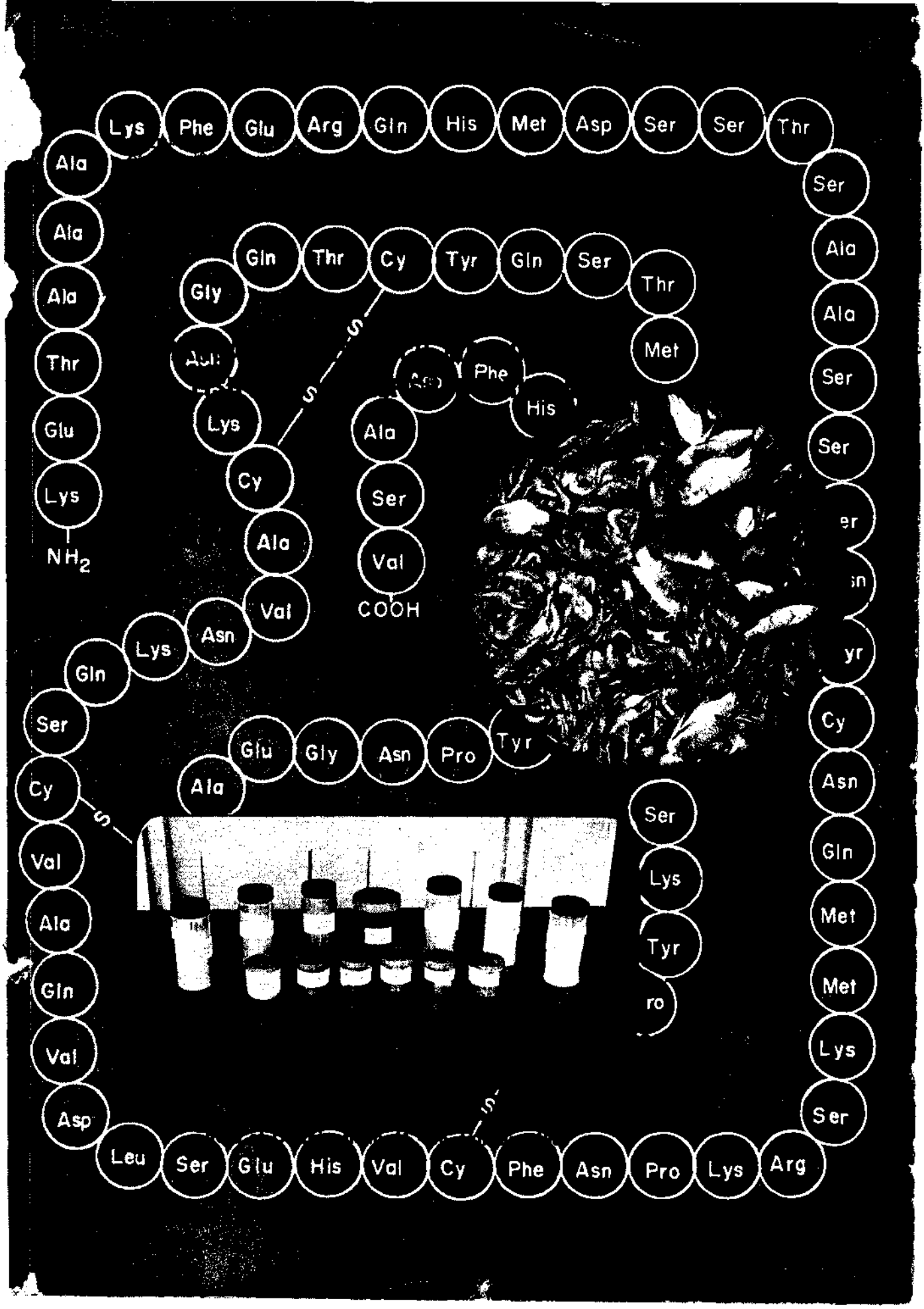
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MANUAL OF RESEARCH METHODS FOR FISH AND SHELLFISH NUTRITION

Issued on the occasion of the Workshop on
METHODOLOGY FOR FISH AND SHELLFISH NUTRITION
organised by
The Centre of Advanced Studies in Mariculture,
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Published by: **E. G. SILAS**
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COCHIN

PREFACE

The Centre of Advanced Studies in Mariculture established at the Central Marine Fisheries Research Institute has been conducting Workshops in Research Methodologies on specialised disciplines with a view to enhance the competence of the scientific workers specialising in researches connected with mariculture. The main emphasis in mariculture research has been directed towards the development of economically viable culture techniques for culturable species of fish and shellfish, with a view to augmenting the fish and shellfish production of the country. In order to develop low-cost technologies the essential operational inputs have to be rationally utilized.

It has been well established that feeding constitutes the major cost of production, often exceeding 50 per cent of the operating costs in intensive aquaculture operations. Two main factors affecting the cost of feeding are composition of the diet and efficiency of feed conversion. In order to develop least-cost formula diets of high conversion efficiency, knowledge of the nutritional requirements of the different species during the different phases of the life cycle and the nutritive value of the complex feed ingredients available in the country to the candidate species is a prerequisite.

The existing information on the nutritional requirements of cultivated species of fish and shellfish in India, is meagre and recently research has been intensified in this area. If researches on this field could be carried out using standardised experimental procedures, the data obtained on the nutritional requirements of the different species could be stored in a fish and shellfish nutrition data bank, from where data could be disseminated to the users such as feed manufacturers, farmers, extension workers and research workers as and when required. It is also necessary that the data collected on the chemical composition of the feed ingredients and their nutritive value for the species should be based on standard chemical methods and experimental procedures so that the data could be stored in

the data bank which eventually could become a National Fish Feed Information Centre.

To undertake studies on the above lines, especially by the technicians and research workers entering afresh into the field, the need of practical guides describing the research techniques and methods, planning of investigations, collection of data and their interpretation need not be emphasized. Keeping this in view, the present manual on Research Methods in Fish and Shellfish Nutrition is issued by the Centre of Advanced Studies in Mariculture on the occasion of the Workshop on Methodology of Fish and Shellfish Nutrition.

Dr. Akio Kanazawa, Professor of Nutritional Chemistry, University of Kagoshima, Japan and Consultant in Fish and Shellfish Nutrition at the CAS in Mariculture, has been kind enough to cooperate with the Scientists of CAS in Mariculture of the Central Marine Fisheries Research Institute in the preparation of this manual. There are chapters in this manual covering various methods on composition analysis of feeds, including growth inhibitors and toxins; determination of digestibility coefficient; protein evaluation; bioenergetics; determination of essential amino acid requirements using radioisotope method; research test diets for fishes and prawns; feed formulation methods; experimental design, etc. Methods of preparation of microparticulate diets, phytoplankton and zooplankton culture methods, etc. are also included to facilitate larval nutrition studies. Many of the methods given in the manual have been standardized for fish and shellfish nutrition studies in India and abroad. The users can also gain maximum benefit by suitable modifications of other methods which are given as guidelines.

I would like to thank all the scientific and technical staff especially Shri S. Ahamed Ali, Dr. K. Alagarswami, Shri D.C.V. Easterson, Shri C.P. Gopinathan, Shri T. Jacob, Shri M.S. Muthu, Dr. R. Paul Raj, Dr. A.G. Ponniah and

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CHAPTER 14

DESIGNING FISH AND SHELLFISH NUTRITION EXPERIMENTS*

1 INTRODUCTION

Statistical method is a powerful tool in experimental investigations. While planning and implementing experimental programmes, this fact, however, has been often ignored by many investigators may be due to lack of familiarity with the subject, as the foundations of statistical science are mathematical. But the logical reasoning and principles underlying the statistical method are not difficult to comprehend and the actual application of these methods in designing experiments and analysing the resultant data are relatively straight-forward and in many cases fairly simple. The present article gives in brief the role of statistical designing in experiments with special reference to fish and shellfish nutrition. A few designs which can be used in nutrition experiments are also dealt with.

2 WHAT IS STATISTICAL DESIGNING TO AN EXPERIMENTER

The formulation and testing of hypothesis are the main features of a scientific method (Kempthorne, 1972). A researcher postulates a hypothesis which he would like to verify. This verification necessitates the collection of observations through an experiment and the designing of experiment is concerned with the pattern of observations to be collected which should be relevant to his hypothesis. Statistical designing involves the formulation of a scheme or lay-out plan where the placements of treatments in experimental units are specified to meet the objectives of the particular problem while keeping in view the statistical requirements like randomisation, replication and local control. (The term 'treatments' is used here in a general way and may mean level of feeding, doses of stimulants, stocking densities, etc.). The experimenter must have such a lay-out plan before administering the treatments so as to enable him to arrive at valid conclusions, the logic of which is acceptable to the concerned scientific community.

* Prepared by T. Jacob, Central Marine Fisheries Research Institute, Cochin-18.

3 WHY STATISTICAL DESIGNING

Variability in experimental material is an inevitable feature in any field of research. Consider for example two fish culture ponds, kept almost under identifical conditions with same species, stocking density, etc. At the harvesting time one would find that the yield of one pond is different from the other. This may be attributed to the uncontrolled or random variation inherent in the production process. Consider again two ponds kept as similar as possible except that in one pond supplementary feeding is provided. Here again at the harvesting time the yields would be found to be different. Can we straight away attribute the difference to the effect of the supplementary feeding? We cannot. May be supplementary feeding did not contribute in any way to the difference in yield and the difference could be purely due to the uncontrolled inherent variation. Differences are expected even when similarity is maintained in the two ponds. One might then say that if the difference is quite high it can be attributed to the level of feeding. But how high the difference should be to attribute it to the level of feeding? The answer becomes quite subjective. Thus variation introduces a degree of uncertainty to the conclusions that are drawn from the results. A mathematical measure of uncertainty is probability, the theory of which enables us to make numerical statements about uncertain outcomes. But this is possible only if the experiment is planned taking into consideration the statistical principles. Only a statistically designed experiment can permit a valid test of significance involving probability statements whether a particular difference is due to chance causes or can be attributed to real difference between treatments.

In practical experimentation one great difficulty is that factors extraneous to those under study mask the real treatment effects. A statistically planned experiment attempts to reduce the effect of extraneous factors from treatment comparisons and also has many desirable properties.

4 CONTROL OF EXPERIMENTAL ERROR

The extraneous variation mentioned earlier is conventionally termed as 'experimental error' where the word 'error' is not synonymous with mistakes but indicate all types of extraneous variation (Cochran et al, 1973). There are two sources of experimental error one refers to the inherent variability in the experimental material or units to which the treatments are applied and the other type refers to the failure to standardise the experimental technique. It is desirable that the experimental error is kept as minimum as possible as otherwise only a large difference in the treatment means will be detected as significant. Reduction of experimental error automatically increases the precision. One way to reduce the error is by ensuring uniformity in the conduct of the experiment. Two other methods to effect the reduction of the error are one by providing more replications and the other by skilful grouping of units in such a way that the unit to which one treatment is applied are closely comparable with those to which another treatment is applied. Some of the general principles governing these methods and other related aspects are elaborated.

5 REPLICATION, RANDOMISATION AND LOCAL CONTROL

Two primary requisites in designing experiments are replication and randomisation. Replication or repetition of treatments provides stability to the mean but more than that makes it possible to estimate the experimental error. It also increases the precision of the estimates of both the treatment mean and the experimental error.

Randomisation which means random allocation of treatments to various experimental units, ensures that a treatment will not be unduly favoured or handicapped in successive replications. It ensures unbiassedness of the estimates of experimental error and provide for valid treatment comparisons against the experimental error (Fisher, 1949). When treatments are replicated and allocated randomly to the various units we are in a position to test the significance of observed treatment differences by

the use of test of significance procedures. Thus it is essential to provide for adequate number of replications and ensure proper randomisation at the planning stage (Panse et al, 1964).

Grouping of Units often help in reducing experimental error. Thus if the experimental units form a very heterogeneous set, try to group them so that units in the same replicate could be large. By this process, from the total variation in the observations the variation between replicates can be removed resulting in the reduction in the error variance (experimental error). The device of reducing errors through suitable groupings is called local control. Looking from another angle, if treatments are allotted to a replication with homogeneous units the observed differences would reflect the real differences between the treatment effects. The principle of local control is the basis for experimental designs such as 'randomised blocks' and 'latin squares'. When the number of treatments to be accommodated in a replicate becomes large, the homogeneity within a replicate tends to be lost and can be restored by dividing the replication into smaller blocks which is the basis of 'confounding' in factorial experiments and also various 'incomplete block designs' (Cochran et al, 1973).

6 SOME USEFUL PLANS

6.1 Randomised block

One of the most commonly used plans is the randomised block design where experimental material is divided into blocks each of which constitute a single replicate in such a way that the units within a block is as homogeneous as possible. The treatments are now randomly allotted to the experimental units within a block. This increases the comparability of treatment effects as they act under conditions which are similar except for the treatments. For instance in an experiment to select an economic supplementary feed mixture from among 4 prepared mixtures for prawn culture, 4 ponds all located by the side of the main water

body like the backwater or estuary, could be grouped as one block or replication and allot treatments at random. The next 4 could be ponds running parallel to the first set but more inside the land so that within a block salinity and associated features are likely to be similar. This arrangement takes care to a good extent salinity gradient likely to be reducing when moved away from the main water body. In the experiment if there are 5 replications there will be totally 20 ponds. If all the 20 ponds are more or less similar no blocking or stratification if required and the treatments could be randomly allotted over the entire range of the 20 ponds. Such a design is called completely randomised design. However, if heterogeneity in the features of the ponds is suspected it is desirable to provide blocks which may help in reducing the experimental error. It may be stated here that if two-way heterogeneity is suspected a latin-square design has to be followed instead of randomised block design which takes care of only one-way heterogeneity.

4.2 Factorial experiments in complete and incomplete blocks

Consider an experiment to study the effect of different levels of protein and energy on body weight of fish in culture ponds. If there are, say, 2 levels for each factor there will be in all 4 (2^2) treatment combinations. A group of treatments which contains two or more levels of two or more factors in all combination is known as the factorial arrangement. The different combinations could be allotted as in a randomised block design. The experimenter could try a one-factor-at-a-time approach. But the advantage in a factorial experiment is that not only the main effects but also the interactions between factors can be studied and tested for statistical significance.

If the number of factors and levels are large say 3 factors at 3 levels each, the number of treatment combinations will be 27. It may be difficult to get 27 experimental ponds, which are more or less homogeneous with regard to factors other than being tested so that the principle of stratification to reduce experimental error cannot be implemented. An

ingenious device to overcome this situation is called confounding where a homogeneous block will not accommodate the full replication. One replication is divided into say, 3 compact blocks such that the units in the smaller blocks are homogeneous. The 27 treatment combinations can be divided into 2 groups of 9 each and allotted to the 3 compact blocks. However some of the treatment comparisons will not be distinguishable from blockdifferences or in other words, get confounded with block differences. Thus some sacrifices have to be made. But at the planning stage this aspect can be considered and the scheme can be so formed that all major and important comparisons are kept free from block differences. Factorial set-up can be easily superimposed in polyculture experiments in pens or in ponds.

6.3 Switch-over

There are occasions in which treatments are applied in sequence over several periods on a group of individuals. Consider an experiment to study the effect of mineral supplementation of two types in lobsters kept in artificial tanks. If there are say six groups of lobsters separated and kept in tanks with sub-partitioning, then the two types of supplementations are given such that half the groups received say, type A and the other half type B in period 1. The lobsters receiving type A in period 1 will be switched over to get type B in period 2 and vise versa. Such a design is called switch-over or change-over design. (Federer, 1967). On the other hand if a time trend is expected in the character under study a switch-back or a double reversal design will have to be used. In these procedures a rest period is to be provided between two treatment periods so that there is no carry over effect or residual effect influencing the treatments during the second period. However if a reasonably long rest period is not feasible or the residual effect is itself a topic of interest the procedure is to be modified so that direct and residual effects of treatments can also be measured.

7 DISCUSSION

The need for statistical designing in scientific experimentation and some common design which can be used in fish nutrition research have been dealt with in the preceding paragraphs. There are several designs available in statistical literature accounts of which are detailed in the references cited.

Once a design is fixed the data collected should be analysed by following procedures relevant to the design. The details of the procedures of analysis corresponding to each design are available in the references mentioned.

The importance of reducing experimental error has been stressed and local control method has been mentioned as a procedure to achieve it. In addition there is a purely statistical procedure to reduce experimental error called analysis of covariance technique where information on a suitably chosen auxiliary variable is used to build up a regression relationship for adjusting the error variance (Snedecar et al, 1967). For instance in a pen culture experiment if the body weights of the fish released into the pens are initially not the same, the treatment comparisons may get vitiated and error increased. The influence if any of the differing body weights on the character under study can be assessed and if found significant necessary adjustments can be made through analysis of covariance procedures.

The cost involved in mariculture experiments will be generally high compared to experiments on land. Replication being one of the essential features of designing, a question often asked is what would be the minimum number of replications required to render test of significance sufficiently sensitive to detect real treatment differences. This depends on the magnitude of variation in the experimental units. If the magnitude is known the number of replications required for detecting a particular difference with a certain level of confidence can be worked out. A fair idea of the magnitude of variation can be obtained from available feed-back data or by conducting a uniformity trial where a particular crop is grown in several contiguous small-sized ponds with uniform treatment and studying

the variation in the yields from these units (Panse et al, 1965). In fact information from uniformity trials would facilitate preparation of contour maps and help in the formation of lay-out plans for the experiment.

There are workers who do not bother to follow a design but want to analyse the data statistically. Some others follow a design but are satisfied with some minimum analysis. It is essential in scientific experimentation to follow a suitable design and make a comprehensive analysis of the valuable resultant data through appropriate statistical procedures. The two aspects go hand in hand.

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9 APPENDIX TO CHAPTER

9.1 Illustration

A randomised block design was employed for carrying out a nutrition experiment to study the comparative effect of

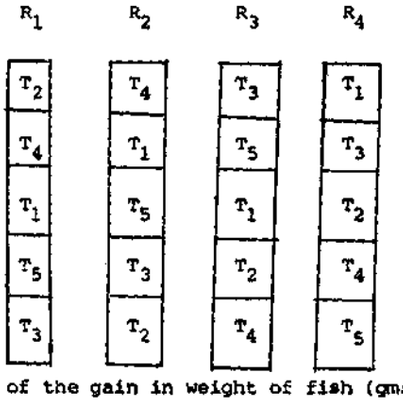
different supplementary feeds (treatments) on growth of sillago fish, keeping factors other than feed uniform.

Number of replications: Four (R_1, R_2, R_3 and R_4)

Number of treatments: Five, one control (T_1) and four supplementary feeds (T_2, T_3, T_4 and T_5)

Total number of ponds used = 4 X 5 = 20

The ponds were grouped into replications such that the five ponds in one replication were as similar as possible. In each replication the treatments were allotted at random. The lay-out plan is shown below:



The figures of the gain in weight of fish (gms./fish) for each of the ponds at the end of the experimental period are given in the following table.

Replication / Treatment	1	2	3	4	Total
1	24	25	23	21	93
2	25	22	24	19	90
3	24	26	21	22	93
4	33	36	32	31	132
5	34	33	31	29	127
Total	140	142	131	122	535

Analyse the data and draw conclusions.

9.1.1 Step-by-step procedure

9.1.1.1 Totals:

(a) The replication totals are:

$R_1 = 24 + 25 + 24 + 33 + 34 = 140$

$R_2 = 25 + 22 + 26 + 36 + 33 = 142$

$R_3 = 23 + 24 + 21 + 32 + 31 = 131$

$R_4 = 21 + 19 + 22 + 31 + 29 = 122$

(b) The treatment totals are:

$T_1 = 24 + 25 + 23 + 21 = 93$

$T_2 = 25 + 22 + 24 + 19 = 90$

$T_3 = 24 + 26 + 21 + 22 = 93$

$T_4 = 33 + 36 + 32 + 31 = 132$

$T_5 = 34 + 33 + 31 + 29 = 127$

(c) The grand total is

$G.T = 140 + 142 + 131 + 122 = 535$

9.1.1.2 Sums of squares (S.S.):

(a) Total:

Crude S.S. $= 24^2 + 25^2 + \ldots + 29^2 = 14811.00$ (20 terms)

Correction factor $= \frac{(535)^2}{20} = 14311.25$

Corrected S.S. = crude S.S. - C.F. = 499.75

(b) Replication:

Crude S.S. $= \frac{1}{5}(140^2+142^2+131^2+122^2) = 14361.80$

C.F. $= 14311.25$

Corrected S.S. $= 50.55$

(c) Treatments:

Crude S.S. $= \frac{1}{4}(93^2+90^2+93^2+132^2+137^2) = 14737.75$

C.F. $= 14311.25$

Corrected S.S. $= 426.50$

(d) Error S.S. is obtained by substraction of corrected S.S. for replication and treatment from the corrected total S.S.

Error S.S. = 499.75 - 50.55 - 426.50 = 22.70

9.1.1.3 Formation of analysis of variance table:

A N O V A

Source of variation	D.F.	S.S.	M.S.S.	F
Replication	3	50.55	16.85	8.94**
Treatments	4	426.50	106.63	56.42**
Error	12	22.70	1.89	
Total	19	499.75		

** Significant at 1% level

It can be seen from the table that the treatment effect is highly significant (1% level). Also the replication effect is highly significant indicating that the grouping of ponds has been effective in reducing the error.

9.1.1.4 Calculation of standard errors (S.E.) for the comparison of two different means:

(a) S.E. of any treatment mean,

$$S.E. = \sqrt{\frac{\text{Error M.S.}}{\text{No. of replications}}} = \sqrt{\frac{1.89}{4}} = 0.69$$

(b) S.E. of difference between any two treatment means.

$$S.E._d = \sqrt{\frac{2(\text{Error M.S.})}{\text{No. of replications}}} = \sqrt{\frac{3.78}{4}} = 0.97$$

(c) Critical difference (C.D.) at 1% level for comparing two means.

= $S.E._d \times t_{0.01}$, where $t_{0.01}$ refers to the 't' value at 1% level for 12 d.f. = 0.97 X 3.05 = 2.96

Note: Comparing two individual means is to be resorted to only, if F test, which is an overall test, is found to be significant.

9.1.1.5 Summary table of means:

Mean gain in weight (gm/fish) for different feeds

T_4	T_5	T_3	T_1	T_2
33.00	31.75	23.25	23.25	22.50

Note: Feeds which do not differ significantly as can be found out with the help of C.D. value are underlined by a bar. Thus T_4 and T_5 are not significantly different as also T_1, T_2 and T_3

9.1.1.6 Conclusions:

The supplementary feeds T_4 and T_5 gave significantly higher gain in weight than rest of the feeds.

Note: If economics is the consideration the cost for unit gain in weight of fish can be computed for each pond and then analysis of variance can be conducted and conclusions drawn by an identical procedure as above.