CHAPTER III

MATERIALS AND METHODS

Sampling

1. Fishing Method

Samples of *Encrasicholina heteroloba* were obtained from commercial catches in the exploitation area off Rayong province, the eastern coast of the Gulf of Thailand. They were from anchovy purse-seining operated at night with 1 or 2 light-luring boats. Anchovy school was detected by echo-sounder or sonar and aggregated by light luring. The fish were then caught by a rectangular, blue polyethylene net of 350-600 m long, 50-80 m deep, and 8.3 mm mesh size. They were landed in the morning at the fishing port near Rayong river-mouth.

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2. Sampling Method

Samples were collected twice each month over one-year period from January to December 1995. About 100 fish were randomly sampled on board and fixed in 10% buffered formalin for histological purpose. Another 100 fish were also randomly collected for macroscopic examination. Both sets of samples were brough

to the laboratory at the Eastern Marine Fisheries Development Center, Ban Phe District, Rayong Province.

At laboratory, 5 pairs of ovaries were subsampled from females in fixative, refixing individually in the new solution of 10% buffered formalin for microscopic examination.

Sample Preparation and Data Collection

1. Macroscopic Examination

1.1 Measurements and Sex determination

Samples were measured for their total lengths (TL) to the nearest 0.1 centrimeters from the tip of snout to the tip of caudal fin, and were weighed for their body wet weights (BW) and gonad wet weights (GW) to the nearest 0.01 grams. Sex was determined by the appearance of gonad in body cavity.

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1.2 Gonad Development Staging

The development stages of ovaries and testes were classified by their sizes and morphological features according to the scale of Holden and Raitte (1974). Details are shown in Table 1.

Table 1. Maturity scales used for shorthead anchovy (adapted from Holden and Raitte, 1974)

Maturity stage	External appearance of ovary	External appearence of testis
Immature	Ovary small, less than 1/3 length of abdominal cavity, pinkish and translucent. No oocytes visible through ovary wall.	Testis small, flat, less than 1/3 length of abdominal cavity, light-grey to whitish, and translucent.
Developing	Ovary about 1/2 length of abdominal cavity, pinkish, soft in texture, and translucent. No oocytes visible through ovary wall.	Testis about 1/2 length of abdominal cavity, flat, whitist, and more or less symmetrical.
Mature (Ripening)	Ovary about 2/3 length of abdominal cavity, becoming broader with rough texture, pinkish-yellow with granular appearance of tiny oocytes.	Testis about 2/3 length of abdominal cavity, becoming broader, whitist to creamy, smooth, and flat.
Ripe	Ovary extends from 2/3 to full length of abdominal cavity, orange-pink with conspicuous superficial blood vessels. Large transparent, ripe oocyte visible.	Testis extends from 2/3 to full length of abdominal cavity, whitish-creamy, soft, and flat with no blood vessels.
Spent	Ovary shrunken to about 1/2 length of abdominal cavity, reddish in colour. Walls loose, may containing remnants of disintegrating opaque and ripe oocytes, darkened or translucent.	Testis shrunken to about 1/2 length of abdominal cavity. Walls loose and flabby with flat shape. No blood vessel visible.

1.3 Fecundity Estimation

As microscopic data showed 3 stages of oocytes developed in the mature ovaries of *E. heteroloba*, therefore batch fecundity was determined based on the most advanced mode of oocytes. The gravimetric method for batch fecundity estimation in multiple spawning fishes by Hunter et. al. (1985) was used.

After macroscopic staging, mature ovaries were preserved individually in a vial of 10% buffered formalin for fecundity estimation. Two way analysis of variance was used to test differences in fecundity estimates between left and right lobes and among three position: anterior, middle, and posterior, within the lobes of 5 pairs of ovaries. No differences were found either among the positions within ovaries or between the left and the right lobes, thus, about 0.01-0.02 gram tissue samples were all taken from the midsection tissue from the preserved ovary, either left or right side. For each sampling period, up to 5 mature ovaries, if there was any, were taken for estimation. Each subsample was weighed and placed on a slide, removed its membrane, then oocytes were loosened by gently tapping the piece of ovary with the blunt tips of forceps. The sample was spreaded over a grid counting slide and covered with a cover slip. Yolked oocytes distinguished from others by their large size and relatively opaque were counted under a 20× stereomicroscope. Number of yolked eggs per unit weight of ovary was calculated for each subsample tissue and extrapolate for the individual fish.

2. Microscopic Examination

2.1 Histological Preparation

After one day of fixation in 10% buffered formalin, ovaries were transferred to be preserved in 70% ethanol. For a minimum of 3 days prior to processing, the preserved ovaries were soaked in the solution of 70% ethanol plus 10% glycerol to soften yolked oocytes. This was done to further facilitate cutting of the specimen block of ovaries which had yolk inside (Hay et al., 1987; Greeley, MacGregor, and Marion, 1988; Marshall, Pullen, and Jordan, 1993).

A piece of 0.3 cm-thick specimen was cut transversely from the central portion of each ovary, left or right. It was dehydrated in a graded series of ethanols, 70%, 90%, 95%, and 100%, cleared in xylene, and infiltrated with paraplast by the tissue processor (Citadel: Shandon Scientific Limited), followed the procedures in Table 2.

The specimens were embedded in Paraplast Plus (Monoject Scientific), then the blocks were cut into serial sections of 5 μm thick with rotary microtome (Yamato Kohki). After floating out on a warm water bath, section ribbons were mounted on microscope slides treated with egg albumen and dried on a slide warmer at 40°C. They were then stained with Harris' haematoxylin and counterstained by eosin Y (Coolidge and Howard, 1979).

Table 2. Automated processing of ovarian tissue of shorthead anchovy (modified from Coolidge and Howard, 1979).

Step	Times (min)
70% ethanol	30
90% ethanol	60
95% ethanol	60
95% ethanol	30
100% ethanol	60
100% ethanol	180
100% ethanol	180
100% ethanol	180
Xylene	30
Xylene	30
Xylene + paraplast (60°C)	60
Paraplast (60°C)	60
Paraplast (60°C)	60

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2.2 Ovarian Development Staging

The development stages of ovaries were determined from the most advanced stage of oocytes. Histological stages were assigned to correspond to the previous macroscopic staging: immature, developing, mature, ripe, and spent with the

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criteria based on West (1990). The prepared sections of the ovarian specimens of *E. heteloroba* were examined *via* 10× to 40× magnification of compound microscope.

2.3 Frequency Distributions of Oocytes-Size and Oocyte-Stage

Oocytes were staged and measured by microscopic inspection of the prepared sections at 10× or 20× magnification. The measurement for diameter was conducted on the major axis of the oocyte. Up to 100 identifiable oocytes sectioned through the nucleus were classified and measured. The presences of postovulatory follicles and atretic oocytes were recorded. Five sets of classifications and measurements for each sampling period were combined to produce the frequency distribution plots.

Data Analyses

Chi-square analysis was used to test the homogeneity of sex ratio for determining the distribution of male and female in the samples.

Gonadosomatic index (GSI) was calculated as $GSI = \frac{\text{gonad weight}}{\text{body weight}} \times 100.$

It was calculated for each fish and the mean was obtained for each sampling period to determine the annual change.

Batch fecundity was calculated from the product of the number of yolked oocytes per unit weight of the tissue subsample and the entire ovary weight, left and

right sides combined. Regression analysis was done to evaluate the relationship between fecundity and length. Least square linear regressions of batch fecundity (F) on length (L) was calculated from log-transformed data: $\text{Log } F = a + b \text{ Log } L$.

Annual development of gonads, ovaries and testes, were determined macroscopically. Oocyte development stages within ovaries were examined for each sampling period by means of histological examination and the two methods were compared.

For frequency distributions, oocytes were classified into 26 size classes with 30 μm interval over the range 12 to 780 μm. Annual patterns of size and stage frequencies were summerized.

Spawning type and periods were determined from the distribution patterns of oocyte-size and oocyte-stage frequencies obtained from histological examination. The presence of postovulatory follicles and atretic oocytes were also used for indicative feature of recent spawning.

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Annual rainfall and atmospheric temperature in Rayong province were summarized from the data obtained from the Rayong Meteorological Station. They were related to the annual variation of mature shorthead anchovy to find out these environmental effects on the maturity of the fish.