasmic vacuoles were considered to develop and spawn along with the larger oocytes. The size of the smallest such oocytes was determined histologically and was found to be 86 µm in diameter. However, the oocyte diameter measurements taken from sectioned material previously fixed in Bouin's showed, when compared to those taken from Gilson's treated ovaries, a size reduction of 10% due to the effect of tissue shrinkage. All oocyte measurements made on sectioned material were thus corrected by taking into consideration the shrinkage factor to obtain the Gilson's equivalent. Therefore, the corrected size of the smallest oocyte with

cytoplasmic vacuoles is 95 µm. For volumetric estimates, all oocytes > 95 µm were counted while, for stereological estimates, all vacuolated and yolked oocytes were counted.

Testing the Accuracy of the Counting Method

To test the accuracy of the counting method, seven replicate oocyte counts from the same ovary were made using both volumetric and stereological methods. The results presented in Table I show that the volumetric method has a coefficient of variation of 6.1% while the stereological method shows a much lower coefficient of variation of 2.7%.

Table I. Seven replicate counts taken from the same ovary using the volumetric and stereological methods to test their accuracy

Replicate	Volumetric counts	Stereological counts
1	99	113
2	106	116
3	96	112
4	110	112
5	108	107
6	115	111
7	106	108
Mean	106	111
S. D.	6.4	3
C.V	6.1	27

Volumetric and Stereological Estimates of Fecundity

Due to the difficulty of obtaining good histological sections from ripe ovaries, only 10 ovaries were successfully sectioned and oocyte counts made both volumetrically and stereologically. By using the equations $N = (V/V^{o})n \times W/W^{o}$ for volumetric counts and $N_1 = (0.95W/(4/3\ Pi\ R3) \times n^{o}/n$ for stereological counts, comparative estimates of the total number of oocytes to be spawned in

the coming season were made and the results are shown in Table II. The comparison of the two estimates made on the ten ovaries show that the volumetric method tended to under-estimate fecundity by an average of 23%. The fecundity of 24 other stage 4 ovaries made using volumetric method were thus corrected by multiplying this percentage by the oocyte numbers obtained and a mean of 476,000 oocytes to be shed in the spawning season following capture were determined.

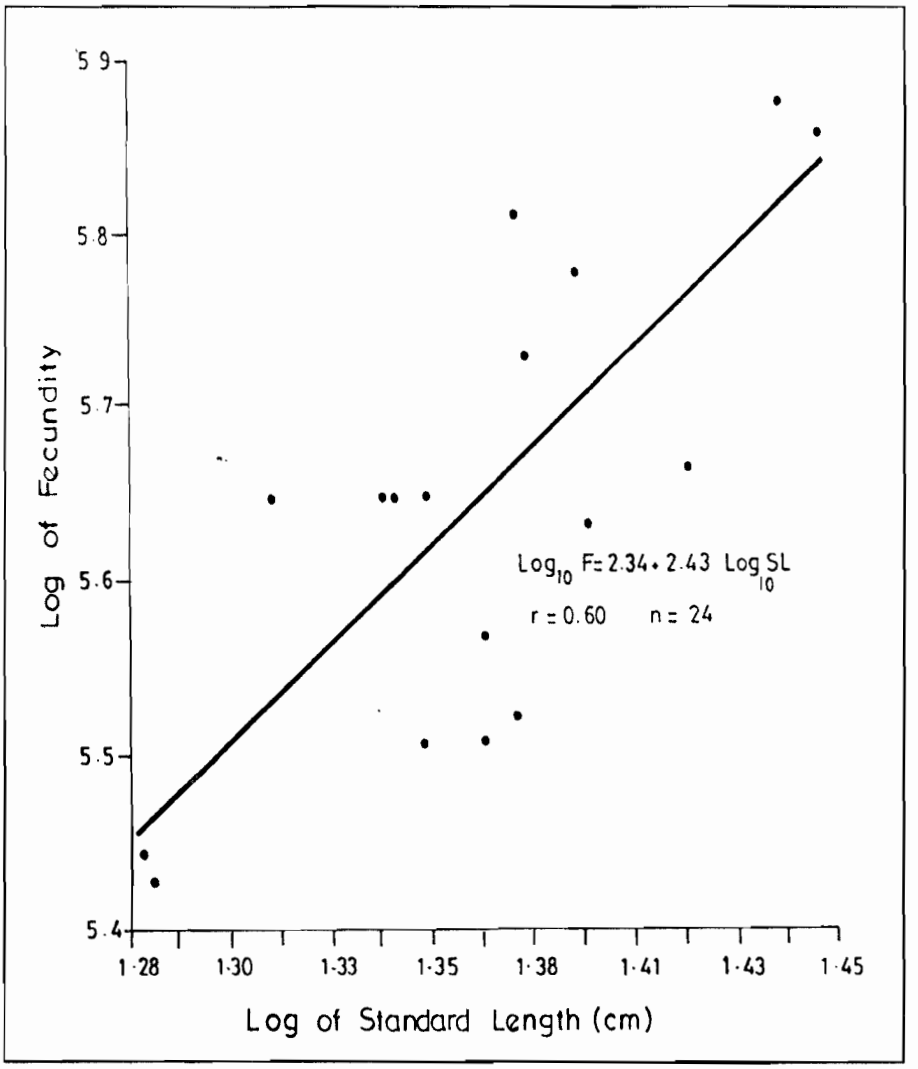
Table II. Comparison of volumetric and stereological estimates made on ten stage 4 ovaries

Number	Stereological	Difference in the	Percentage
Volumetric	estimates (V)	estimates (S)	two estimates (S-V)	Difference
1	227,000	298,669	70,669	24
2	231,016	321,683	88,667	28
3	257,560	278,348	19,788	7
4	266,526	280,850	12,324	5
5	250,286	261,929	7,643	4
6	354,656	488,538	123,882	27
7	352,600	628,758	256,158	44
8	355,200	410,394	55,194	13
9	566,088	906,931	310,843	38
10	616,960	983,764	366,804	37
Mean	**347,789**	**485,986**	**138,197**	**23**
			S.D.	15

Fecundity in Relation to Fish Length, Fish and Ovary Weights

The relationship between fecundity (F) and standard length (SL) shown in figure 2 indicates that fecundity increases with an increase of fish length. This relationship is found to be linear and is expressed by the formula:

Fig. 2: Relationships between fecundity and standard weight



Similarly, fecundity increases with weight (figure 3). The following equation represents fecundity-total weight (TW) relationship:

$$\text{Log (F)} = 3.90 + 0.69 \text{ Log (TW)}, \; r = 0.58 \; n = 24$$

Fig. 3. Relationships between fecundity and total weight.

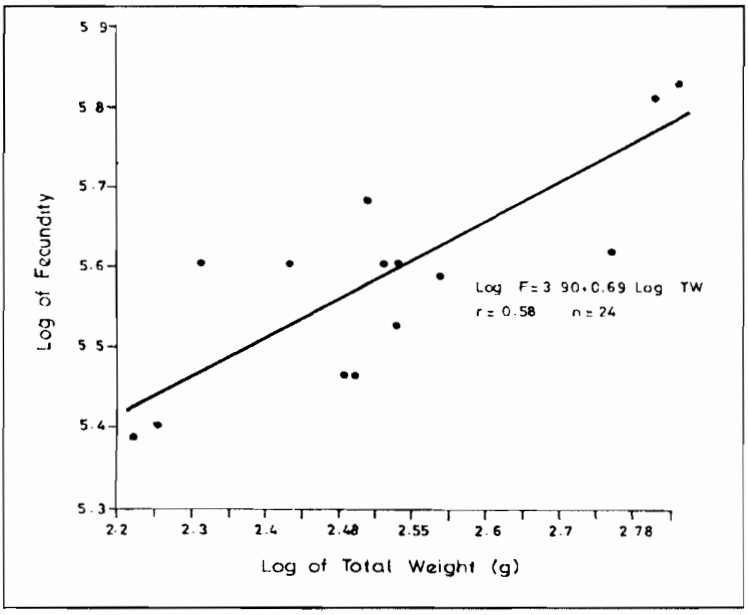
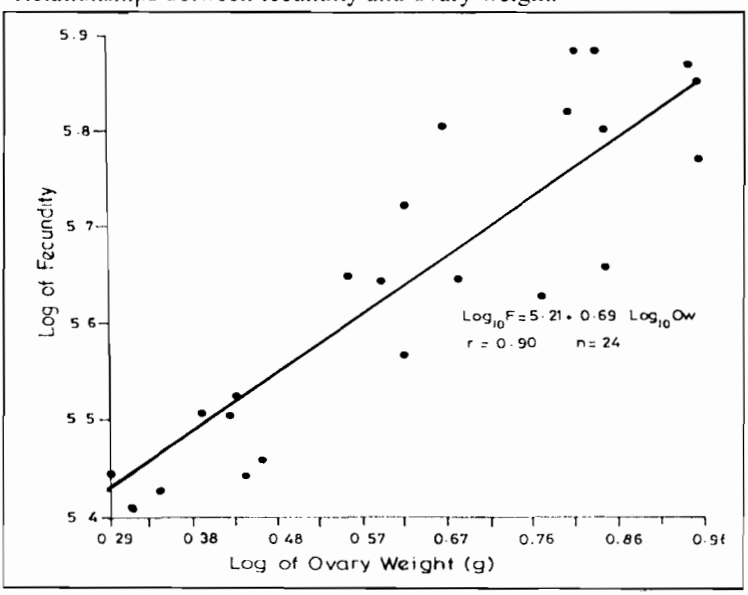


Fig. 4. Relationships between fecundity and ovary weight.



Fecundity and ovary weight (OW) also show a linear relationship (figure 4) and is expressed by the equation:

$$\text{Log (F)} = 5.21 + 0.69 \text{ Log (OW)}, \; r = 0.90 \; n = 24.$$

DISCUSSION

The comparative fecundity estimates made using volumetric and stereological methods showed that the volumetric method not only gave the least counting accuracy but also tended to under-estimate the fecundity by an average of 23%.

The inconsistency of sub-sample counts made volumetrically stemmed mainly from the oocyte disturbance by the finlet pipette when taking the sub-sample and the sinking rate of the oocytes which made collection of a representative sample of all oocyte groups rather difficult. May (1967) cited in Whitthames and Walker (1987) reported that the high variability and tendency for the volumetric method to under-estimate fecundity is probably due to the difficulty encountered in obtaining an even suspension of eggs of different sizes and densities.

The finlet pipette disturbance and the inter-sub-sample variability can be reduced by using the Bogorov pipette (Bogorov and Zenkevich, 1947) which, unlike the stempel and finlet pipettes, encloses the sample by falling over it and in the process causes less disturbance, and by using saturated salt solution which, having a higher density than water, reduces the sinking rate of the oocytes (Alvarez-Lajonchere, 1982). Ntiba (1989) working with the long rough dab, *Hippoglossoides platessoides* in the North Sea, has also used saturated sugar solution with good results.

Witthames and Walker (1987), comparing an automated egg counting method with the conventional volumetric and gravimetric methods, found that the volumetric method under-estimated the fecundity of plaice by 6% with a coefficient of variation of 5.3%. However, most of the granular eggs they counted were much larger (200-2500 μm in diameter) than the oocytes we have used in the present study and they would have probably ended up with a higher percentage had they counted the smaller eggs below 200 μm in diameter.

Apart from producing higher counting accuracy (2.7%) and hence more accurate fecundity estimates, the stereological method excels in saving crucial time by eliminating the need to sub-sample the oocytes. It also overcomes the problem of treating small and large oocytes in a sub-sample separately when the former are extremely numerous. One major problem, which can greatly influ-

ence the stereological estimates unless rectified, is the effect of tissue shrinkage. All oocyte measurements taken from sectioned material previously fixed in Bouin's fixative for histological sectioning have to be corrected to pre-shrinkage size values before any estimates are carried out.

Ovarian atresia characterizes both pre- and post-spawning stages of many fish species (Macer, 1974; Cyrus & Blaber, 1984; Hunter & Macewicz, 1985; Ntiba & Jaccarini, 1990). In *L. harak*, it is post-spawning artesia that affects spent ovaries mainly. The estimated mean fecundity of 476,000 oocytes is expected to be spawned as no atretic oocytes were seen in stage 5 ovaries.

Information on the reproduction of *L. harak* other than the present study is not available. However, data on other related species can be used for comparison. Wassef and Bawazeer (1992), working with *L. elongatus*, found that the absolute fecundity ranged from 10,400 for 23-cm fish to 621,000 for 49 cm fish. There was a gross under-estimation of the fecundity since they counted only the yolked eggs. In *L. harak*, where the length range of fish specimens studied was narrower than that of *L. elongatus* (Wassef and Bawazeer, 1992), fecundity was much higher and ranged from 318,000 for 27.7 cm to 907,000 for 34-cm fish.

In the present study, positive correlation is found to exist between fecundity and three variables namely: standard fish length, fish weight and ovary weight. However, the highest correlation exists between fecundity and ovary weight, which is not surprising since production of eggs (fecundity) is the dominant function of an ovary (Hickling, 1940; Somvanshi, 1985). A close relationship should be expected between ovary weight and the number of oocyte produced. Ntiba and Jaccarini (1992) suggested that the ovary may also function as a storage organ which would probably be one reason for high levels of atresia observed in some fish species under varying environmental conditions.

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