Chapter II

METHODS AND MATERIALS

Green mussels (Mytilus viridis L.) used in this experiments were collected from the mussel beds in Cholburi Bay near the Bangpakong River delta. At high tide, the maximum depth of this area is between 4-6 metres. The bottom is mostly muddy sand. This experiment is carried out both in the laboratory at Chulalongkorn University Marine Laboratory at Ang Sila, Cholburi Province and in the field at the above mentioned mussel beds. As for the field work, specimens were collected once a month from October 1974 to September 1975 for biochemical analyses and biological analyses. The spats of this mussel were newly settled sometimes before October 1974. At the first observation, the mussels were 1.0-1.5 cm in length. In September 1975, they became matured and were 7.0-8.0 cm in length. Some had already spawned. In Laboratory, mussels of mean lengths 5.0-5.5 cm were reared for biochemical analyses in comparison to those in the field of the same length. The rearing experiment lasted one month. This experiment is divided into three main parts

1. Biochemical Analyses in Field Specimens

In this part seasonal changes in some biochemical composition of the mussels were determined. The changes

related to growth were also be detected. In order to analyse for various biochemical composition the body of the mussel was divided into mantle part, non-mantle part by dissecting. As for total part, the entire soft body was ground up.

1.1 Estimation of Total Solid.

1.2 Estimation of Proteins-Modified macro-Kjeldahl method.

1.3 Estimation of Lipids-Petroleum ether Soxhlet extraction Method.

1.4 Estimation of Carbohydrates-as glycogen equivalent by Colorimetric Micro-Method.

2. Biochemical Analyses in Laboratory Specimens

Animals of mean length of 5.0-5.5 cm. were kept in laboratory from April 1975 to May 1975 for about one month. They were fed with cultured algae namely: green algae, Chlorella sp. and diatom, Chaetoceros calcitrans. Biochemical analyses as in field specimens were done after the rearing was over in order to compare with those in field of the same length.

3. Biological Analyses in Field Specimens

Two main topics were studied namely: reproductive state and the nutrition of the mussel. These were done at monthly intervals as in biochemical analyses.

3.1 Reproductive state-as Gonad Condition Index.

3.2 Nutritional state-Stomach contents Analysis and Degree of fullness of the stomach.

Part I : Biochemical Analyses in Field specimens

Method of specimens collection

Mussels were removed from the wooden stack and immediately frozen for biochemical analyses. Some specimens were kept alive for reproduction study in the laboratory. As for food study, mussels were first washed under tap water and immediately preserved in formalin in order to stop food digestion in mussel.

Preparation of samples

The outside of the mussel-shells was first freed from barnacles and any foreign matter. Then they were opened by cutting the adductor muscle. The flesh was excised and the mantle dissected to give separate samples of mantle and non-mantle tissue for each mussel. Some were left undissected and the entire body were ground up to be determined as total tissue. The tissues were homogenized by kitchen blender and kept in the refrigerator.

1.1 Estimation of Total Solid

Total solid was determined by standard method (A.O.A.C.,1970) given in percentage. Percentage of water content was converted from total solid content.

1.2 Estimation of Protein

The total nitrogen in the flesh was determined by modified Kjeldahl-method (A.O.A.C.,1970) of approximately 3 gm of homogenized flesh. The nitrogen was determined as ammonia by absorption in a 4 % saturated solution of boric acid followed by titration with N/10 hydrochloric acid. The percentage of proteins was taken as 6.25 timed the value of total nitrogen. This must be only an approximation because it was based on an assumed average figure of amino-acid composition of protein and the non-protein nitrogen has not been determined. The proteins determined were simply called crude proteins.

1.3 Estimation of Lipids

Lipids were determined as crude fat which was defined as substances extracted by petroleum ether. After extraction, lipids were determined gravimetrically (Jacobs, 1958)

1.4 Estimation of Carbohydrates

Carbohydrates were determined as glycogen plus "glucose" equivalent. Barnes and Heath (1966) have shown that the total carbohydrate (glycogen plus "glucose") in tissues can be extracted with hot 5% trichloroacetic acid and then estimated by a colour reaction with sulphuric acid. Amount of glycogen was read from standard curve constructed from pure glucose. Standard Glucose Curve is shown in Figure 1.

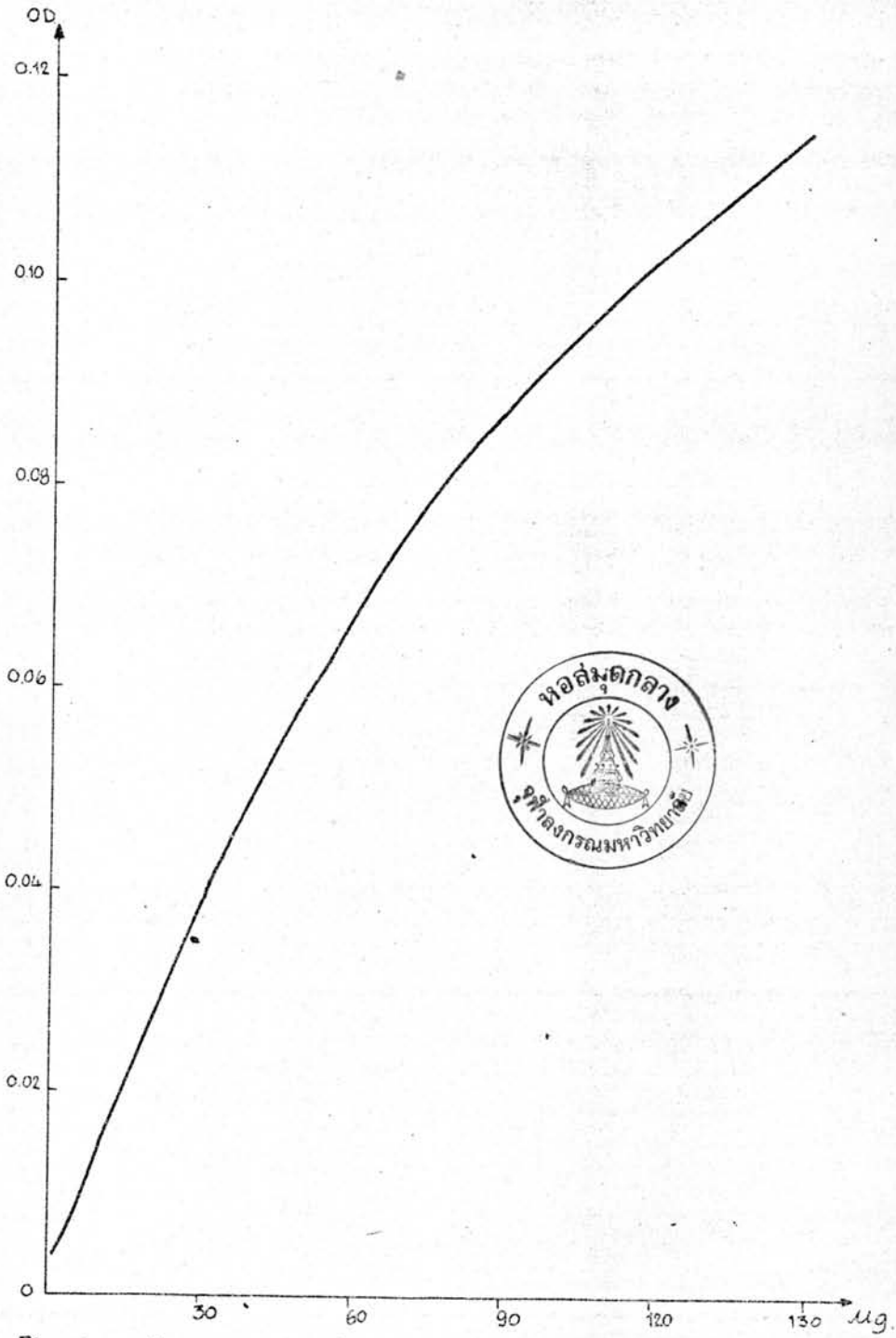

Fig 1 Glucose Standard Curve

All determinations for biochemical analyses were done in duplicate for each of the mantle, non-mantle and total tissue. Mean value and standard deviation for each determination were calculated. The percentage of each composition was given both in wet weight and in dry weight level.

Part 2 : Biochemical Analyses in Laboratory Specimens

Mussels of mean length of 5.0-5.5 cm were kept in the laboratory for a short period of one month from April 1975 to May 1975. Specimens were carefully removed from the wooden stack by cutting the byssus. They were kept in cages made of mylon net and cylindrical in shape. Each cage has a diameter of 16 cm and a height of 30 cm. They were hung in the aquarium which was 66 cm in width, 123 cm in length and 67 cm in depth. The water in each aquarium was circulated and aerated continuously. Mussels were fed two times daily: in the morning and in the late afternoon. Green algae (Chlorella sp.) and diatom (Chaetoceros calcitrans) were used to feed the mussels. These algae were cultured by using media (Robert Guillard, 1962), which was consists of major elements, vitamins and trace metals.

Major elements :

$NaNO_3$	150	mg
$NaH_2PO_4.H_2O$	10	mg
Fe sequenstrene	10	mg
$Na_2SiO_3.9H_2O$	10	mg

Vitamins :

Thiamin HCl	0.2	mg
Biotin	1.0	mg
B_{12}	1.0	mg

Trace Metals :

$CuSO_4 \cdot 5H_2O$	0.0196	mg
$ZnSO_4 \cdot 7H_2O$	0.044	mg
$CaCl_2 \cdot 6H_2O$	0.022	mg
$MnCl_2 \cdot 4H_2O$	0.360	mg
$Na_2MoO_4 \cdot 2H_2O$	0.0126	mg

Sea water to one litre

These algae were cultured in a 1000 c.c. erlenmyer flask with continuous aeration and the lighting. Thus the algae increased rapidly in number.

After one month rearing, mussels were removed from the aquarium. They were opened by cutting the adductor muscle. The flesh was excised and the mantle dissected to separate mantle and non-mantle tissue from each other. Some were left undissected for the total tissue determination. Biochemical analyses were done in comparison to those in the field of the same size.

Statistical Analyses

1. Mean value ($\bar{X}$)

For each determination, mean value of the percentage

of the biochemical composition in various tissues was calculated both for wet weight and dry weight. As for the dry weight level, the percentage of the biochemical composition was calculated from the percentage of wet weight level and the percentage of total solid. Since all determinations were done in duplicate thus

$$\bar{X} = \frac{X_1+X_2+\ldots+X_n}{N}$$

$X_1, X_2 \ldots X_n$ = mean value of each sample

N = total number of samples

2. Standard deviation (σ)

Standard deviations were calculated for each mean value of the percentage of the biochemical composition (in wet weight level) in various tissues.

$$SD = \sigma = \sqrt{\frac{\Sigma(X-\bar{X})^2}{N}}$$

3. Differences in biochemical composition in various tissues

Biochemical determinations were done in mantle, non-mantle and total tissue. Analysis of variance (F-test) was applied here to determine whether the mean values of the biochemical composition in each season for various tissues were the same. Hypothesis was set up that all mean values were equal, assuming that the variances of each group were

equal, according to Snedecor (1956)

$$Ho : \mu_1 = \mu_2 = \mu_3$$

μ = mean value of each biochemical composition in various tissues

$$F = \frac{\text{Mean square of sample means}}{\text{Mean square of individual}}$$

If the value of F was found to be less than the one from analysis of variance Table at the same degree of freedom, the hypothesis was accepted. There would be no statistical difference in biochemical composition in each season for various tissues. If the value of F calculated was greater than the one from the F-test table, the hypothesis was rejected. That is, for various tissues in each season there would be significantly different amount of the biochemical composition.

4. Seasonal changes in biochemical composition

This experiment was carried out from the very early stage in the mussel life cycle when they were first settled in October 1974 to the stage when they were mature in September 1975. This experimental period covered the three seasons of Thailand, winter season (from October to January), summer season (from February to May) and rainy season (from June to September). Analysis of variance (F-test)was again applied here to determine whether the mean values of each biochemical

composition in various tissues for each season were the same. Hypothesis was the same as the afore-mentioned one

5. Comparison between the biochemical composition of the laboratory specimens and those collected in the field.

Student's t-test was applied here to determine whether there was no difference in the biochemical composition in mussels reared in controlled environment and those of the same size reared in the field. Hypothesis was set up that all mean values are equal and the equations according to Snedecor (1956) are:-

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

$$S^2 = \frac{(X_1 - \bar{X}_1)^2 + (X_2 - \bar{X}_2)^2}{n_1 + n_2 - 1}$$

$$= \frac{(n_1 - 1)\, S_1^2 + (n_2 - 1)\, S_2^2}{n_1 + n_2 - 1}$$

If the value of t-calculated was less than the one from the t-test table at the same degree of freedom, the hypothesis was accepted. If the value of t calculated was greater than the t value from table, the hypothesis was rejected.

Part 3 : Biological Analyses in Field Specimens

3.1 Reproductive state

This was determined as gonad condition index.The stages of gonad development were assesed from the colour and thickness of the mantle, and the degree of development of egg and sperm ascertained from the microscopic examination of smears. Ten Mytilus viridis were taken from field specimens collected at monthly intervals and a piece of mantle tissue examined for the determination of the gonad index. The tissue was squashed on a slide and graded into the following stages according to Chipperfield (1953) and Tan (1973).

Stage 0 mantle was thick and no gametes were observable. The mantle characteristics varied according to the amount of reserve food material stored. When the mussel was considered "fat", the mantle was thick and of very smooth appearance. No egg or sperm coulb be seen. In poor "fattening" areas, the mantles in specimens were thin and semi-transparent.

Stage I mantle was thick, the ovarian and testicular follicles could be distinguished. The colour of the mantle in each sex varied considerably, depending on the degree of development of the follicles and the amount and distribution of glycogen in the connective tissue. It was creamy for the male and the female was usually red or orange.

Stage II mantle was thick, sperms and eggs were presented but the sperms were not active when released into

sea water. At this stage, the colour of the male and female mantle were distinctly different. The male has creamy white colour while the female was reddish orange.

Stage III the gametes were activated on release into sea water. Morphologically stage III was similar to stage II.

Stage IV recently spent stage. The mantle in both sexes was semi-transparent, only a few relict eggs or sperms could be seen.

The numbers of animals that fall into each category were multiplied by the number of the stage and the sum was divided by the number of animals in the sample.

3.2 Nutritional state

This was determined from stomach content analysis. Degree of fullness of stomach of each mussel was also determined. Ten mussels from each sample were removed from the formalin, washed under tap water. They were opened immediately by inserting a sharp scapel blade between the shell valves and cutting the posterior adductor muscles. Then the digestive diverticula covering the stomach was removed. Longitudinal slit was made in the stomach wall and a visual assessment of the degree of fullness of each stomach was made. Four classes were recognised: empty, little, moderate and full. The contents were withdrawn from the stomachs on to a watch glass by means of a

dropper. The contents in the watch glass were throughly mixed. One drop was pipetted onto a glass slide and covered with a cover glass. Ten random fields were examined under the microscope (X100, X400) for the plankton organisms and detritus, after the method of Tan (1973). Only the diatoms and some dinoflagellates were recognized. Thus this were given in percentage according to the whole stomach content. Unidentified species and detritus were excluded from these given percentage.