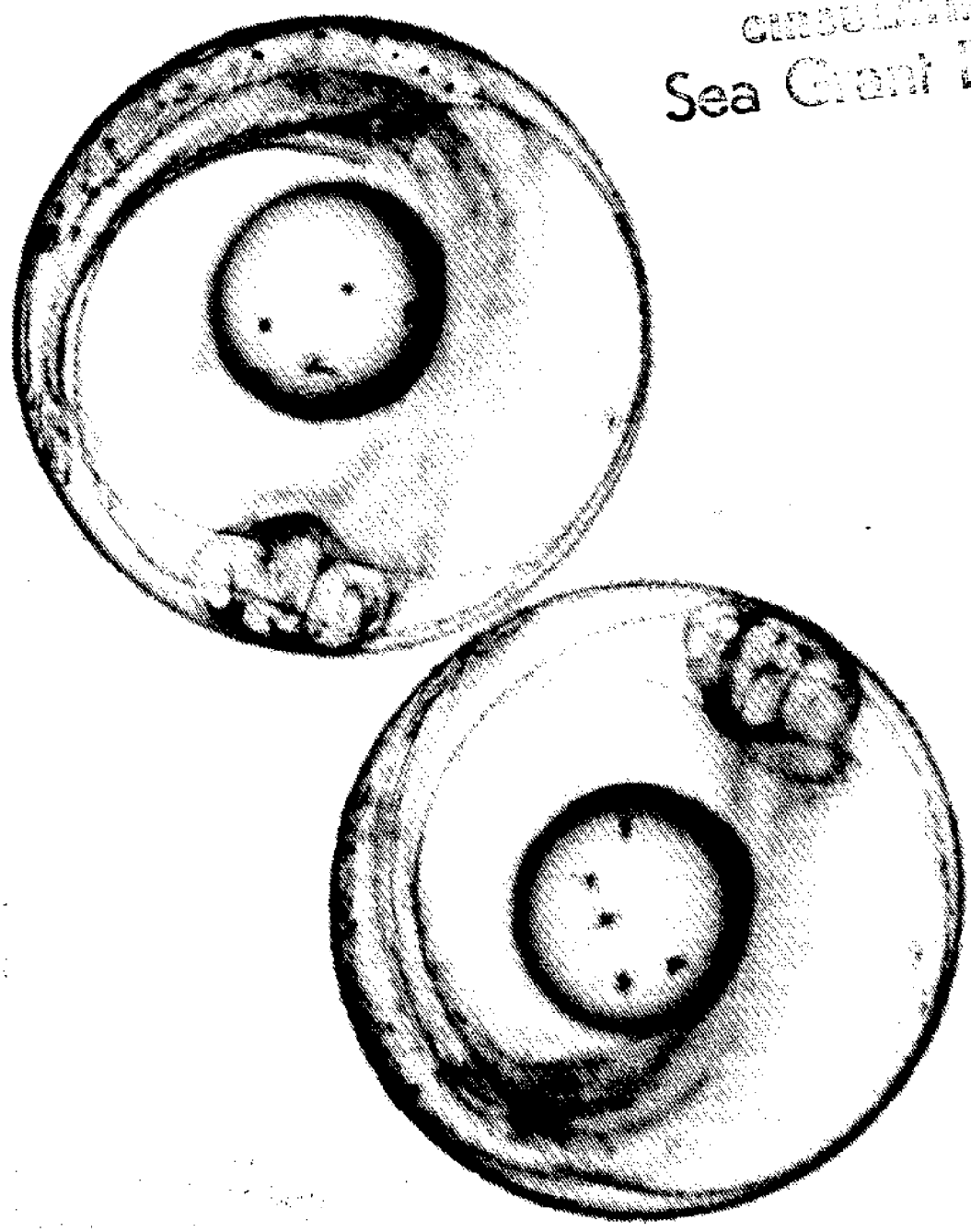
THE GREY MULLET

Induced Breeding and Larval Rearing

1972-73 VOLUME II

Oceanic Institute • Waimanalo, Hawaii



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THE GREY MULLET (Mugil cephalus L.):

INDUCED BREEDING AND LARVAL REARING RESEARCH

1972-73 Volume II

by

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Front cover: Laboratory spawned mullet eggs just prior to hatching. Photo by Chuck Peterson.

Preface

The 1972-73 program on the induced breeding and larval rearing of the grey mullet, Mugil cephalus L., had certain defined objectives. Specifically these were:

- o refine out of season spawning techniques,
- o total replacement of the purified salmon gonadotropin with mammal hormone to induce breeding,
- o further develop mass-propagation techniques to increase larval survival to a commercial level by the use of (a) better live diets, (b) simplified and efficient rearing apparatus, and (c) improved handling methods,
- o continue chemical and biochemical studies relating to natural maturation and oocyte hydration,
- o test the technical and economic feasibility of the extraction and partial purification of gonadotropins from trash fish and/or fish waste with simplified procedures.

The majority of these objectives have been realized and the results are described in this report which comprises seven technical papers.

Work continues with the extraction of gonadotropins from fish waste materials and will be reported in Volume III. Although larval survival in the past year was increased to record levels, the low survival rate through the first fourteen days and uncertain repeatability with eggs from different female fish indicate that there is a great deal of work still to be performed to make mass-propagation attainable.

Included in the report are two papers which summarize experiences and results with hypophysation and culture techniques developed in the last three years, and which recommend the best procedures for the benefit of other workers.

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A PROCEDURAL GUIDE TO INDUCE SPAWNING

Ching-Ming Kuo, Ph. D.,
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Abstract

This procedural guide is for the benefit of colleagues and fish culturists working on the induced spawning of the grey mullet and other species. The paper describes the standard practices developed and repeated successfully to induce spawning annually under controlled conditions.

The method for sampling ova in vivo and identification of the stages of egg development are both described and illustrated. Hypophysation of the gravid females is begun when intraovarian oocytes are at the tertiary yolk globule stage. For the best results the mean egg diameter is preferably larger than 650 μ, but at least 600 μ, before inducement is begun. For small and less developed eggs, hypophysation can be induced by varying dosage and injection sequence as outlined, but the chances of completion are reduced.

Partially purified salmon gonadotropin required to induce spawning is given in two injections in a ratio of 1:2 with a 48-hr interval. The total dose is inversely proportional to the initial egg diameter of recipient females and varies between 12-21 μg/g body weight. An illustrated diagram for calculating dose level is provided.

Fertilization procedures are also described.

INTRODUCTION

The technique of hypophysation has produced rapid progress in the induced breeding of cultivated fishes, particularly for those species which do not readily breed in captivity. Despite prolonged usage, hypophysation practices are impeded by a lack of standardization amongst workers. The dosage, latency period and preferred mode of injection (intramuscular or intraperitoneal) have not yet been defined accurately for any one species, and individual investigators continue to adjust these variables according to circumstances at the time. Such procedures have caused widely differing results, unnecessary duplication of effort and often total failure when repeating techniques used successfully by other investigators.

Quantifying dosage appears to be the major problem in fixing standard procedures. The efficacy of any dose is related to the sexual maturity of both donor and recipient, their phylogenetic relationship, the gonadotropic content of the injected preparation and the physiological state of the recipient (Pickford and Atz, 1957).

The potency of a pituitary preparation or extract must first be specified and standardized. Due to the variety of test animals being used and the bioassay techniques and standard units employed, attempts to standardize the potency of preparations have been ineffective to date. The requirements for standard test animals suggested by Pickford and Atz (1957) and Clemens and Sneed (1962) have not been widely accepted. More precise bioassay methods such as the incorporation of radio-phosphorus by the gonads (Fontaine, 1967) would be more useful.

The phylogenetic specifics of fish and vertebrate gonadotropins in general are controversial and as yet undefined. However, it can be concluded for fish that, although optimal results are obtained with homoplastic pituitaries, quantitative differences which exist within phyla are of little practical consequence to the success of hypophysation procedures. In cases where donor and recipients are phylogenetically distant, the practice has been to increase the applied dosage to overcome any physiological resistance.

The sex-related differences in the gonadotropic activity of fish pituitaries have been reviewed by Pickford and Atz (1957), and more recently by Clemens and Sneed (1962); Sundararaj and Goswami (1969) and Ibrahim (1969). The literature is therefore extensive but at times contradictory. Despite the unequivocal presence of such sex-related differences, the effects can usually be minimized in practice by the preparation of mixed pituitary extracts from both sexes and injecting doses in excess of threshold requirements to induce spawning (Clemens and Sneed, 1962).

Seasonal fluctuations in gonadotropic activity are not uncommon for fish species. High activity has been reported during the spawning season in Heteropneustes (Sundararaj and Goswami, 1969) and Cyprinus carpio (Yashouv et al., 1968) but Clemens and Sneed (1962) reported the absence of any relationship between activity and season in a number of donor species. They found the pituitaries of carp (C. carpio) and buffalofish (Ictiobus sp.) to be active throughout most of the year. In general, however, most pituitaries collected for hypophysation have been taken from mature fish during their natural spawning season (Hickling, 1966).

The effectiveness of the hypophysation technique depends ultimately on the selection of suitable recipient fish at the proper stage of ovarian development. For species of fish which undergo normal gonad development but fail to spawn in captivity, identification of this stage is critical. Replication of efforts by individual investigators can be reduced if the developmental stage of intraovarian oocytes is expressed objectively and accurately.

To date, selection of recipients has been largely subjective. The external anatomical characteristics have been described and used, e.g. depth and fullness of the belly, color and state of swelling of cloaca, softness and resiliency of the belly, roughness of pectoral fins, presence of head tubercules, etc. More complicated approaches describe the microscopic appearance of oocytes (Sundararaj and Goswami, 1969), histological structures of eggs (Chen et al., 1969), or other histological data. Physiological parameters associated with sexual maturation such as elevated plasma proteins and calcium concentration (Booke, 1964; Woodhead, 1968) have also bee used but are of little practical importance. Shehadeh et al. (1973b) described a method for the assessment of ovarian maturity in vivo which is accurate and reliable and replaces all subjective selection methods for Mugilidae.

This paper describes the standard procedures which have been developed and are applied regularly to induce the spawning of the grey mullet (Mugil cephalus L.) under controlled conditions. The methods for the determination of the stages of egg development and required dosage of partially purified salmon pituitary gonadotropin (SG-G100) for spawning are illustrated and emphasized so that the procedures can be readily used by other culturists. The potency of the salmon pituitary gonadotropin has been described by Donaldson et al. (1972) and is given as 1 mg equivalent to 2250 IU human chorionic gonadotropin.

DETERMINATION OF SEXUAL MATURITY OF BROODSTOCK

For grey mullet, Mugil cephalus L., anatomical characteristics are not a reliable indication of maturity. In many cases females with soft and enlarged bellies are in the early stage of oocyte development, even when collected during the spawning season. Enlarged abdomens are often due to engorged intestines and a marked accumulation of visceral fat.

Ovarian maturity, i.e. the stage of development of intra-ovarian oocytes, is measured by the method described by Shehadeh et al. (1973b). The intra-ovarian oocytes are removed in vivo from an unanaesthetized female through a polyethylene cannula. The cannula is inserted into the oviduct for a length of 6-7 cm from the cloaca, and oocytes are sucked orally into the tube by the operator as the cannula is withdrawn. The distance to which the cannula is inserted depends on the length of the ovary. Oocyte samples from the mid-portion of the ovary are the most representative and any sampling error can be minimized by avoiding the extremities.

The oocytes are removed from the cannula and washed and preserved in a solution of 1% formalin in 0.6% NaCl. They are then placed on a small plexi-glass plate and measured under an ocular micrometer. Fine grooves cut in the plate align the oocytes and facilitate measurement. Egg diameters are measured along the horizontal axis and the measurements grouped for 50-u class intervals. The sexual maturity of the fish is expressed by the mean egg diameter calculated from the egg-diameter frequency distribution.

The oocytes of the grey mullet develop in synchrony. Ovarian development can therefore be determined accurately and quickly without sacrificing female fish. The method also provides a means to observe and record oocyte development in individual fish and thus precludes variation between females in the broodstock. Furthermore, it replaces the need for any histological processing and examination of oocytes.

No spontaneous spawning of the grey mullet in captivity has been recorded. The onset of sexual maturation in captivity has been recorded through observation of vitellogenesis of the intraovarian oocytes, but the oocytes have always failed to develop beyond the yolk-laden stage. Consequently, the sequence of oocyte development described and illustrated here focuses on the vitellogenic oocytes.

The oogenesis of oocytes can be readily divided by observation into three general stages. Corresponding developmental stages of oocytes used and described by Yamamoto (1956) are in parentheses. Figure 1 shows the comparison of the microscopic appearance and histological details of oocytes.

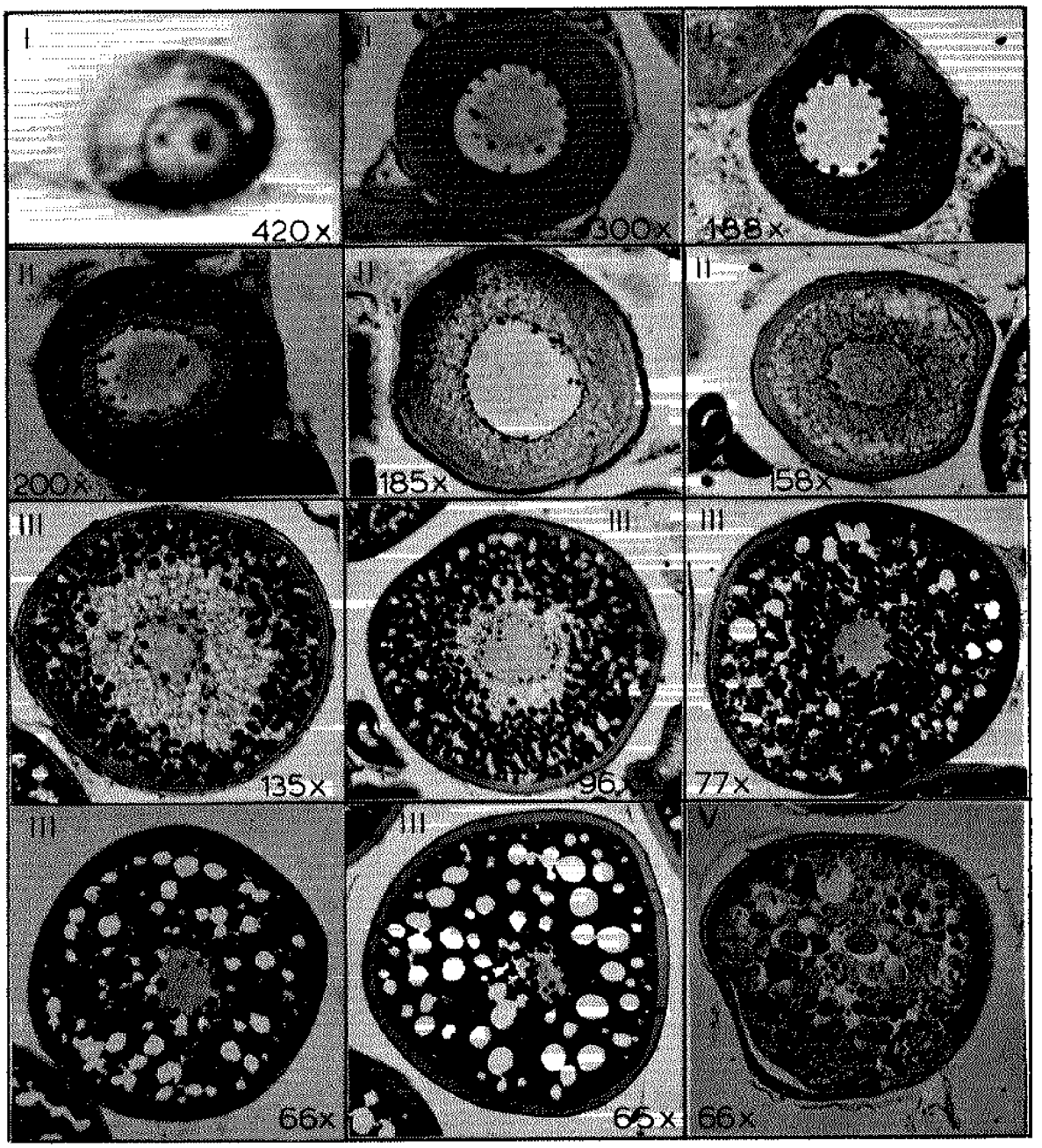


Fig. 1.a Histological details of oocytes, stages I, II, III and V (atresia).

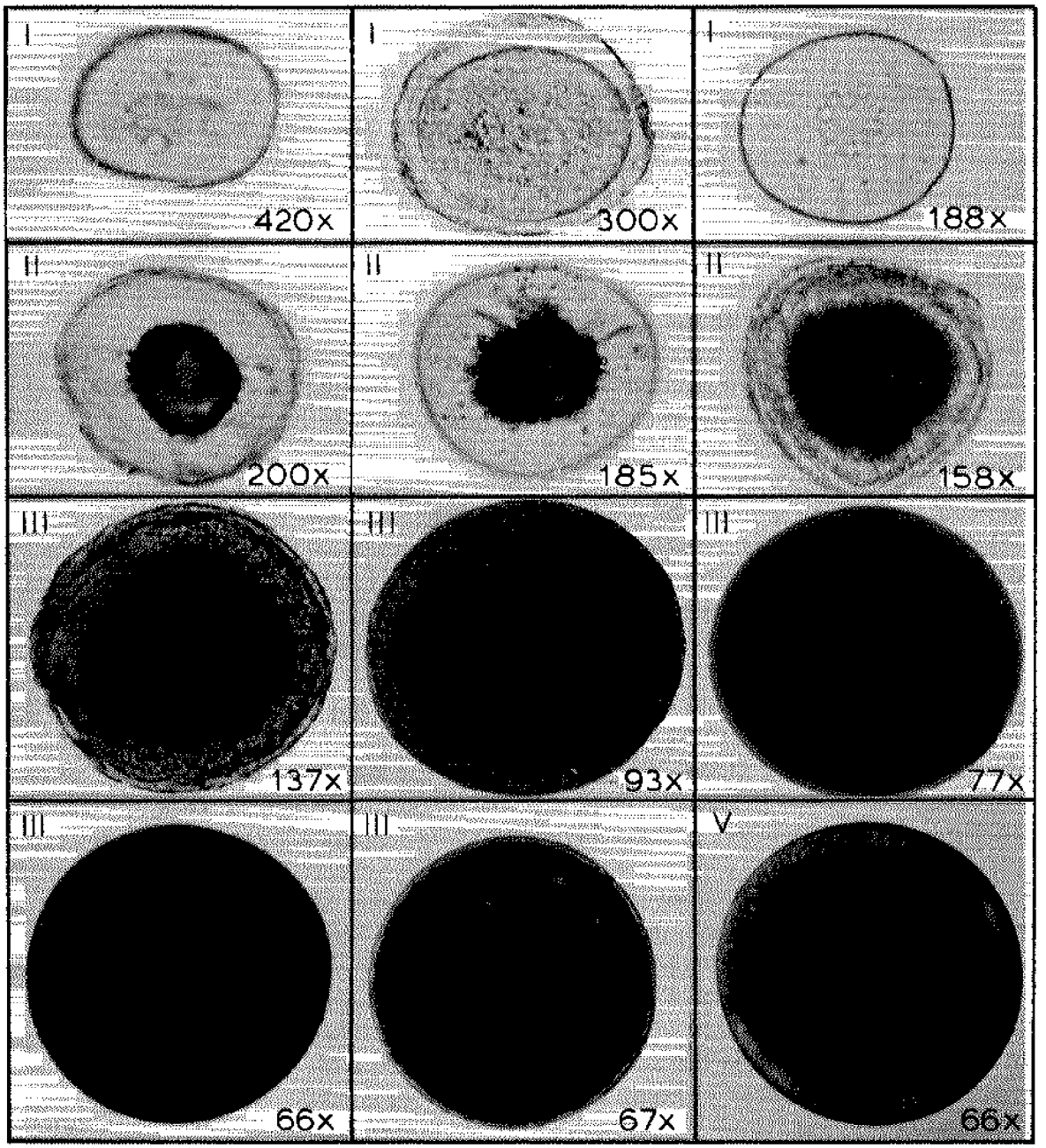


Fig. 1.b Microscopic appearance of oocytes, stages I, II, III and V (atresia).

1. Primary oocyte stage (chromatin-nucleolus and peri-nucleolus stages): 12 - 170 μ in diameter

Primary oocytes are numerous and found in the ovaries throughout the year. At the commencement of the primary oocyte stage, the oocytes are small and spherical or oval in shape. They are transparent and vacuole-like nucleoli can be seen. (Fig. 1, I).

The spherical nucleus of each oocyte, through which one or more chromatin-nucleoli are distributed, occupies the greater part of the cell body within a thin layer of cytoplasm. With the growth of the oocytes, the cytoplasm increases considerably in relative volume and becomes more basophilic. The chromatin-nucleoli also increase in number and move toward the periphery of the nucleus. The oocytes become enclosed by a single thin layer of follicle cells.

As the primary oocyte stage progresses the cytoplasm of the oocytes increases, becoming greater in volume than the nucleus and gradually loses its basophilic nature. The zonation of ooplasm is evident as the outer part of the ooplasm is distinct from the germinal vesicle (Fig. 1, II). The chromatin-nucleoli lie close to the nuclear membrane. Oocytes in the later part of the primary stage are irregular and vary between spherical and tetragonal in shape.

2. Yolk vesicle stage (yolk vesicle stage): 170 - 210 μ

At the beginning of the yolk vesicle stage the appearance of each oocyte is characterized by the gramular cytoplasm and darker zone surrounding the germinal vesicle. There are several outer layers of cells but the zona radiata is not distinct.

The general histological structure of the nucleus has not changed greatly from the late primary stage and chromatin-nucleoli are distributed close to the nuclear membrane in a single row. The nucleoli usually vary in shape from spherical to elliptical, but are at times very irregular. The oocyte is characterized by the appearance of yolk vesicles which, in the early development period of the stage, form a thin layer outside the nucleus. When stained with hematoxylin these yolk vesicles are lighter in color than the ooplasm.

As the oocytes develop, the yolk vesicles increase in number and size gradually filling the cytoplasm from the center of the oocyte toward the periphery. The zona radiata becomes apparent between the ooplasm and the follicular layer. At first it is narrow, compact and homogeneous but it thickens with the growth of the oocyte. The outer layer of follicle cells around the oocyte continues to thicken.

3. Yolk globule stage (primary, secondary and tertiary yolk globule stages): 200 - 700 μ

In the early part of this stage the yolk vesicle occupies the entire ooplasm and the appearance of each oocyte is characterized by a granular appearance (Fig. 1, III). As each oocyte develops, the internal definition is slowly obscured from the center towards the periphery as the deposition of yolk material increases evenly. Finally, when the fusion of yolk globules is complete, the central portion of the oocyte appears to clear.

From histological examination, each oocyte at this stage is characterized by the appearance of minute spherical yolk globules which stain deep black with Heidenhain's hematoxylin. The yolk globules first appear in the outermost part of the ooplasm and increase in number and size as the oocyte grows, and the yolk layer continues to become thicker in proportion to the adjacent layer of cytoplasm. Finally the layer reaches the surface of the germinal vesicle.

The oocytes become nearly spherical or oval in shape. The nucleoli do not change in size and number but begin to lose their peripheral arrangement and become scattered randomly in the nucleus. The follicle layer becomes broader as the follicle cells increase in size and the zona radiata thickens notably. The striation of the zona radiata is now clearly visible. As the oocyte grows, the yolk globules appear to accumulate very rapidly in the inner part of the ooplasm. The germinal vesicle does not change but the chromatin-nucleoli become more regular in shape.

In the final phase of this stage, the yolk globules accumulate throughout the ooplasm. Fusion of yolk globules and the numerous oil globules is apparent. The germinal vesicle becomes irregular in outline and consequently the nucleoli assume an irregular arrangement along the periphery. The nuclear membrane disappears slowly followed by the nucleoli which first become vacuolated and move toward the inner part of the germinal vesicle.

All egg samples can be preserved temporarily in Bouin's fixative and later dehydrated with isopropyl alcohol for histological examination. Shrinkage of oocytes is detectable during this time. Figure 2 illustrates the degree of oocyte shrinkage following preservation in Bouin's fluid. For oocytes between 325μ and 682μ, the anticipated shrinkage for preserved oocytes is between 1 - 5%. For dehydrated oocytes it is about 12.5%. The degree of dehydration through the alcohol series is proportional to the size of preserved oocytes. No significant difference has been found in the slope for shrinkage of oocytes with size but the distance between the regression lines for dehydrated and preserved specimens tested by analyses of covariance is significant at 1% level (Table 1).

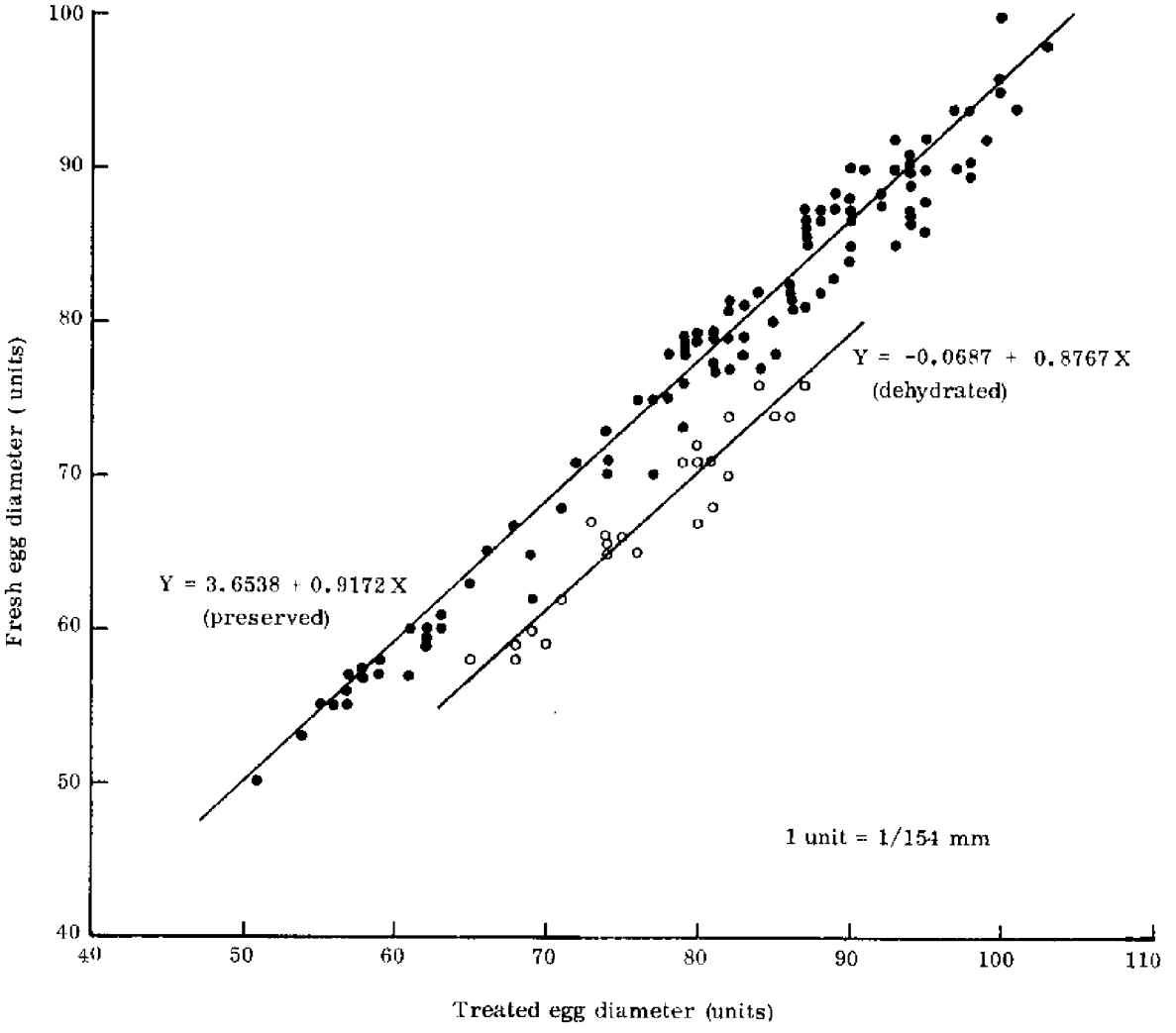


Fig. 2 Shrinkage for preserved and dehydrated oocytes.

Table 1 - Analysis of covariance on the preserved and dehydrated egg diameter
(egg diameters in units: 1 unit = 1/154 mm)

Sample	N	f	x^2	xy^2	y^2	b	f	d^2	M. S.	F
preserved	104	103	18143.04	16641.67	15708.53	0.917	102	443.98	4.35	1.45
dehydrated	24	23	909.33	797.22	764.96	0.877	22	66.03	3.00	
pool							124	510.01	4.11	0.35
reg Coeff.							1	1.42	1.42	
common		126	19052.37	17438.89	16473.49		125	511.43	4.09	
adj. mean							1	893.90	893.90	218.48 ($P<0.0005$)
TOTAL	128	127	19459.72	18421.50	18844.00		126	1405.33		

preserved $Y = 3.6538 + 0.9172$ X unit ($Y = 0.0232 + 0.9172$ X mm)

dehydrated $Y = -0.0687 + 0.8767$ X unit ($Y = -0.00044 + 0.8767$ X mm)

THE PROCEDURE FOR INDUCING SPAWNING

Hypophysation of the gravid females is begun when intraovarian oocytes are filled with yolk, i.e. at the tertiary yolk globule stage (Fig. 1), and the mean egg diameter is preferably larger than 650 μ but at least 600 μ. The hormone used to induce spawning is partially purified salmon gonadotropin described by Donaldson et al. (1972) as being 1 mg equivalent to 2250 IU of HCG. The relationship between the total quantity of hormone required to induce the ovarian maturation expressed by the mean egg diameter is presented in Fig. 3.

The dose of gonadotropin required to induce spawning is inversely proportional to the initial egg diameter of recipient females and varies between 12 and 21 μg/g body weight. The necessary dose, estimated from the regression (Fig. 3) is applied in two injections: one third of the total dose is followed 48 hr later by the remaining two thirds. This sequence appears to be critical to avoid partial spawning. Administration of the first dose does not alter mean egg diameter or diameter-frequency distribution. No change in the morphology is detectable within 24 hr but the central portion of the egg becomes translucent by 48 hr and before the second injection is given.

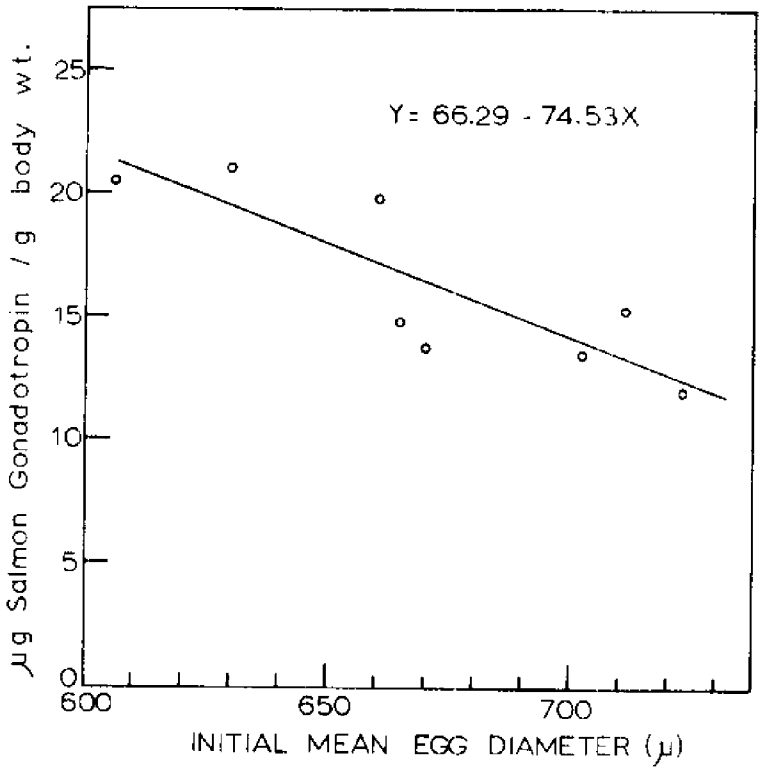


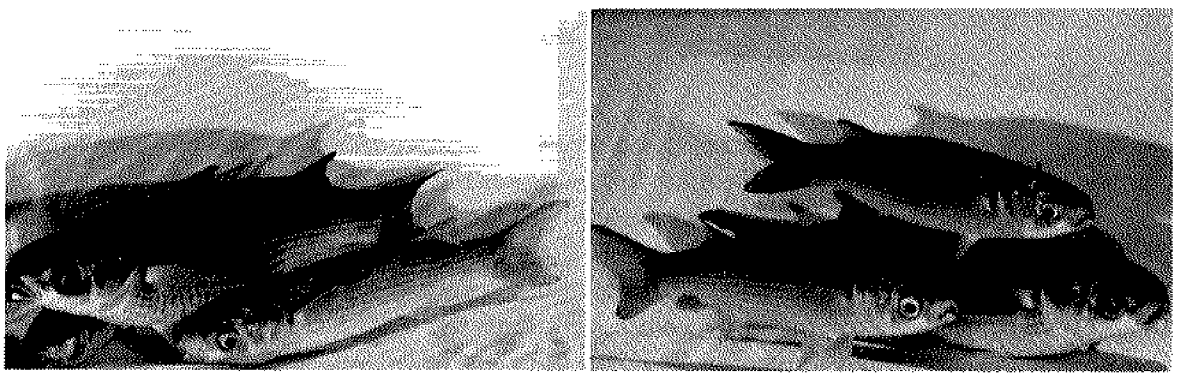
Fig. 3 The relationship between initial mean egg diameter of recipient females and the amount of gonadotropin required to induce spawning. (After Shehadeh et al., 1972)

FERTILIZATION

The abdominal distension of the female is first observed 8 hr after the second injection. Progressive abdominal distension and protrusion of the cloacal region increases as a result of hydration. The water content of the eggs increases from 72.5% to 83.5% at the time of spawning, and osmolarity increases from 250 mOsm/kg to 665 mOsm/kg. The cloacal opening changes from a horizontal (related to the base line of the anal fin) to a vertical position.

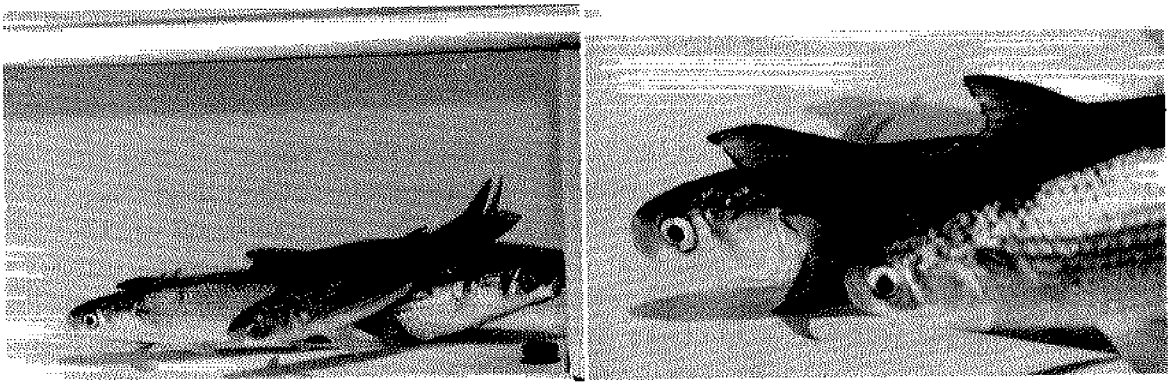
Two or three males are placed in the 50-gal aquaria with the female about 2 hr after the second dose. As hydration progresses, indicated by the belly distension and frequent excretion of calcium deposits, the males become more active and remain in close contact with the female (Fig. 4.a-d). Occasionally the males will spin around the female or nudge her cloacal region (Fig. 4.e-f).

The first release of a small number of ripened eggs stimulates the males to liberate spermatoza and fertilization follows. The first cleavage of the fertilized eggs can be observed in about one hour if water conditions are at 24°C and salinity of 32.2‰. The percentage fertilization rate is determined by microscopic examination of a sample of eggs one hour after spawning.

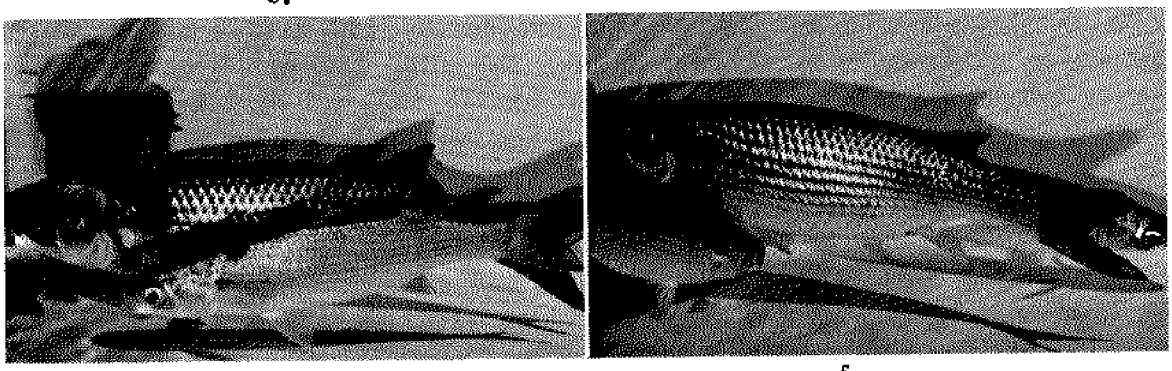


a. b.

Fig. 4 Courtship behavior prior to spawning.



c. d.



e. f.

ADDENDUM

Vitellogenesis of intraovarian oocytes of captive grey mullet has been observed to proceed naturally prior to the breeding season up to the tertiary yolk globule stage, but it can also be induced out-of-season by the manipulation of photoperiod and temperature regimes (Kuo et al., Chapter VI). However, no instance of complete development or spontaneous spawning in captivity has been observed. The procedures described above are effective for spawning females when the mean diameter of their oocytes is beyond 600μ.

At the end of the breeding season several females remain in the broodstock but, although their oocytes have progressed to the tertiary yolk globule stage, the egg size is below an effective range for injection, i.e. below 600μ. By careful procedures, spawning can still be induced in these females. Growth of the oocytes is accelerated readily from 500μ by daily injection of pituitary gonadotropin in increasing doses from 0.12 to 2.4 μg/g body weight (Fig. 5) The series lasts from 6 - 10 days. A decrease in water temperature can also assist oocyte development (Fig. 6). The last portion of hypophysation then follows as described.

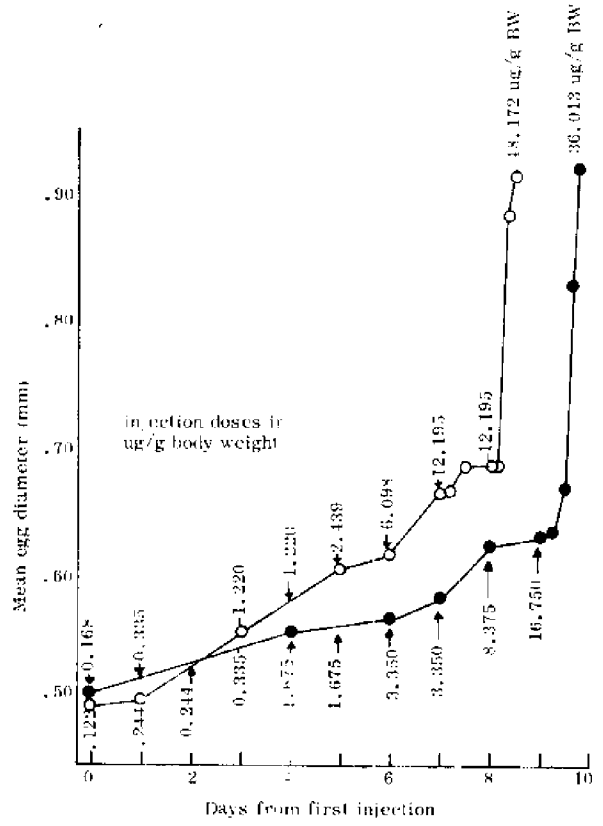


Fig. 5 Growth of oocytes with increasing gonadotropin dose.

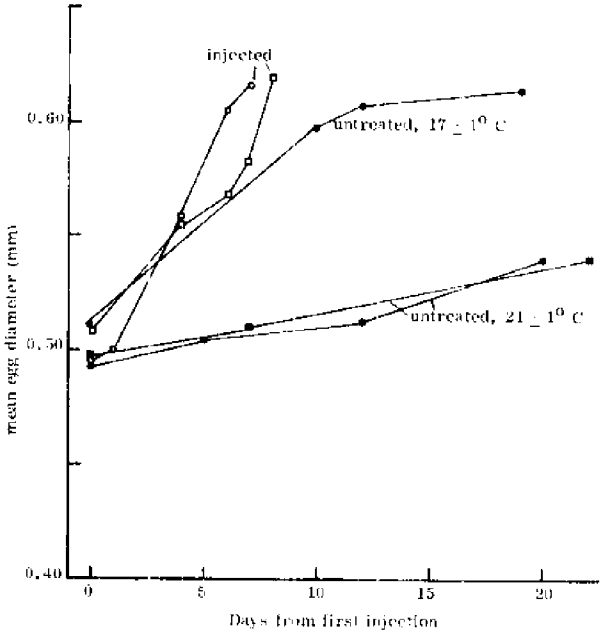


Fig. 6 Effect of water temperature on oocyte development.

II

INDUCED SPAWNING OF CAPTIVE FEMALES BY INJECTION OF HUMAN CHORIONIC GONADOTROPIN (HCG)

Ching-Ming Kuo, Ph.D.,
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Abstract

HCG can be used effectively to induce the spawning of the grey mullet, Mugil cephalus, replacing the more expensive purified salmon gonadotropin. The best procedure for the use of HCG is to inject a priming dose of about 20 IU/g body weight when the mean oocyte diameter for that female is 0.600 mm. This is then to be followed by a second and larger dose (about 40 IU/g body weight) after an interval of 24 hr. Spawning can be anticipated within 24 hr.

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Pickford and Atz (1957) summarized attempts to induce maturation and ovulation of teleostean fishes with mammalian gonadotropin, but to date successful induced spawning is confined to a few teleostean species, namely Cyprinus carpio, Perca fluviatilis, Carassius auratus and Heteropneustes fossilis. Techniques for inducing spawning in teleostean fishes have been reviewed by Shehadeh (1970).

Spontaneous spawning of captive grey mullet, Mugil cephalus, has never been recorded. Although captive males complete spermiogenesis, the vitellogenesis of ovarian oocytes in captive females has been observed only up to the tertiary yolk globule stage. Spawning of the grey mullet during the natural breeding season (December through February in Hawaii) has been induced by injection with the homogenate of salmon pituitaries, preserved in ethyl alcohol, combined with Synahorin [Teikoku Hormone Mfg. Co., Ltd., Japan] (Shehadeh and Ellis, 1970).

Shehadeh et al. (1973a) reported the successful induced spawning of captive mullet using purified salmon gonadotropin. In addition, they advanced the spawning season by four months through the artificial control of photoperiod and temperature.

This section describes the results obtained in an attempt to replace the salmon pituitary gonadotropin with the cheaper and more readily available mammalian hormone, human chorionic gonadotropin (HCG).

Six female mullet (Table 1, no. 1-6) from a captive population maintained in running seawater (32 $^{o}/_{oo}$) were used in the first experiment. Before each injection sequence, oocytes were sampled in vivo using a polyethylene cannula and were measured with an ocular micrometer. All measurements (see Table 1) indicated that the oocytes were at the tertiary yolk globule stage of vitellogenesis and the mean oocyte diameter was greater than 0.600 mm.

The HCG hormone injection treatment consisted of an initial priming dose followed by a larger dose after a period of 24 hr. In the interval between injections, the mean oocyte diameters of all females increased (see Table 1).

Ten hours after the second injection, two ripe males were placed with each female in separate aquaria. The first release of a small number of ripened eggs stimulated the males to liberate spermatozoa and mass liberation and immediate fertilization followed. All females spawned between 11 and 21 hr after the second injection.

Table 1 Dose rates, schedules, and reactions of Mugil cephalus females to HCG injection.

Time (hr)		0		12		24		48				
Fish No.	Body wt. (g)	Dose (IU/g body wt.)	$\bar{X}$* (mm)	Dose (IU/g body wt.)	$\bar{X}$ (mm)	Dose (IU/g body wt.)	$\bar{X}$ (mm)	Dose (IU/g body wt.)	$\bar{X}$ (mm)	Total dose (IU/g body wt.)	Fertilization (%)	Time of spawning (hr)
1	634	28	0.607			50	0.630			78	45	45
2	819	21	0.617			48	0.631			69	54	35-1/2
3	872	16	0.617			33	0.630			49	60	40
4	908	19	0.621			39	0.630			58	98	37-1/4
5	759	20	0.645			30	0.651			50	84	39
6	1092	14	0.645			42	0.659			56	92	37-3/4
7	795	13	0.641	20	0.649	16	0.654			49	+	42-1/3
8	1032	13	0.625	23	0.639	18	0.648			54	94	41
9	876	12	0.616			24	0.633	16	0.632	52	+**	70-1/2

* Mean oocyte diameter

** Cloacal opening plugged

\+ Partial spawning, no fertilization rate determined

A sample of 200 eggs was examined microscopically one hour after mass fertilization to observe early cleavages, and the percentage of fertilization success for each fish was determined.

Subsequent experiments attempted to maintain the same overall HCG dosage for each female fish to induce spawning, but to vary the injection sequence. Two female fish (Table 1, no. 7-8) received two additional low-level doses at intervals of 12 hr after the priming dose. The mean oocyte diameter for both fish increased after the priming and second injection, and spawning occurred about 18 hr after the third injection. In one case, no. 7, spawning was not totally successful.

Finally, another female received two additional low-level injections at 24- and 48-hr intervals after the initial priming dose. The mean oocyte diameter increased after the priming injection but not after the second. Spawning was again only partial and after a further 24-hr interval.

It appears from the data that a priming dose as low as 13 IU/g body weight of HCG is as effective as doses up to 30 IU/g body weight in initiating oocyte development beyond the critical point. The mean oocyte diameter of all experimental fish had reached the range 0.630-0.660 mm before the final HCG injection. A similar result was reported by Shehadeh et al. (1973a) when the priming dose of purified pituitary gonadotropin caused an increase of mean oocyte diameter to the range of 0.650-0.700 mm.

Frequency of HCG was not a determining factor inducing spawning. Liberation of eggs occurred some time after the total dose of HCG injected reached an effective level, say above 50 IU/g body weight of recipient females. However, the spawning and fertilization was not always successful.

III

OPERATIONAL PROCEDURES FOR REARING

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and
Susan C. McConnell, B.A.

Abstract

Larvae of the grey mullet, Mugil cephalus L., can be reared successfully using careful procedures following induced spawning of the adults with purified salmon gonadotropin or human chorionic gonadotropin.

Preliminary incubation of the eggs to the gastrulae stage at high density for 12 hr at 22° C is preferred using stirred irradiated seawater (32 °/oo) and the antibiotics penicillin (80 IU/ml) and streptomycin (0.05 mg/ml). The eggs are transferred before hatching to circular rearing tanks at a density of 250 eggs/l.

The mouths of the larvae are open and ossified and eyes sufficiently pigmented to begin feeding on the third day. The larvae are fed cultured phytoplankton and rotifers supplemented with any available natural plankton. The nauplii of Artemia are fed on the seventh day.

The migratory behavior of the larvae during early development and their physiology are also discussed, being relevant to the procedures and the techniques applied.

Mass-propagation in very large (over 12 ft diameter) circular tanks with total light control is recommended.

INTRODUCTION

The artificially induced spawning of Mugilidae has been studied for many years because of the potential of these widespread and desirable fishes to increase the available supply of aquatic protein resources for developing nations.

Sanzo (1936), Anderson (1957), Yang and Kim (1962) reported successful artificial fertilization of species of Mugilidae using ripe adults captured at sea. Tang (1964) described the first hypophysation-induced spawning of the grey mullet, M. cephalus, using homogenates of mullet pituitary glands. This has also been achieved with the pituitary homogenate of carp (Yashouv, 1969); of mullet again (Liao et al., 1971); and of Pacific salmon (Shehadeh and Ellis, 1970).

Shehadeh et al. (1973a) developed a spawning procedure for the grey mullet after establishing a method for determining ovarian development, and delineated dose rates and frequencies using purified salmon gonadotropin. Subsequently they obtained data for procedures using a variety of mammalian hormones, such as human chorionic gonadotropin (HCG) (Kuo et al., 1973b) and Synahorin (Liao et al., 1971).

Since the breeding and spawning of the grey mullet became controlled, attention has been directed towards the intensive rearing of the larvae. Liao et al. (1971) and Kuo et al. (1973a) report successful rearing of the emergent larvae through the 42-day larval period. Survival was very low (below 5%) and both groups noted that the greatest mortality (above 90%) occurred within the first eleven days of life.

This report combines the best techniques which have been developed to date to increase the survival of the larvae in the early and most delicate stages of development and presents the operational procedures recommended for successful mass-propagation.

PRODUCTION PROCEDURES

1. Fertilization

The female mullet are induced to spawn naturally in captivity by the procedures established by Shehadeh et al. (1973a) using purified salmon gonadotropin or HCG (Kuo et al., 1973b). The eggs must be in the tertiary yolk globule stage and larger than the 'critical' mean egg diameter of 600 μ as determined by in vivo sampling and measurement. A priming dose of the hormone initiates the final stages of vitellogenesis and induces successful spawning. For purified salmon gonadotropin, a total dose of between 12-21 μg/g body weight is required (inversely proportional to the initial mean egg diameter) and applied in two injections. Time interval between injections is either 24 or 48 hr depending on observed development after further in vivo sampling. A second dose (about twice the volume of the first injection) after 48 hr requires less hormone to initiate spawning and is therefore more economical to use.

If the eggs are only developing slowly through the tertiary yolk globule stage and the mean egg diameter is less than 600 μ, a series of ten injections of salmon pituitary increasing in dose and frequency, can initiate spawning. The total dose for the series increases to between 36-48 μg/g body weight.

Males do not require gonadotropin injections for spermiogenesis to be completed if breeding is within the natural spawning season (December to March in Hawaii.

Fertilization will occur naturally in small aerated fiberglass tanks (140-l. capacity) with one glass side for observation. Two or three males are used with each conditioned female and all may release sperm when the female spawns. The fecundity of the female grey mullet is estimated to be 849± 62 egg/g body weight. Consequently, about 1 million eggs are released from each female and fertilized at spawning. Microscopic examination of the eggs 55 to 65 minutes after spawning enables percentage fertilization to be determined. Usually it is above 90 percent.

After spawning, the adults are removed from the tank and the aeration intensified to maintain the fertilized eggs in constant motion. A period of 20 minutes is allowed to elapse whilst fertilization is assured. The eggs are then distributed evenly into two large circular polyethylene tanks (800 l.)

containing filtered and irradiated seawater and where aeration is again vigorous. The eggs remain in these containers for a further period of 12 hr.

2. Incubation

The embryonic development of the eggs of the grey mullet has been described both by Liao et al. (1971) and Kuo et al. (1973a). Mean egg diameter is about 930 μ and the eggs are characterized by a single large oil globule which gives the eggs buoyancy. Development proceeds rapidly and gastrulation is reached 12 hr after spawning.

The behavior of the eggs during incubation has been described at length because of the differing observations and results. Sanzo (1936) observed that all the fertile eggs sank soon after fertilization, whilst Yashouv (1969) reported sinking towards the end of incubation. Kuo et al. (1973a) recorded that the majority of eggs which sank were unfertilized or undeveloped. All these workers, together with Perceva-Ostroumova (1951) and Yashouv and Berner-Samsonov (1970), observed that eggs could have more than one oil globule, but Kuo et al. (1973a) inferred this to be directly related to premature inducement by manual extrusion. Spontaneous release of eggs by the female produces eggs with a single oil globule.

Recent observations confirm that many eggs do sink during the first 12 hours if not maintained in suspension by strong aeration. However, unlike the eggs of some flatfish (Shelbourne, 1964; Flüchter, 1965) a percentage of these eggs are viable and will complete incubation on the bottom of the container if left. It is probable that the majority of mullet eggs which sink would develop if distributed evenly but that high mortality is increased by contact, agglutination and bacterial attack.

After 12 hrs, aeration is suspended. The majority of eggs rise to the surface and float as water movement in the tanks ceases. The eggs which sink immediately are opaque and unviable (up to 10 percent). The buoyant viable eggs are removed from the surface of the container in a cotton hand net in reasonable quantities, washed under running irradiated and filtered seawater, and then dipped for 1 minute in a seawater bath containing the antibiotics potassium penicillin G (80 IU/ml) and streptomycin sulfate (0.05 mg/ml). They are then distributed into the rearing kreisels at a density of about 250 eggs/l. and incubation is continued for the remaining 24 hr.

The kreisel (Fig. 1) has proved to be an effective rearing apparatus and can be constructed with many dimensions. Aeration of the water and circulation

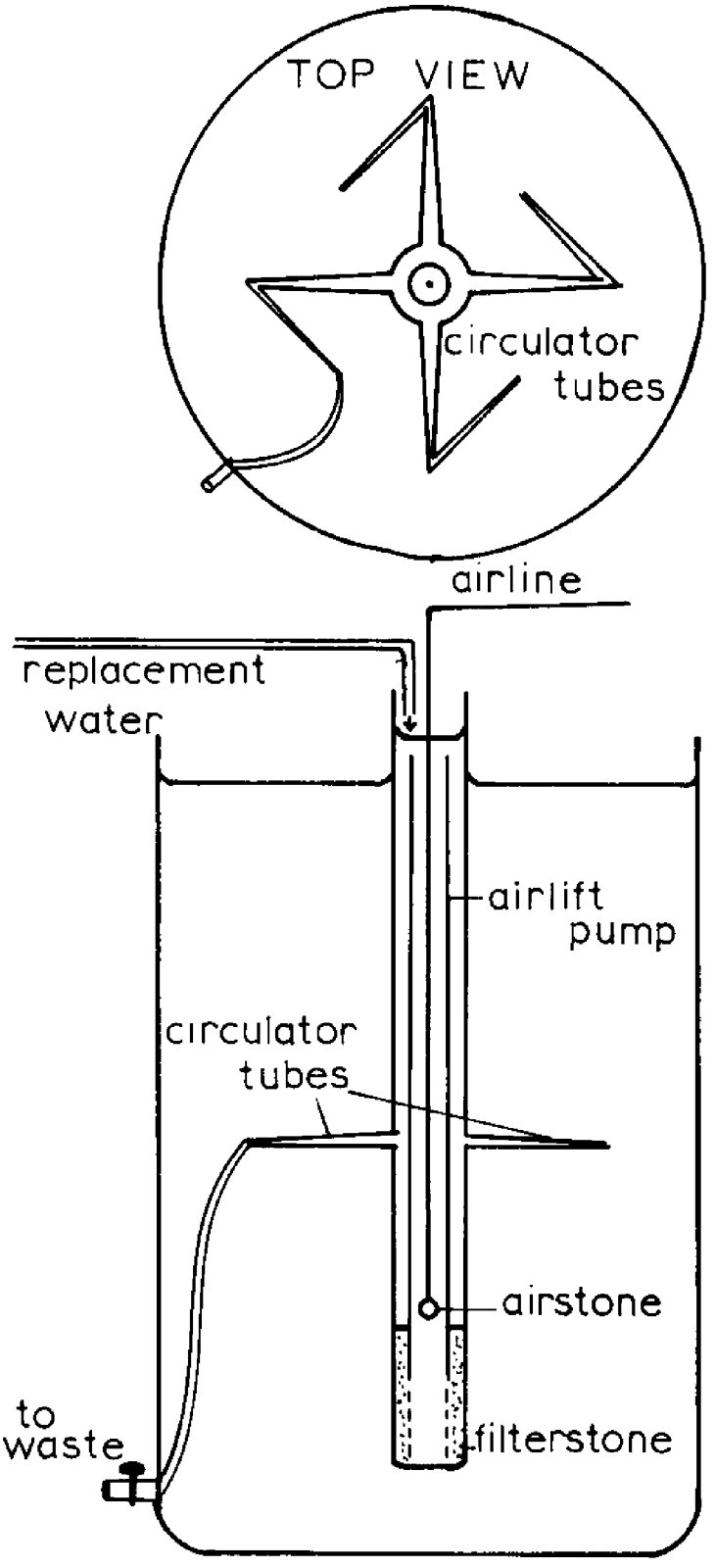

Fig. 1 Rearing kreisel

are maintained by an air-lift pump located in a larger center column. The water is drawn by the air-lift pump into the column through a large filterstone, aerated and passed back down the outside of the pump and redirected into the container. A connection on one of the discharge arms of the column directs a fraction of the water to waste and the volume is replaced by an incoming supply fitted above the column.

The seawater in the kreisel (32 $^{o}/_{oo}$ salinity) is previously filtered and irradiated and treated again with antibiotics. Both penicillin (10 IU/ml) and streptomycin (0.01 mg/ml) are added daily and are effective in controlling marine bacteria.

Continuous circulating motion keeps all the eggs in suspension for the remainder of the incubation period. Incubation time is temperature dependent and temperature is a major limiting factor. Figures 2 and 3 illustrate the relationship between incubation time and temperature, the optimum temperature (22^{o} C) and effective temperature range (10-24^{o}C). No data is available as yet on the influence of salinity on incubation. All other known work to date uses fully saline water (30-35 $^{o}/_{oo}$).

3. Larval Rearing

Highest incubation survival occurs within the temperature range 21-24^{o} C with an optimum at 22^{o} C. Within these temperatures hatching occurs around 36 hr after spawning. The larvae are immediately free swimming and average 2.6 mm in length. During early development at these temperatures the larvae utilize the yolk sac and oil globule for nutrition but absorption times are different (Fig. 4). The yolk sac is fully utilized by day 5 but the oil globule can still be present at day 9 (24^{o}C) or day 14 (22^{o}C).

The presence of the oil globule continues to influence the activity of the larvae. Kuo et al. (1973a) described and illustrated the resting and swimming movements of newly hatched larvae, and described the changes in vertical distribution during the first fifteen days. They reported two major larval descents, at 60 hours and 156 hours after hatching, both accompanied by changes in specific gravity of the larvae and by major mortality. The mortality after the second descent was greater than that of the first and usually total. They observed that the vertical migrations were not phototrophic reactions, and were controlled by internal rhythm.

A change in rearing technique now prevents mortality following the descent on the third day. It is believed that on the 2-3 day the larvae respond

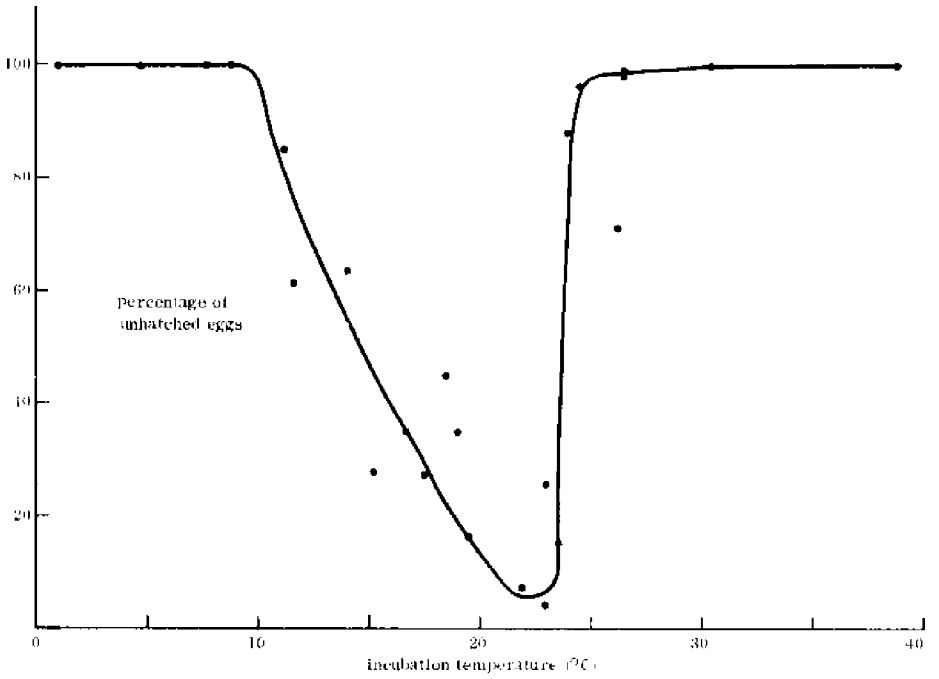

Fig. 2 Eggs: mortality (%) and temperature (^{o}C).

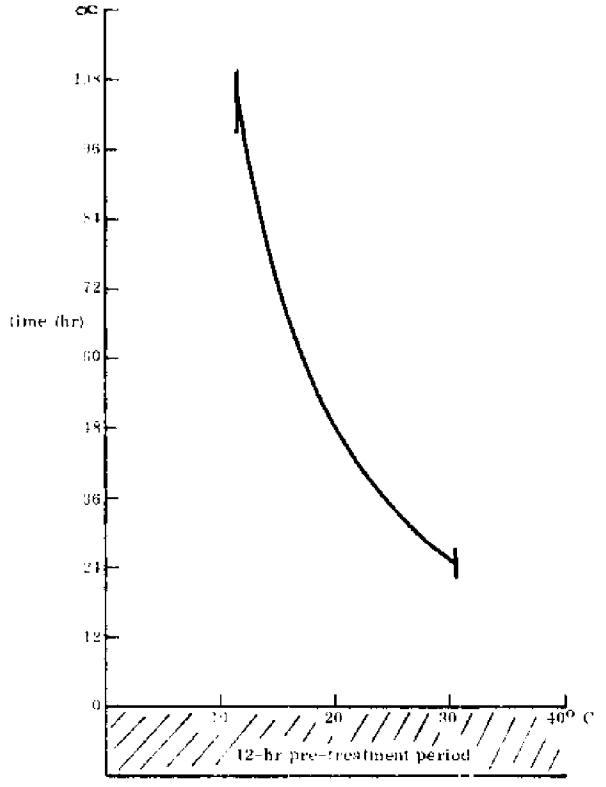

Fig. 3 Eggs: temperature (^{o}C) and incubation period (hr) from gastrula stage.

Figures 2 and 3 by courtesy of EPA Grant R-800924 (after Nash and Sylvester unpublished data).

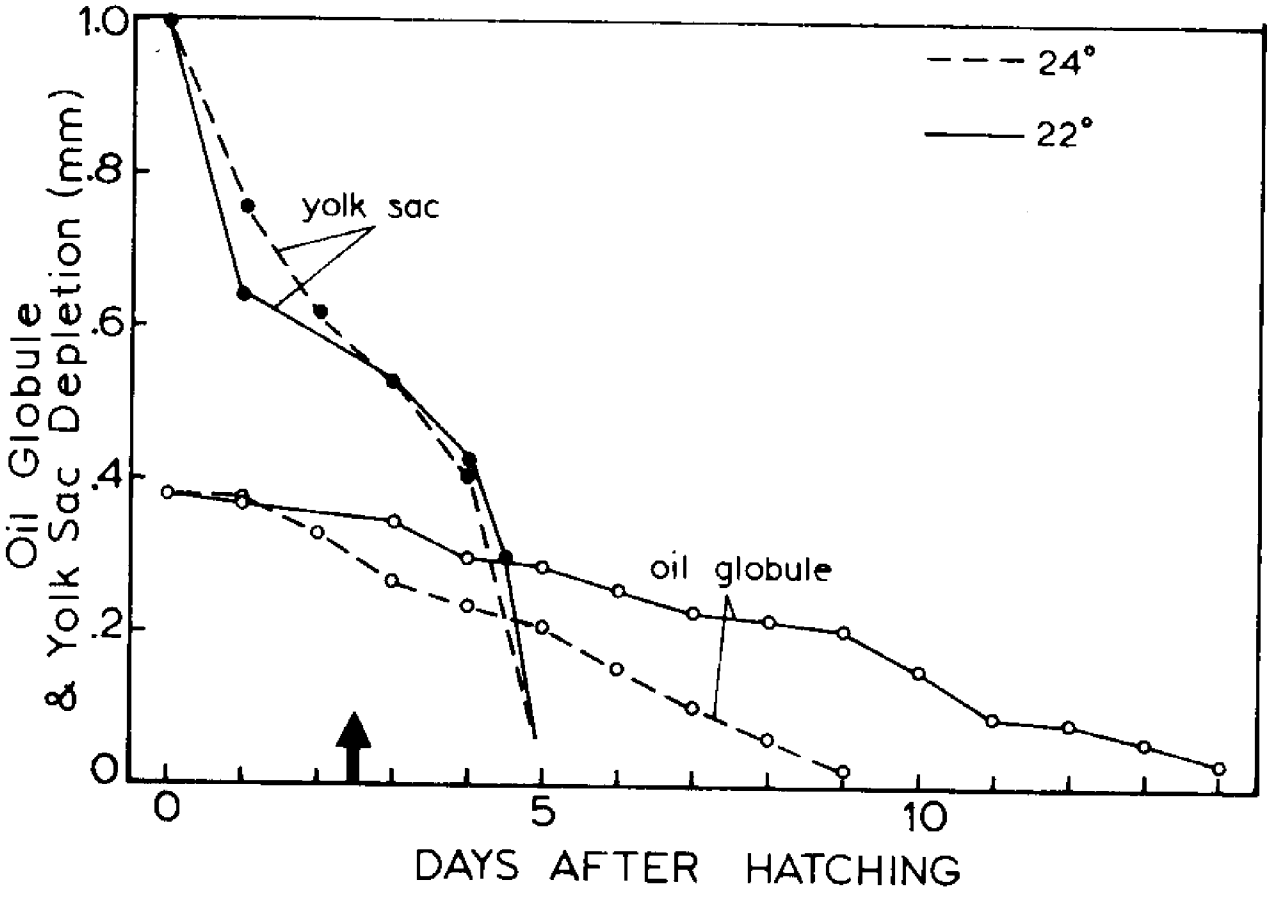

Fig. 4. The relationship between yolk sac and oil globule depletion and larval growth at 22 and 24 °C. Arrow indicates time of mouth opening. Yolk and oil globule measurements indicate length and diameter respectively. (From Shehadeh et al., 1972)

positively to morphological and internal physiological changes and sink to the bottom. However, in the kreisel they are not exposed directly to intense and damaging aeration. Shelbourne (1964) used minimum aeration to circulate the water for the mass-propagation of marine flatfish. Emergent larvae make little demand on the available oxygen in the seawater, and the use of aeration to replace the oxygen consumed is not necessary. Water movement, on the other hand, is important to prevent interference and crowding. After the larvae sink on the third day they can ascend soon after and appear to have muscular control. Although the active larvae are incapable of sustained swimming and rest a great deal, they are sufficiently motivated to move up and down the full water column at will.

After the third day, when the larvae are independently active, water circulation in the kreisel can be intensified. The mouths of the larvae are open and the eyes are pigmented sufficiently for food to be presented. Work by Blaxter (1969) revealed the importance of light intensity for the feeding and rearing of teleostean larvae and each kreisel is fitted with overhead illumination.

The second vertical migration, which is observed between 7-8 days after hatching, is associated again with a change in specific gravity of the larvae (Kuo et al., 1973a). Morphological observations of the larvae show that the pneumatic duct of the air bladder is occluded but the significance of this is not known. Mortality after the second vertical migration is high, but recent observations indicate that two factors are concerned: one is associated with the morphological and physiological changes; the other with nutrition.

Observations show that there are differences between feeding and non-feeding larvae by day 7 after hatching. Established larvae are longer (Fig. 5), are more active and are more broadly and intensely pigmented. The unestablished larvae usually remain in the surface water layers, often using the increased surface tension along the sides of the container to support themselves. Development of the pigment is slow and little or no growth is evident. Between days 7 and 10 they sink to the bottom and die.

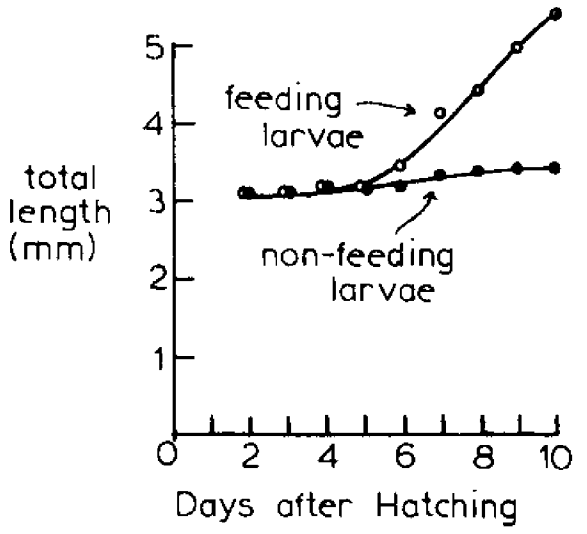

Fig. 5 Growth data for selected feeding and non-feeding larvae.

Therefore, between days 7 and 10 after hatching, all larvae descend for one of two purposes. The under-developed are too weak to sustain themselves after the yolk sac is utilized and consequently sink and die. The developed still have to undergo a physiological or morphological process which must be critical. As with the flatfish, which become passive as they undergo a major metamorphosis after seven weeks (at 10° C), the mullet larvae become inactive and sink. Not all complete the transition or transformation so again there is mortality.

Survival has been increased in the early larval stages by increasing the size of the rearing kreisel (12' diameter) and increasing aeration and circulation. A percentage of the larvae do not live through the weaning stage, but the chances for them to survive have been increased by keeping them in suspension with the food instead of giving them opportunity to rest in the surface tension layers or along the sides of the container.

4. Larval Feeding

The mouths of the larvae open on the second day and food is offered on the third day when the mouths are known to be ossified and eye pigment is sufficiently developed. Although emergent larvae can survive without food beyond day 5 and even to day 10, both Blaxter and Hempel (1961) and Lasker et al. (1970) have demonstrated an irreversible starvation level which it is necessary to avoid. In view of the previous observations on vertical migration it is believed that such a critical period for the mullet occurs early in its development, probably about day 5.

May (1970) reviewed the larval food used by fish culturists for many species and most have been tried on mullet larvae with some degree of success. Liao et al. (1971) used oyster veligers; Kuo et al. (1973a) used both oyster and sea-urching veligers followed by day-old nauplii of Artemia salina. In addition, many of the more common phytoplankters have been tried, such as Gymnodinium, Chlorella, Dunaliella, Isochrysis, etc. Rotifers (Brachionus sps.) and Isochrysis have proved to be the most successful to date during the early days of development, followed by Artemia nauplii on day 7 after hatching.

Survival has been increased if the feeding regime is supplemented by the addition of marine plankton from day 3 onward. By day 7, the most advanced larvae can be observed to feed off the bottom of the kreisel. By day 14 the larvae are firmly established on Artemia nauplii. By day 42, when the larvae have become established juveniles and fully scaled, the diet can consist of commercially available feed formulations (flakes then pellets) supplemented with larger Artemia nauplii. A feeding program is illustrated in Fig. 6.

5. Survival

The most successful survival estimated in the largest kreisel used is 10,000 larvae on day 14 after all known critical phases had been passed. High survival through the first fourteen days still does not guarantee large numbers of juveniles fully established at day 42. Poor management can still cause high mortality. There is evidence that survival is dependent on tank

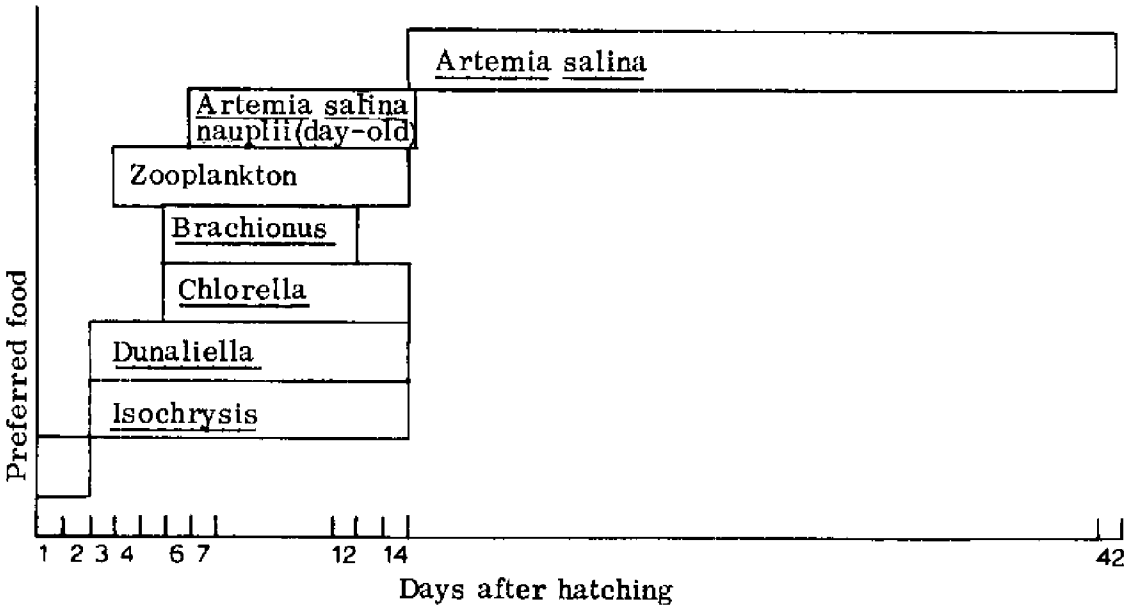

Fig. 6 Feeding schedule for larvae and post-larvae.

size and larval density. Survival has been increased dramatically by operating in 12' diameter kreisels and in all probability can be increased further by using even larger units. Depth is not as important as diameter and probably a depth of 1 m is adequate in spite of the migratory behavior of the larvae.

Light control throughout the entire larval rearing period is very essential. Direct sunlight on the larvae during the first five days is fatal and artificial lights are preferred. Phytoplankters are added to the kreisels. Although gut examinations have revealed that little or no use is made of such organisms nutritionally, they are known to have chemical value by controlling levels of pH and removing metabolites. These and other algal organisms quickly dominate a rearing unit if the light intensity is not controlled.

Larvae up to four weeks old are vulnerable to the empty cysts or shells of Artemia if ingested. The production of the nauplii without the cysts is still a vital feature for all culturists (Nash, 1973).

Finally, there is also some danger from ectoparasites being introduced from the natural plankton which is used as a food supplement. Amphipods have been observed on larvae six weeks old and which have subsequently died.

IV

BRIEF EXPERIMENTAL NOTES ON LARVAL SURVIVAL

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and
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Abstract

Results from a series of larval rearing experiments are reported. Attention was paid specifically to survival of larvae at day 14 for changes of diet, water temperature, light intensity, stock density, and water quality. Details are given for rate of food uptake following gut content analyses.

Isochrysis is taken by day 3 and readily consumed by day 5 after hatching. Natural zooplankton (copepods below 150 μ) cannot be utilized before day 5, and then only in small quantity.

The results of maintaining larvae under 24 hr light conditions for continuous feeding and increased survival are not clear. Under 24 hr light conditions, highest survival was achieved at a stock density of 12 larvae per liter, compared with 8 and 16 larvae per liter.

For parameters of nitrate, nitrite, sulfide, phosphate and ammonia, only sulfide concentration could be correlated with mortality of the larvae.

INTRODUCTION

Broodstock of the grey mullet, Mugil cephalus L., have been established satisfactorily in captivity (Shehadeh et al., 1973c) and gonadal maturation has been accelerated by manipulation of environmental conditions out of the natural breeding season (Kuo et al., Chapter VI). The induced spawning of captive grey mullet by hypophysation techniques (Tang, 1964; Liao, 1969; Shehadeh and Ellis, 1970; Yashouv, 1969; Shehadeh et al., (1973a) has raised hopes that future demands for juveniles by farmers can be met by controlled breeding and mass-propagation methods in hatcheries. The constant supply of fertile eggs to hatcheries can be readily controlled, but the survival of larvae after incubation to the post-larval stage is still low. Both Liao et al. (1971) and Kuo et al. (1973a) record larval mortalities of over 95 percent within the first fourteen days of life.

Two periods of high mortality were originally observed within the early days of larval development; the first between day 2-3 after hatching and the second between day 7-9. Both periods were correlated with certain behavioral patterns involving vertical movements within the rearing tanks. Refined procedures have reduced the first mortality period (Nash et al., Chapter III), but the second and greater one is still proving difficult to overcome.

These brief notes contain the results of a number of rearing experiments conducted during the season to investigate some of the problems believed to be associated with low larval survival.

METHODS

All eggs were obtained from resident female fish induced to spawn in captivity following injections with partially purified salmon pituitary gonadotropin after the method of Shehadeh et al., (1973a). The potency of the gonadotropin is given as 1 mg equivalent to 2250 IU of HCG (Donaldson et al., 1972).

Fertilized eggs were incubated in 800-l. aerated circular polyethylene kreisels at 24° C and salinity 32°/oo. Details of incubation procedures have been described by Nash et al. (Chapter III). Larvae were then transferred from the kreisels to each experimental unit as required within 24 hr of hatching.

A series of larval rearing experiments were performed with controlled variables within each series. Particular attention was paid to survival for

changes of food, temperature, light, stock density and water quality. Other experiments were conducted to study food uptake by the larvae. Each experiment lasted for a period of 14 days as mortality after that time was known to be negligible.

All experimental units were conducted in small glass aquaria containing static seawater ($32^{o}/_{oo}$) and fitted with suitable temperature control mechanisms. All units were cleaned daily and dead larvae removed and counted. Natural zooplankton (mostly copepods below 150 μ) was used to feed the larvae from day 5 on.

RESULTS

1. The uptake of food

Beginning on day 5 after hatching, food uptake determinations were made by gut content analysis two hours after food was provided.

The mouth of the mullet larva is known to be open on day 2 after hatching and is ossified by day 3. Liao et al. (1971) reported that for their most successful results that fertilized oyster eggs were added to the rearing tanks containing cultured diatoms on day 3. Yeast and albumin were then supplemented on day 4 and rotifers and copepods on day 6. Kuo et al. (unpublished data) showed that feeding first on days 4 or 5 was better in terms of survival and management than that on either days 3 or 6. Furthermore, copepods smaller than 150 μ could not be utilized until day 5.

Four series of experiments were performed using Isochrysis, Brachionus, Chlorella and natural zooplankton. Observations of stomach contents during development between days 5-11 are presented in Table 1. Isochrysis was taken first of all food organisms tested, being readily consumed in quantity on day 5 after hatching.

The food preference by mullet larvae for natural zooplankton or Isochrysis as an initial food was further examined. The food organisms were given singly and in combination and the gut contents examined daily as before. No food preference was indicated except Isochrysis was again consumed readily on day 5.

Table 1. Observations of stomach contents of mullet larvae.

Days after hatching	5	6	7	8	9	10	11
Zooplankton							
No. fish feeding	1	6	7	11	14	10	12
% of sample feeding	6	35	47	61	82	59	67
No. food organisms in fish stomach (sample range)	1	1-3	1-6	1-6	1-7	1-3	1-8
Isochrysis							
No. fish feeding	10	7	10	10	6	8	9
% of sample feeding	91	58	100	83	50	73	82
No. food organisms in fish stomach (sample range)	35-160	25-100	10-95	5-35	2-112	(full stomach)	(15-full stomach)
Brachionus							
No. fish feeding	0	0	1	1	5	5	2
% of sample feeding	0	0	10	8	42	50	18
No. food organisms in fish stomach (sample range)	-	-	1	2	1-3	1-4	1
Chlorella							
No. fish feeding	0	0	1	4	1	5	1
% of sample feeding	0	0	10	33	8	45	9
No. food organisms in fish stomach (sample range)	-	-	1	3-6	4	3-8	40

2. Effect of light period on larval survival

The results of experiments rearing larvae under natural and 24 hr artificial light at an intensity of 600 ft-c. at the water surface were inconclusive for temperatures of 22 and 24° C (Fig. 1). Whilst good survival (23%) was recorded under natural light conditions at 22° C, total losses were recorded at 24° C. Survival at both 22 and 24° C under 24 hr artificial light was similar. The value of maintaining light for 24 hr to enable the larvae to feed has been clearly demonstrated by Shelbourne (1964) and Blaxter (1969).

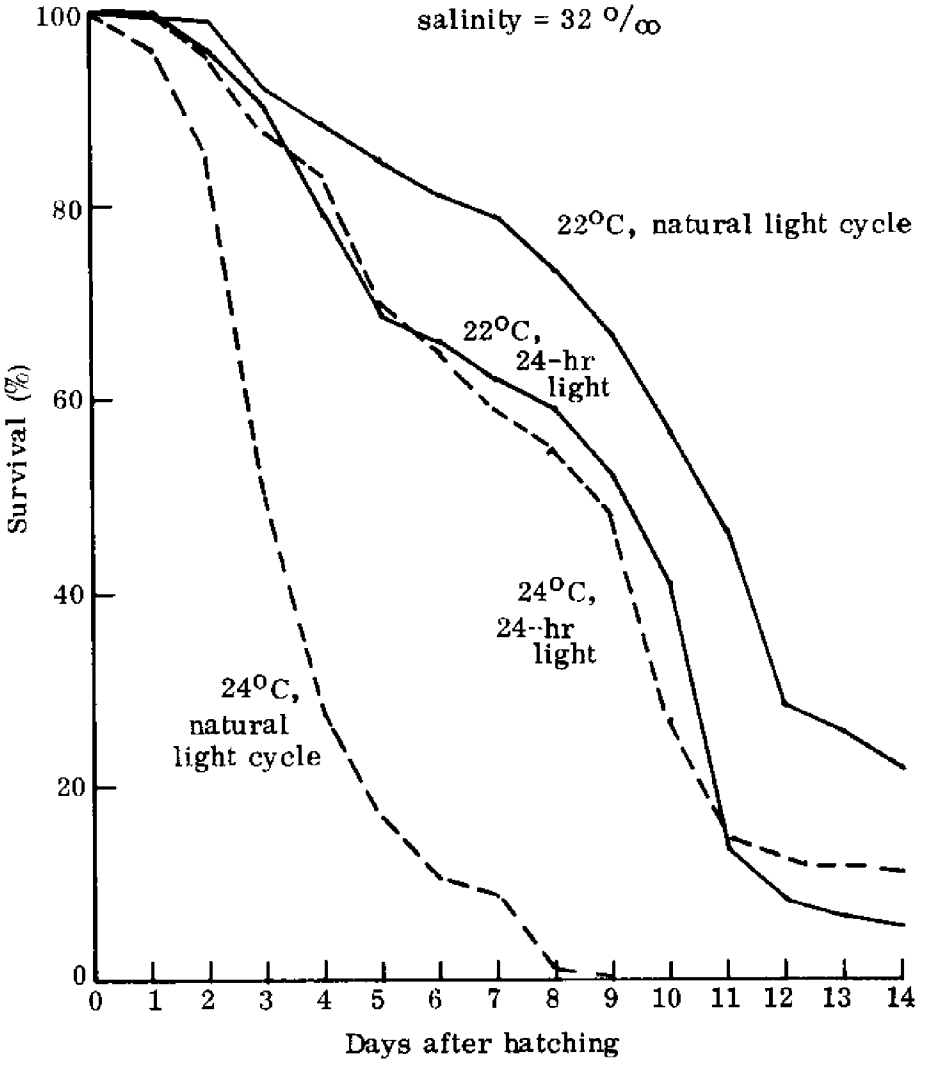

Fig. 1 Percentage survival of larvae for differing photoperiod and temperature.

3. Effect of density on larval survival

In small aquaria (10-l. capacity), and under constant conditions of salinity (32°/oo), 24-hr light period (600 ft-c. at the surface), and static water conditions, results for larval survival at differing densities at 22 and 24° C showed that highest survival was achieved at a density of 12 larvae per liter (Fig. 2). Differences for survival at densities of 8 and 16 larvae per liter at either temperature were not significant.

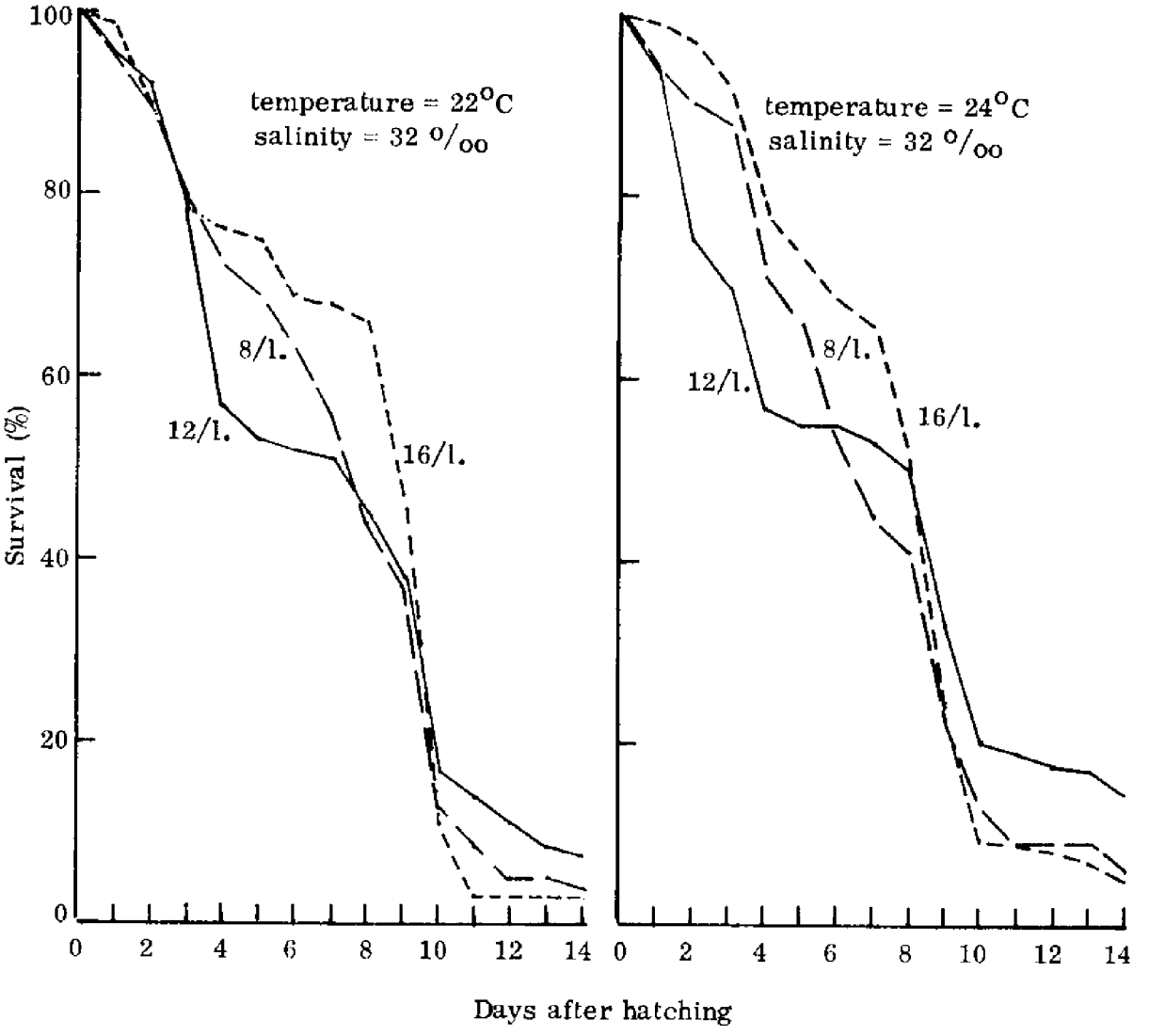

Fig. 2 Percentage survival of larvae for differing density (number of larvae per liter).

4. Effect of food on larval survival

Results of larval survival following feeding with natural zooplankton (mostly copepods below 150 μ) and Isochrysis were inconclusive (Fig. 3). Isochrysis alone appeared to be better (7.3%) than natural zooplankton (2.2%), but the difference was not significant. Previous experiments showed that Isochrysis was readily consumed by larvae at day 3, whereas natural zooplankton (or cultured copepods) were not taken in quantity before days 5 to 7.

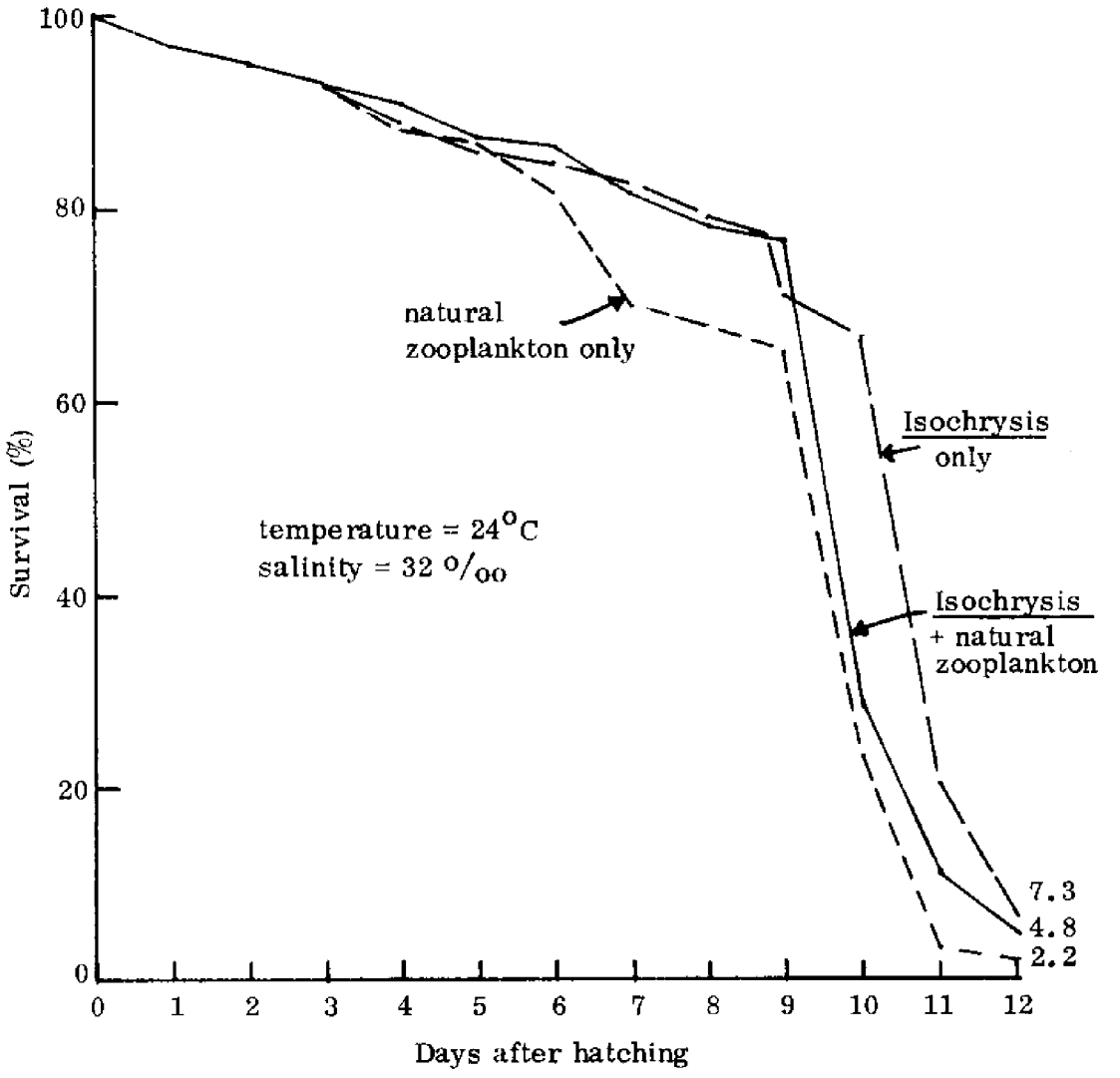

Fig. 3 Percentage survival of larvae for differing feeds.

5. Effect of water quality on larval survival

Larval survival related to water quality was examined under static water conditions at 24° C only. Duplicate experiments were conducted in four glass aquaria (32-l. capacity). From each tank a sample of 1 liter of water was removed daily for analysis and the volume then replaced. Two of the tanks were cleaned each day with a hand siphon. A total volume of 10 l. of water was removed during cleaning and the volume again replaced. The other two tanks were not cleaned.

Physical parameters of temperature and dissolved oxygen were recorded daily in each tank before sampling. The sample of water removed from each tank was analysed for nitrate, nitrite, sulfide, phosphate and ammonia. The concentrations of nitrite, nitrate and sulfide were determined by the procedures described by Strickland and Parsons (1968); phosphate by Murphy and Riley (1962), and ammonia by Newell (1967).

The results of the separate analyses performed daily throughout the experimental period are illustrated in Fig. 4.

From examination of the data, only the concentrations of sulfide and mortality were highly correlated. All other chemical concentrations decreased in general through the experimental period. The larval survival figures at the end of the fourteen days (17.7 - 25.5%) for the four experimental tanks were the best ever recorded for the grey mullet larvae to date. Cleaning the tanks did not produce the highest survival figures, but the fact that all tanks received some fresh seawater daily, as the sample volume was replaced, was probably influential.

* All quantities in μg-at/l. unless otherwise indicated.

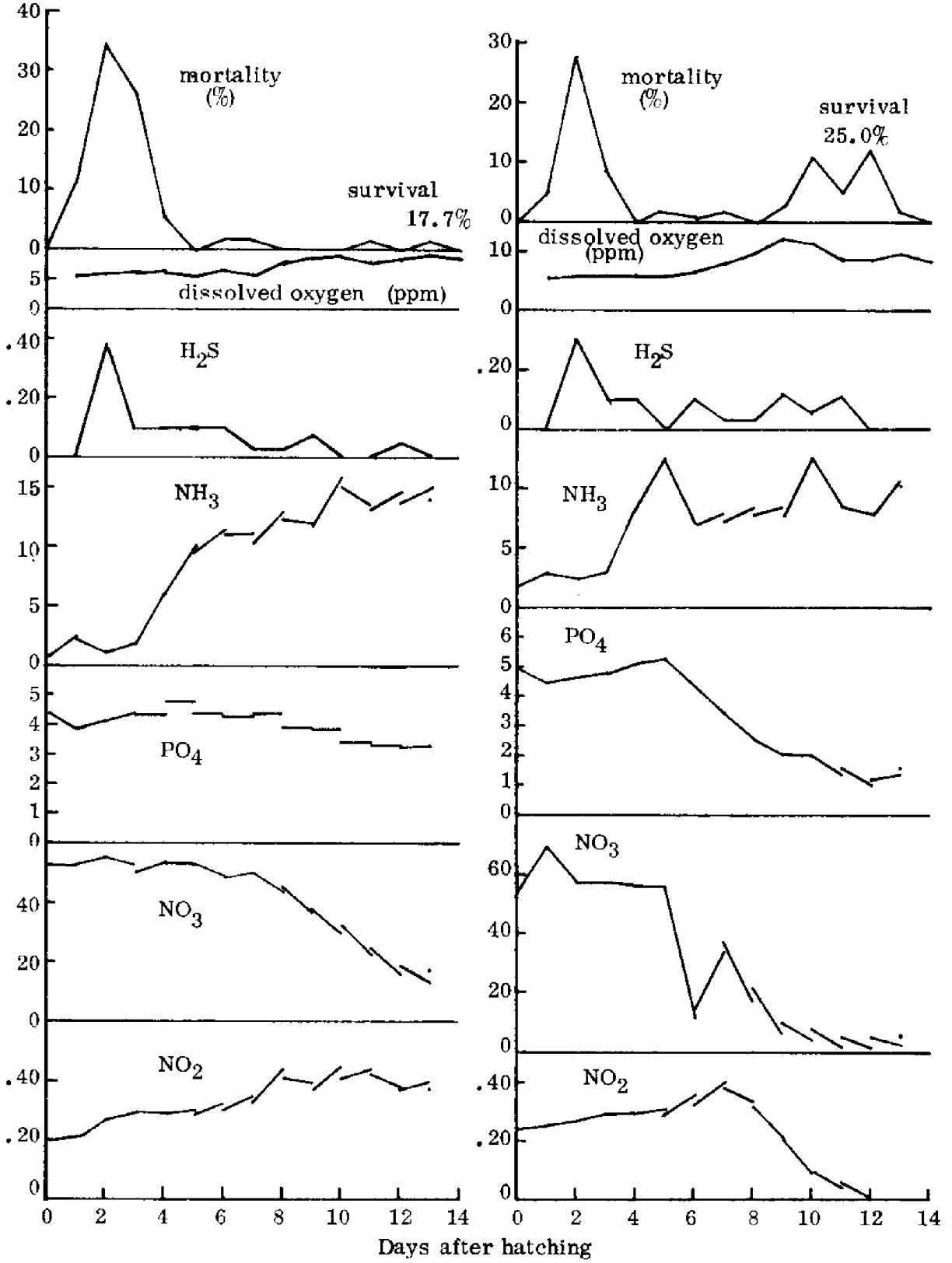

Fig. 4.a Daily changes in concentrations of nitrite, nitrate, ammonia, sulfide, phosphate, and dissolved oxygen for uncleaned tanks.

*All quantities in μg-at/l. unless otherwise indicated.

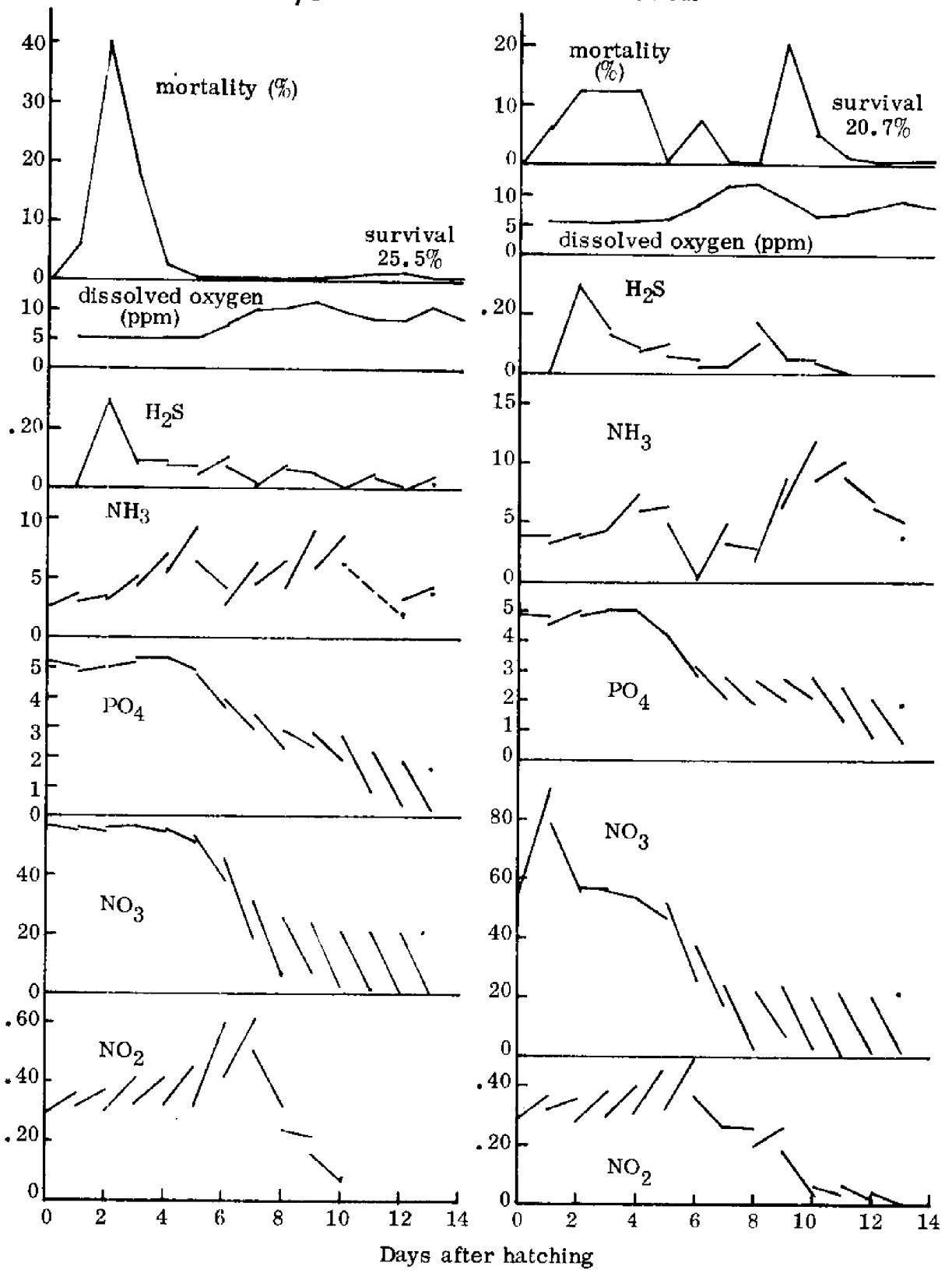

Fig. 4.b Daily changes in concentrations of nitrite, nitrate, ammonia, sulfide, phosphate, and dissolved oxygen for cleaned tanks.

V

PRELIMINARY EXPERIMENTATION WITH LARVAL FEEDS

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Abstract

The rotifer, Brachionus plicatilus, has been suggested as a suitable larval food for marine fish species. Experiments are described which test the value of various food organisms for the culture of the rotifer in the laboratory in support of larval rearing programs. A nutrient medium formula, which produces high yields of the test organism, is included.

The report describes experiments on the responses of grey mullet larvae to prepared artificial feeds. The results of the experiments with the nineteen feeds or feed combinations, based on larval survival up to day 14 after hatching, are divided into three categories compared with the unfed controls. Six feeds tested produced significantly better daily survival compared with starved controls. These were freeze-dried mullet ovaries, freeze-dried zooplankton, freeze-dried zooplankton with Artemia nauplii, and two prepared feed mixtures with and without Artemia nauplii supplements.

INTRODUCTION

Rearing larval marine fishes on laboratory cultured feeds has not been totally satisfactory to date.

Few attempts to use phytoplankton have proved successful (May, 1970). Farbe-Domergue and Bietrix (1905) gave the naked green flagellate, Dunaliella salina, to freshly hatched sole larvae, Solea solea, and recorded that it was eaten in preference to zooplankton for the first few days. A similar result was obtained by Kasahara et al. (1960) using the unarmored dinoflagellate Oxyrrhis, supplemented with other marine organisms; thereafter the larvae became entirely carnivorous. Anchovy larvae, Engraulis mordax, were reared in the laboratory by Lasker et al. (1970). The larvae maintained good growth and survival when offered only the naked dinoflagellate Gymnodinium splendens. Growth and survival was improved further when both the dinoflagellate and veligers of Bulla gouldina were provided.

For all the laboratory attempts to culture marine fish larvae on phytoplankton, the larvae often feed heavily on them at first, but finally survive no better than starved controls. It is known that most marine larvae can be reared successfully using natural zooplankton. During early post-yolk sac periods, teleost larvae readily consume copepod nauplii followed by larger zooplankton. More than forty species of marine and brackishwater fish have been reared successfully through metamorphosis on natural zooplankton alone. This would indicate that the large majority of fish larvae are carnivorous and that zooplankton is their staple diet. However, laboratory experiments show that 2-4 edible organisms per cc of the rearing medium must be present for larvae to survive (O'Connell and Raymond, 1970). Densities of this magnitude are not reported to occur in the ocean, even in the vicinity of upwellings, and this leads many investigators to suspect that other materials may also be acceptable feeds in nature.

In the laboratory, where predation and environmental parameters can be controlled, survival as high as forty percent at metamorphosis has been reported with natural zooplankton fed at high density. Potentially, the production of juveniles from the average pelagic marine fish is high, but mass culture has not yet succeeded relying on zooplankton as the larval food. The reasons are several, among which are the quantities of natural zooplankton required, its undependable nutritional quality and its dynamic composition and abundance. The effects of such variability on any aquaculture practice are obvious: variable quantities and qualities of foods can only produce variable yields of culture products. For aquaculture to be successful, dependable feeds must be developed.

Current research is concentrating on methods for continuous cultivation of acceptable zooplankton, the development of artificial feeds, and the testing of concentrated algal diets. Theilacker and McMaster (1971) demonstrated that the rotifer Brachionus plicatilus was nutritionally equivalent to natural zooplankton when fed to the northern anchovy, E. mordax. Other workers have demonstrated the organism suitable for other larval marine fishes.

This paper describes experiments testing the value of various food organisms for the culture of B. plicatilus. Media are described which produce high cell densities of the more suitable feeds and techniques used for media evaluation are presented. In addition, this report describes experiments for the responses of grey mullet larvae to prepared artificial feeds. Larval survival is used as the measure of feed suitability and daily survival curves are evaluated to illustrate the more desirable feed properties. Some techniques for food preparation are also presented.

MATERIALS AND METHODS

1. The culture of B. plicatilus

Food organisms tested for the culture of B. plicatilus were Isochrysis galbana, Monochrysis lutherii, Pavlova gyrens, Dunaliella, sp., and Chlorella sp. Single egg-bearing females of B. plicatilus were placed in 100-ml. Thomas media (Theilacker and McMaster, 1971) containing unialgal logarithmic growth phase cultures of each of the above foods. The rotifers were permitted to grow on these feeds for two transfer periods (approximately two weeks). Having established identical nutritional baseline characteristics, 10 egg-bearing females were then transferred to cultures of each single food organism, including that which they had used previously. Daily aliquots were taken from the cultures and visual counts of the density of the rotifers were made using an AO brightline hemacytometer. Cultures were grown at 25°C under continuous 24-hr illumination (500 ft-c.) using cool white fluorescent lamps. The length of time each culture was maintained was used to indicate the value of each food, either alone and in mixture. Routine transfers of cultures were made when they entered the stationary production phases.

2. Media evaluations

Holes were drilled in two 12" x 12" plexiglass plates (1" thick) to form a grid to support Bausch and Lomb cylindrical cuvettes (5 ml) with a 1 cm light path. Each cuvette was filled with 3 ml of medium (Table 1) and the meniscus etched on the outside of the cuvette with a diamond pen. The cuvettes were sterilized in an autoclave for 30 min. at 15 psi, and the medium for 15 minutes. The medium was then transferred to each cuvette under sterile conditions.

The medium in each cuvette was inoculated aseptically with .05 ml of I. galbana (in exponential growth phase) previously cultured in an artificial seawater medium (Jones, 1960). The nutrient composition of all media tested are fully described in Fig. 1 with the results.

The plates supporting the inoculated cuvettes were placed in a standard culture chamber and positioned for maximum illumination on each cuvette. Continuous illumination was provided by cool white fluorescent lamps in the chamber (500 ft - c.) and temperature was maintained at 22.5 ± 1°C throughout.

Daily growth rates were monitored using a Turner spectrophotometer (Model 350) at 730 nm. Each cuvette was shaken before a reading was taken. Any volume changes of media caused by evaporation were carefully monitored by comparing differences between the observed and etched meniscus heights. Necessary corrections were made by adding an appropriate amount of sterile media. Growth rates were measured daily until all cultures demonstrated stationary growth phase characteristics.

3. Feed preparation

The following feeds were prepared and evaluated by recording larval survival. Experimental controls were fed live nauplii of Artemia salina or natural zooplankton:

a) Ripe mullet ovary

Fish larvae are nutritionally supported by egg yolk during their early development. Yolk provided orally to larval fish after the yolk sac stage is potentially of value. Ovaries from a mature female mullet were removed a few hours before it was due to spawn. At this yolk globule stage the oocytes are almost entirely yolk. The excised tissue was immediately freeze dried, sealed in vacuum vials and stored in a refrigerator. Before offering the freeze-dried ovary preparation to the larvae, the material was warmed to room temperature and forced through a 125μ mesh screen to produce particles of edible size.

b) Brewer's yeast (Saccromyces cerevisae)

Fresh brewer's yeast was obtained from a brewery. A wet concentrate was made by centrifuging small volumes of the suspended yeast through a Deval cream separator. The concentrate was freeze-dried and stored. Before use, the concentrate was powdered using a Wyllie laboratory mill fitted with a .5 mm screen.

c) Dried zooplankton

Natural zooplankton was collected and freeze-dried. The concentrate was powdered using the laboratory mill, and finer particulates were produced when required by passing the powders through a 125μ mesh screen.

d) Feed mixture I

A mixture was constituted from several potentially useful organisms and supplements. If the mixture proved to be a successful feed, the active components could later be identified by elimination. Mixture I had the following composition:

	%
Freeze-dried mullet ovary	12.4
" " I. galbana	2.5
" " Dunaliella sp.	3.7
" " Chlorella sp.	1.6
" " Hansenula anomala	4.0
" " Saccromyces cervisae	12.3
Oven-dried Synechocystis sp.	7.4
Wheat germ oil	6.2
Cod liver oil	0.5

combined with the diet found suitable for juveniles and adult fish (49.4%)

I. galbana was produced in the best medium indicated by the experimental work Chlorella sp. and Dunaliella sp. were cultured in the medium of Thomas (Theilacker and McMaster, 1971), and H. anomala in Difco Bacto YM broth. All media were sterilized in an autoclave for 15 minutes at 15 PSI. All cultures were harvested with a Deval cream separator and freeze-dried and stored before use. The composition of the juvenile and adult dietary mix used as a filler was as follows:

A. Dry ration	
Fish meal	400 g
Soybean meal	250 g
Chicken starter mash	100 g
Fish bone meal	50 g
Dairy whey	50 g
Wheat germ	50 g
Wheat middlings	50 g

B. Liquid ration	
Water	900 ml
Choline chloride	0.5 g
Urea	0.5 g
Propylene glycol	5.0 g

Mix A and B separately, then together before use. This mix is used routinely by the laboratory as a feed for the grey mullet for growing juveniles, adults and broodstock.

After preparation, feed mixture I was passed through a Wyllie laboratory mill (.5 mm screen) and then through a 125μ mesh screen.

e) Feed mixture II

Some dietary mixtures, which were developed to satisfy the nutritional requirements of salmonids (Halver, 1968) were prepared for tests with mullet larvae. The constituents were:

A. Carbohydrates
- 4.4 g galactose
- 13.1 g glucose

B. Proteins (amino acids)
- 5.0 g Arginine
- 1.4 g Histidine
- 2.0 g Isoleucine
- 3.0 g Leucine
- 4.3 g Lysine
- 1.0 g Methionine
- 4.0 g Phenylalanine
- 1.5 g Threonine
- 0.4 g Tryptophan
- 3.0 g Valine

C. Fats (oils)
- 3.5 g Wheat germ oil
- 1.0 g cod liver oil
- 100 units vitamin E

D. Fatty acids
- 1.25 g β-glycerolphosphate
- 5.0 g linoleic acid

E. Vitamins
- 50 mg Thiamin HCL
- 200 mg Riboflavin
- 50 mg Pyridoxine HCL
- 5000 mg Choline chloride
- 750 mg Nicotinic acid
- 500 mg Ca-pantothenate
- 2000 mg Inositol
- 5 mg Biotin
- 15 mg Folic acid
- 1000 mg Vitamin E
- .01 mg Cobalimine

The mixtures were then combined in the ratio of 60% protein, 35% carbohydrate, and 5% fats. Protein and carbohydrate mixtures were dissolved in heated 4% Nobels non-nutritive agar and autoclaved for 15 min. at 15 psi. The vitamin and fatty acid mixtures were then added to the main mixture as it cooled. After solidifying it was passed under pressure through a 900μ mesh screen placed in a Büchner funnel. Finally, the oil mixture was drawn over the fine threads falling from the screen to provide a thin, water resistant coating. Feed mixture II was then stored in a refrigerator until required.

4. Feed evaluation trials

Twelve open-ended rearing tanks (1' x 1' x 1') were constructed of plexiglass (5 mm thick) and the inner walls coated with a non-acrylic flat black paint.

Nitex screen (10μ) was used to form the base of each tank so that water could be exchanged from below. Each tank was leached for two weeks by total immersion in running seawater (32 ‰) to remove any toxic components in the materials.

Three circular fiberglass containers (30" in depth and 6' in diameter) were used to hold the small tanks. Seawater flowing into the containers entered via baffles which produced sufficient turbulence to maintain dissolved oxygen concentrations at saturation, and eliminate dangers of supersaturated or low oxygen conditions. Water levels in each container were controlled by external adjustable standpipes.

Four rearing tanks were suspended in each container and were flushed continuously by water exchanged through the Nitex screen. During the experiments, the water level in the rearing tanks was manipulated daily by adjusting the external standpipe of the container to exchange half the water volume in each tank.

All experiments were conducted in temperature controlled laboratories. Water temperature was maintained at 25.5 ± .5°C. Continuous illumination was provided by an incandescent lamp (500 watt) suspended above the center of each container. Salinity of the seawater was constant (32 ‰) and dissolved oxygen concentrations remained at saturation.

Approximately 1000 embryos were distributed in each of the rearing tanks. Twelve hours after hatching all dead eggs, larvae and other debris were collected by pipette and counted.

Each prepared feed was tested in duplicate. Feeds were offered for the first time on day 2 after hatching, and given twice daily. Before feeding, all dead larvae were removed and counted, the tanks were cleaned and the water exchanged. As the water level was dropped, internal surfaces of the tanks were rinsed gently with seawater to prevent isolating larvae.

RESULTS

1. Culture of B. plicatilus

Table 1 shows the differences in feed quality of certain algae for the culture of the rotifer, B. plicatilus.

Table 1 B. plicatilus culture longevity as related to feed and feed history and measured by the number of transfers possible before diminutive culture conditions were observed.

Previous feed	Completed transfers of: I. galbana	M. lutherii	P. gyrens	Chlorella sp.	Dunaliella sp.
I. galbana	1	7	1	6	5
M. lutherii	8	3	2	14	14
P. gyrens	0	4	0	11	9
Chlorella sp.	6	33	9	4	16
Dunaliella sp.	6	36*	10	28*	5

* Additionally, M. lutherii and Chlorella sp. were grown separately and mixed immediately before rotifer inoculation, yielding 43+ possible transfers.

M. lutherii was the best single food offered after the rotifer was fed initially with either of the chlorophytes; Chlorella sp. was the best after chrysophytes had been fed initially. Dunaliella sp. was also a suitable feed.

The growth of B. plicatilus was increased by any change in feed. For example, if the rotifers were first offered Chlorella sp. before transfer to I. galbana cultures, they sustained good growth for six transfers. If they were first offered I. galbana and transferred to it again, only one further transfer period was possible.

Although influenced by the initial feed, all algae tested gave rotifer growth without producing any physiological changes suggesting that their chemical constituents supplement one another. It is likely that many of the nutritional deficiencies of single organism feeds are compounds required by B. plicatilus in trace amounts only.

2. Media evaluations

Figure 1 illustrates the matrix of responses of I. galbana to the media tested. Data are presented for differences between initial and maximum optical density. Contours superimposed on the figure show regions of similar growth and degrees of media specificity for I. galbana. The nutrient composition of the best medium tested is given in Table 2.

Table 2 Nutrient composition of the best medium tested for I. galbana (amounts per liter of doubly distilled water for final medium)

Trace X		S1 metals	
$ZnCl_2$	16.00 µg	$SrCl_2 \cdot 6H_2O$	11.88 mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	147.29 µg	RbCl	84.72 µg
$CuCl_2 \cdot 2H_2O$	13.60 µg	LiCl	367.50 µg
$MnCl_2 \cdot 4H_2O$	160.00 µg	KI	19.638 µg
H_3BO_3	240.00 µg	NaBr	25.08 mg
Trace Y		Salts	
$FeCl_3 \cdot 6H_2O$	340.00 µg	NaCl	32.10 g
Na_2EDTA	2.976 mg	$MgCl_2 \cdot 6H_2O$	5.49 g
		$MgSO_4 \cdot 7H_2O$	6.66 g
Solution 5		$CaCl_2$	1.11 g
KNO_3	404.00 mg	TRIS buffer	20.00 ml
KH_2PO_4	27.20 mg		

Stock solutions

Trace X (µg/ml)		Solution 5 (mg/ml)	
$ZnCl_2$	4.00	KNO_3	101.00
$(NH_4)_6Mo_7 \cdot 4H_2O$	36.80	KH_2PO_4	6.80
$CuCl_2 \cdot 2H_2O$	3.40		
$MnCl_2 \cdot 4H_2O$	40.00	S1 Metals (µg/ml)	
H_3BO_3	60.00	$SrCl_2 \cdot 6H_2O$	1980.00
		RbCl	14.12
Trace Y (µg/ml)		LiCl	61.25
$FeCl_3 \cdot 6H_2O$	24.00	KI	3.27
Na_2EDTA	186.00	NaBr	4.18

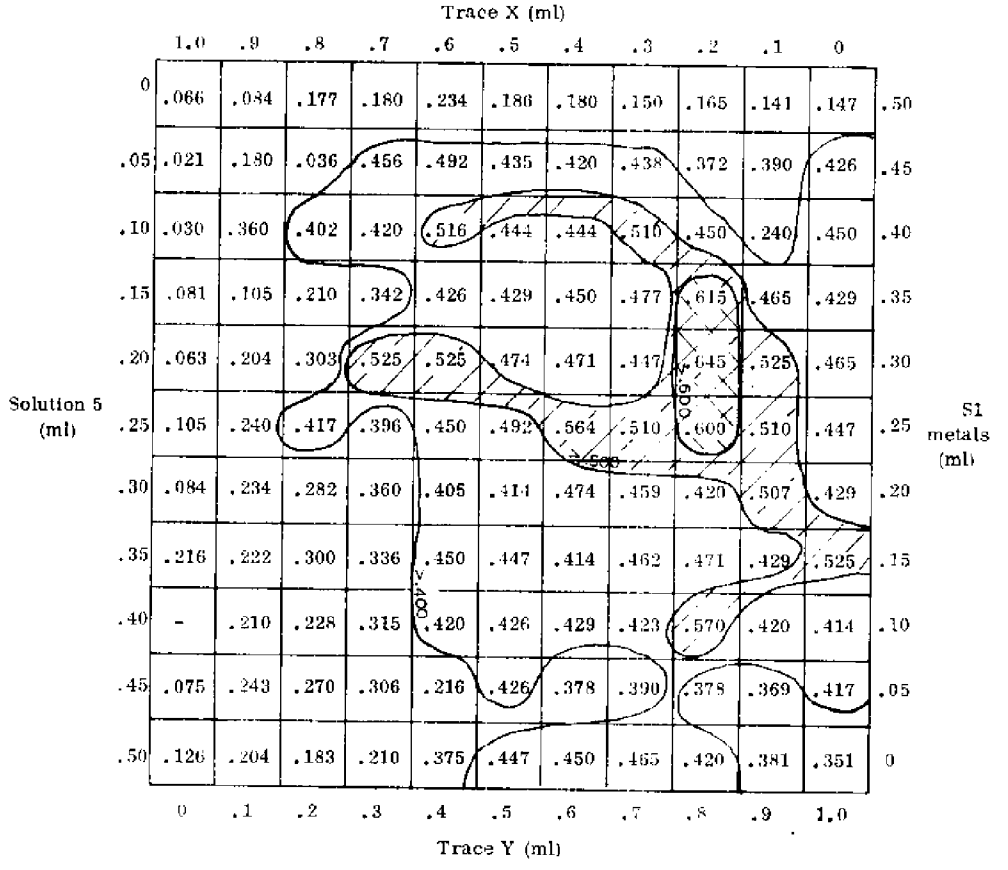

Fig. 1 Changes in optical density (OD) shown by cultures of I. galbana exposed to a variety of media. Contours indicate regions of similar response. Axis values are volumes of stock solutions 5, X, Y, and S1 added to a basic salt solution to give 50 mls of each final medium. Concentrations of each nutrient per ml of stock solution are given below; the basic salt solution is given in Table 2.

The medium of Jones (1960) was high in nitrogen and phosphorus and low in iron according to the data. While nutrient ratio and salinity modifications might further improve media characteristics, yields of 9.0×10^7 cells/ml were obtained when the nutrient adjustments indicated by this experiment were employed. At these cell densities, the capacity of TRIS buffer to maintain pH was tested and additions of sodium bicarbonate proved beneficial.

3. Feed evaluation trials

None of the feeds or feed mixtures tested produced significantly increased larval survival. Poor survival under a variety of experimental conditions infer that some basic technical problems exist in larval culture which influence the data and cannot be related directly to the experimental variable. The different feeds have therefore been evaluated in general terms to aid future investigations.

None of the fish larvae were offered food before day 2 after hatching. During this period eyes become pigmented and much of the yolk is absorbed. Mortalities at the time resulted from poor conditions ratherthan dietary deficiency. The experiments indicated that emergent larvae could be divided into two categories; namely those which exhibited insignificant mortalities during the first three days, and those which died at a rate equivalent to unfed controls. No explanation can be offered for this observation as differences in food quality were not indicated until after day 3.

The data (Figs. 2, 3 and 4) are presented in three categories, namely

1. feeds which produced no differences in survival rates compared with unfed controls;

2. those which yielded a moderate but indefinite response; and

3. those which showed significant improvements in survival figures over unfed controls.

Included in the first category were the highest and lowest daily rations of feed mixture I and live phytoplankton and zooplankton in various combinations with dried zooplankton preparations. In category two were mullet ovary supplemented with cod liver oil alone or in combination with vitamins and amino acids, brewer's yeast with and without supplementations, and moderate daily rations of feed mixture I. Feeds which produced encouraging results included mullet ovary without supplementation, feed mixture II alone and in combination with Artemia nauplii, feed mixture I in combination with Artemia nauplii, and freeze-dried zooplankton alone and together with Artemia nauplii.

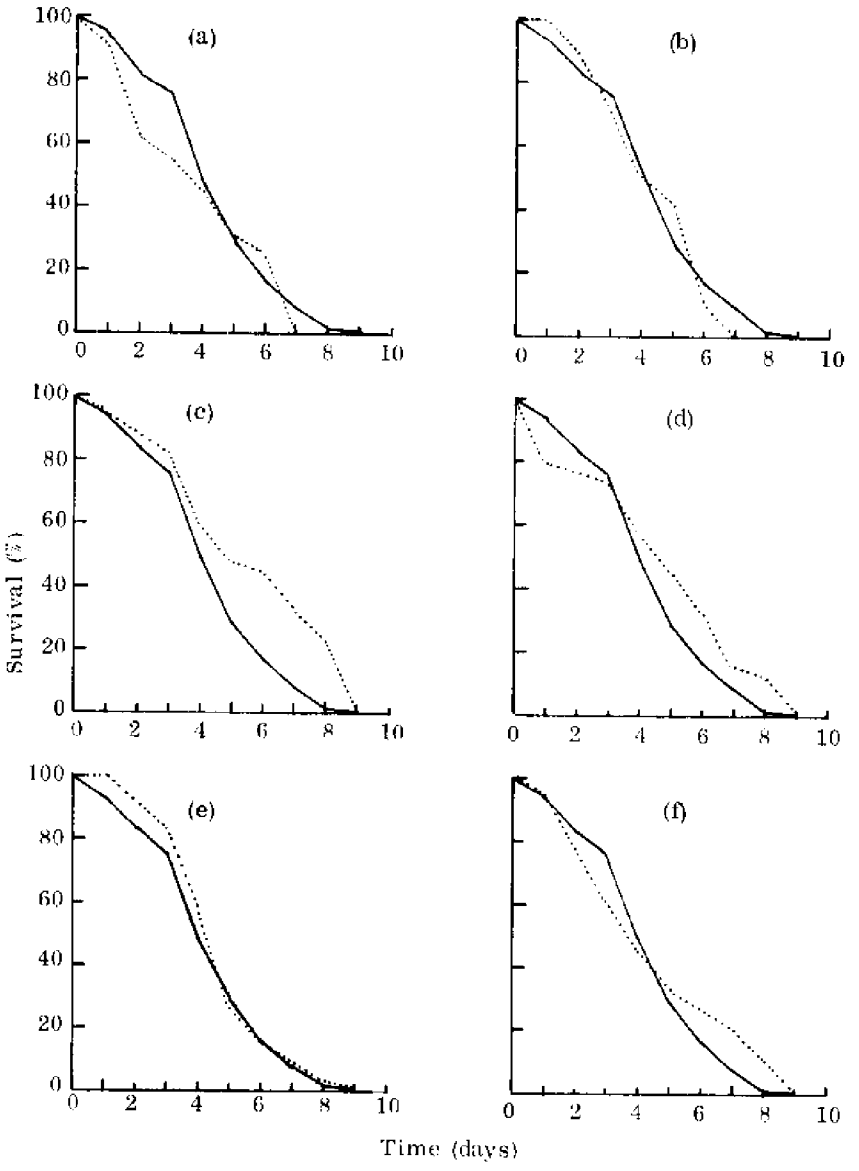

Fig. 2 Feeds (broken line) which produce no differences in survival rates compared with unfed controls (solid line).

(a) 0.01 gm/day feed mixture I
(b) 0.40 gm/day feed mixture I
(c) live phytoplankton (Chlorella sp.) and zooplankton at day 2 followed by freeze-dried zooplankton each day
(c) live phytoplankton and zooplankton daily through day 5; live phytoplankton and freeze-dried zooplankton thereafter
(e) live phytoplankton at day 2 only, live zooplankton through day 5, and freeze-dried zooplankton thereafter
(f) repeat experiment of (c)

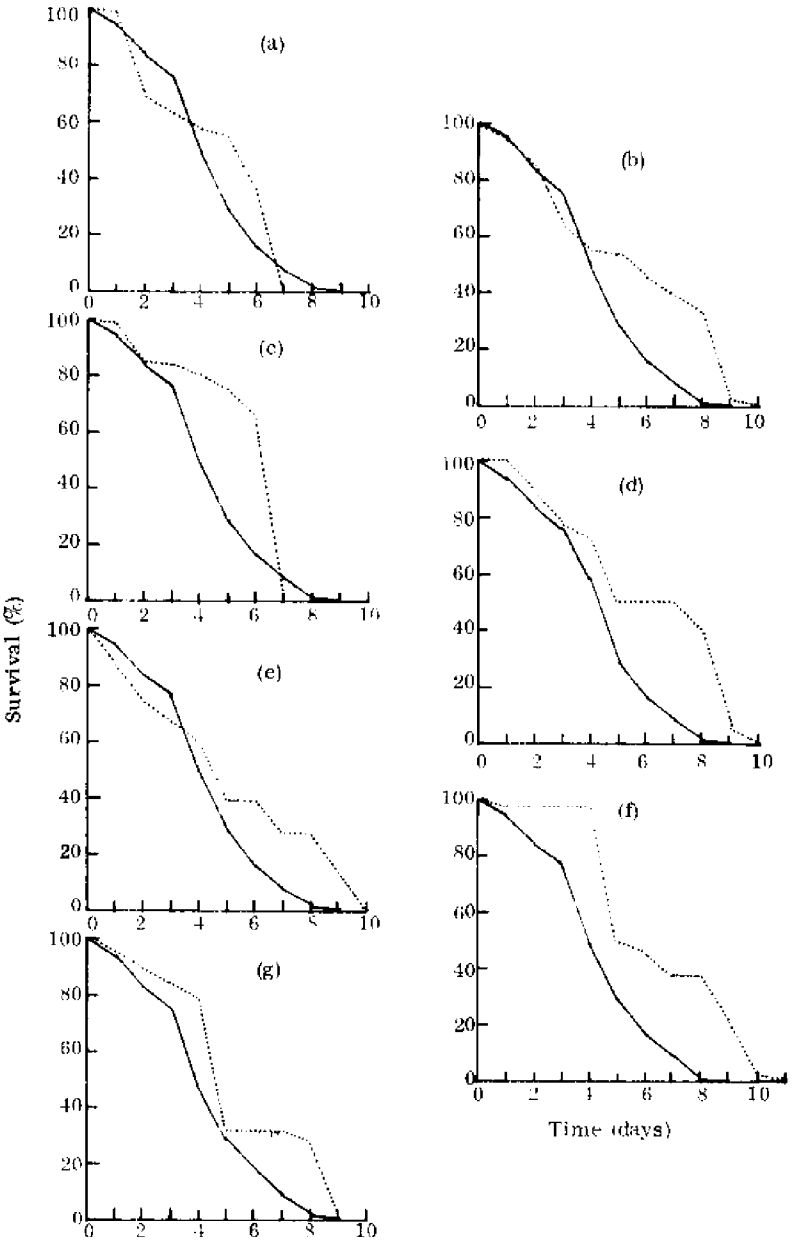

Fig. 3 Feeds (broken line) which produce a moderate but indefinite response in survival rates compared with unfed controls (solid line).

(a) 0.1 gm/day feed mixture I
(b) repeat experiment of (a)
(c) 0.05 gm/day feed mixture I
(d) mullet ovary + 2% cod liver oil
(e) mullet ovary + 2% w/w vitamin mix (feed mixture II) + 2% cod liver oil
(f) brewer's yeast + 2% vitamin mix (feed mixture II)
(g) brewer's yeast alone

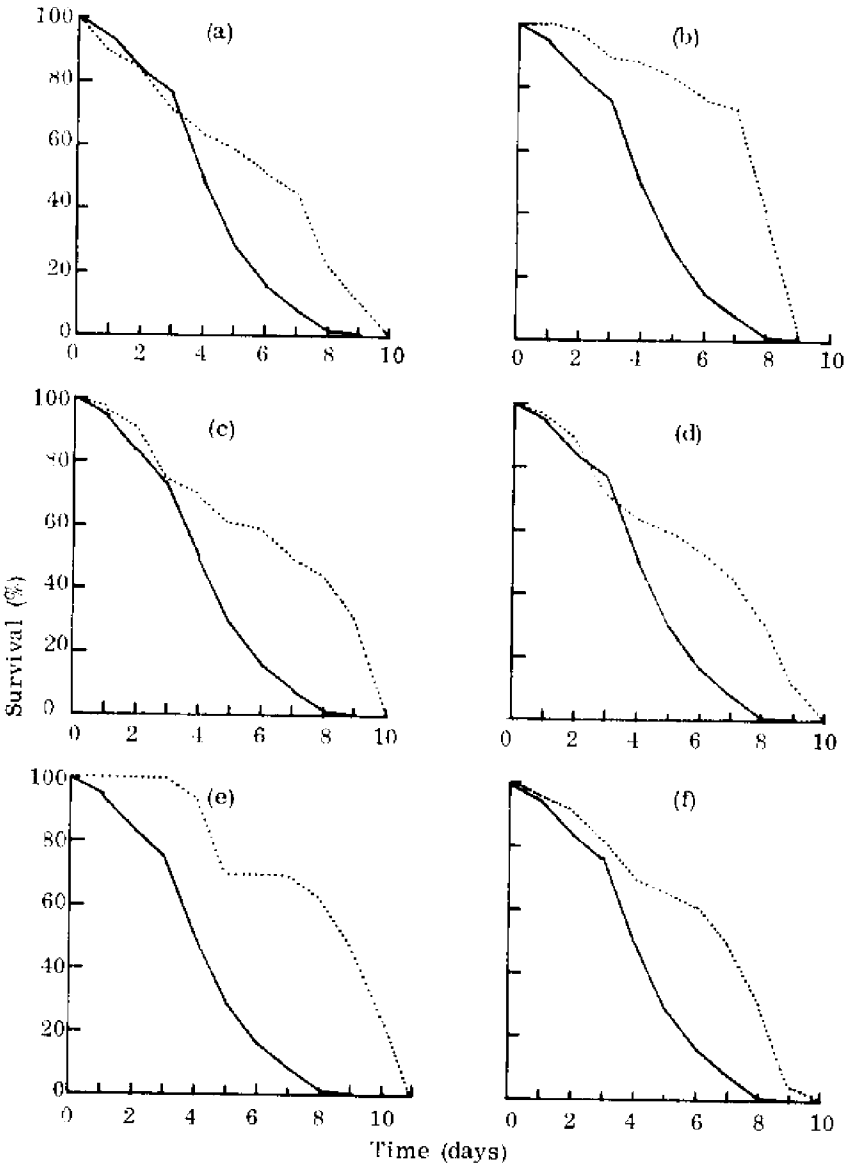

Fig. 4 Feeds (broken line) which showed significant improvements in survival rates over unfed controls (solid line).

(a) 0.1 gm/day feed mixture I to day 5

(b) 0.2 gm/day freeze-dried zooplankton to day 5, Artemia nauplii thereafter (4/cc)

(c) 0.2 gm/day freeze-dried zooplankton

(d) 0.5 gm/day feed mixture II

(e) mullet ovary

(f) 0.5 gm/day feed mixture I to day 5, and Artemia nauplii thereafter

VI

THE EFFECTS OF TEMPERATURE AND PHOTOPERIOD ON OVARIAN DEVELOPMENT

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Abstract

A series of sixteen environmental experiments are described, under controlled laboratory conditions during the refractory period in the reproductive cycle of the grey mullet, to determine the effect of photoperiod and temperature on the vitellogenesis of intraovarian oocytes. Fish subjected to the natural light cycle and ambient water temperatures (24 - 26°C) serve as controls. A classification of stages of vitellogenesis (I - V) is used to determine the percentage composition of oocytes for each fish at intervals throughout the experiment following sampling in vivo.

Onset of vitellogenesis is timed by the environmental conditions. The retarded photoperiod, irrespective of preconditioning photoperiod, plays a dominant role in stimulating oocyte growth. Temperature regulates vitellogenesis towards functional maturity. The combination of retarded photoperiod (6L/18D) and constant temperature of 21°C is the most effective for the completion of vitellogenesis of oocytes to functional maturity.

Regular injections of pregnant mare's serum gonadotropin, (PMSG), (1 IU/g body wt.) are effective in initiating vitellogenesis compared with non-injected controls.

INTRODUCTION

The endocrine system of chordates forms the main link between the reproductive organs and environmental regulators. Fluctuating regulators, such as photoperiod and temperature, mediated through the central nervous system, trigger neurosecretions which regulate, in turn, the activities of the pituitary gland. As one of many target organs the gonads are influenced accordingly, and the reproductive cycles are thus regulated intimately by the trophic hormones of the pituitary.

Many experiments with fishes have been designed to interpret the effects of certain environmental regulators on the reproductive cycle, by observing gonad development. Among the factors concerned, temperature and photoperiod are the two most important which initiate pituitary activity for fish in temperate and sub-temperate regions (Hoar, 1959), but the relative importance of each varies with different species of teleosts. Photoperiod has been reported as the dominant factor influencing the seasonal cycle of Enneacanthus obesus, Notropis bifrenatus and Fundulus confluentus (Harrington 1956, 1957, 1959a, 1959b); Gasterosteus aculeatus (Baggerman, 1957); Salvelinus fontinalis (Henderson, 1963) and Oryzias latipes (Yoshioka, 1962). Temperature has been shown by other workers to be the dominant influence for Fundulus (Matthews, 1939); Phoxinus laevis (Bullough, 1940); Apeltes quadracus (Merriman and Schedl, 1941); Gambusia affinis (Medlen, 1951); and Couisius plumbeus (Ahsan, 1964). It was concluded for Salvelinus fontinalis (Henderson, 1963) that the influence of an environmental regulator varied with the stage of gonad maturation. The most responsive period to a regulator could in fact differ between males and females of the same species, and the gametogenetic process may be independent of environmental regulators at certain stages of maturity.

The most appropriate time for testing effects of environmental regulators on successive phases of the annual reproductive cycle is the refractory or non-breeding period, when the gonads undergo their maximum annual regression. The refractory period can be short, particularly for fish in temperate and sub-temperate latitudes. The refractory period for Mugil cephalus in semi-tropical Hawaii is long, lasting from the end of March through to November. The effects of certain regulators on the reproductive cycle can therefore be interpreted accurately if the regulators are manipulated in the refractory period and spawning is subsequently induced.

Experiments in fish reproduction on the interaction of environmental regulators and the endocrine system indicate that influences on the reproductive cycle are reflected more readily by changes in the ovaries rather than in the testes. Most of the work has therefore been confined to females of the species.

The resources of female breeders for aquaculture is vitally important, particularly when linked to mass-propagation of juveniles in hatcheries. Female grey mullet have been induced to spawn by hypophysation techniques (Tang, 1964; Liao, 1969; Shehadeh and Ellis, 1970; Yashouv, 1969). Captive broodstocks have been maintained for long periods satisfactorily and the spawning later induced (Shehadeh et al., 1973c). In an attempt to prolong the breeding season of the grey mullet, and eventually to supply fertile eggs to a hatchery throughout the year, experiments have been made on the effects of two environmental regulators on ovarian development. The aim of the study was to determine the levels of influence of photoperiod and temperature on the reproductive cycle of female grey mullet indicated by vitellogenic changes in the intraovarian oocytes.

This paper reports the effects on gonad development indicated by the stage composition of oocytes during vitellogenesis following controlled photoperiod and temperature manipulation, and a comparison with the natural situation for fish exposed to ambient seasonal and diurnal variations. In addition, observations are recorded on the use of pregnant mare's serum gonadotropin (PMSG) to stimulate vitellogenesis.

MATERIALS AND METHODS

All experiments were performed in the Environmental Control Laboratory with control experiments in tanks located outdoors and subjected to natural photoperiod and temperature rhythms. The Environmental Control Laboratory has been described in detail by Bacon (Chapter VII). Briefly, eight circular tanks (42 x 48 in. i.d.) were available in each of the three rooms of the laboratory, and the environmental conditions, including light intensity, photoperiod, water temperature and air temperature, were all controlled.

An incandescent lamp (300 watt), suspended above the center of each holding tank, was adjusted vertically to provide an intensity of 75 ft-c. at the water surface throughout all experimental periods. The lamps in each room were controlled by separate timers and dimmer units, operated automatically.

The water system consisted of a number of supply and recirculation lines, any of which could be used. Water temperature was maintained by adjusting the flow rate of either pre-chilled or pre-heated seawater. The salinity of the water throughout all the experiments was 32 $^{o}/_{oo}$.

Air temperature in each room was maintained by an air conditioner regulated by a separate thermostat. This minimized any sudden fluctuation of water temperature in the tanks.

Female grey mullet adults were selected from established broodstock, originally collected in Kaneohe Bay, Oahu, but held captive for more than two years in dirt ponds lined with butyl rubber. Only females were used which had been induced to spawn in the natural breeding season preceding the experimental period or which could be induced to spawn in the next natural breeding season. The minimum fork length of all experimental fish was 31 cm.

No sexual dimorphism has been noted in the grey mullet species. Sex was therefore determined by examining gametes which were sampled in vivo by the technique described by Shehadeh et al. (1973b). The natural breeding season of grey mullet in Hawaii is generally between December and March. Spawning in captivity without inducement has never been recorded, although well developed oocytes can be identified in captive females during the seasonal active breeding period.

In order to be certain that the effects of the environmental regulators on intraovarian oocyte development were not reflecting the natural reproductive cycle, all experiments were conducted in the refractory period during which only recruitment crops of oocytes are observed.

The number of fish maintained in each tank and used for each experiment was small. For identification, the fish were at first tagged in the operculum with metal tags, or by 'hot marking'. Neither method proved satisfactory. By limiting the number of fish in each tank, individuals were recognized either by size of fin incision.

The fish were fed daily with a prepared artificial diet. The formulation is described in Table 1. Uneaten food was removed regularly and the tanks kept clean.

Table 1 Formula for the feed for juvenile or adult fish.

A. Dry ration		B. Liquid ration	
Fish meal	400 g	Water	900 ml
Soybean meal	250 g	Choline chloride	0.5 g
Chicken starter mash	100 g	Urea	0.5 g
Fish bone meal	50 g	Propylene glycol	5.0 g
Dairy whey	50 g		
Wheat germ	50 g	Mix A and B separately, then	
Wheat middlings	50 g	together before use.	

The oocytes were sampled in vivo monthly at first, and more frequently as they matured during the latter part of the experimental period. Individual records of oocyte development, as indicated by the classified stages of vitellogenesis, were therefore obtained. The sampled oocytes were preserved in Bouin's fixative, dehydrated in alcohol and cleared in xylol. They were then embedded in the tissuemate and later cut into sections of 10 μ in thickness. The sections were then stained with Heidenhain's iron-hematoxylin, counter-stained in eosin, and then mounted.

The work was conducted between the months of March and November 1971, and repeated the following year. Three general procedures for the experimental work were established:

1. Subject fish to the natural photoperiod and ambient temperatures throughout, thus establishing the control.

2. Precondition fish to the natural photoperiod and ambient temperatures first, followed by manipulation of regulators in the laboratory.

3. Subject the fish to manipulated regulators in the laboratory throughout.

For simplicity, all the photoperiod cycles were manipulated in units of three hours. The realistic photoperiod for simulating extreme conditions was judged to be 6L/18D, and this regime was the lowest used in the experiments. The operating range for water temperature was selected as 17-26^{o} C, the range of coastal temperatures in Hawaii to include both surface and deeper waters.

VITELLOGENIC DEVELOPMENT AND CLASSIFICATION

The histological details of the intraovarian oocytes during vitellogenesis have been described and illustrated by Kuo et al. (Chapter I). Three development stages were named, the primary oocyte stage, the yolk vesicle stage and the yolk globule stage. The latter is a prolonged development period and can be further subdivided.

The simple classification of oocytes based on gross observation utilizes five categories. Briefly these are:

Stage I: Primary oocyte stage, oocytes 12-170 μ in diameter. Oocytes in this stage are numerous and found in the ovaries throughout the year. At the beginning of the stage, the oocytes are small and spherical or oval in shape. They are transparent

and vacuole-like nucleoli can be identified. As the stage progresses the cytoplasm of the oocytes increases, becoming greater in volume than the nucleus. In the later part of the stage, the oocytes are irregular and vary between spherical and tetragonal in shape.

Stage II: Yolk vesicle stage, oocytes 170-210 μ in diameter.
At the beginning of stage II the appearance of each oocyte is characterized by the granular cytoplasm and darker zone surrounding the germinal vesicle. As the stage develops, the yolk vesicles increase in number and size gradually filling the cytoplasm from the center of the oocyte toward the periphery.

Stage III: Yolk globule stage, oocytes 200-700 μ in diameter.
In the early part of stage III the yolk vesicle occupies the entire ooplasm and the appearance of each oocyte is characterized by the granular appearance. As the stage proceeds, the internal definition is obscured from the center towards the periphery as the deposition of the yolk material increases evenly. Finally, when the fusion of yolk globules is complete, the central portion of the oocyte appears to clear. In the final phase of stage III, the yolk globules accumulate throughout the ooplasm. Fusion of the yolk globules and the numerous oil globules is apparent. The germinal vesicle becomes irregular in outline and consequently the nucleoli assume an irregular arrangement along the periphery.

Stage IV: Ripe stage, oocytes 825 μ in diameter and over.
Stage IV oocytes occur immediately before ovulation. The duration of the stage is short as the induced female is undergoing the final rapid development of oocytes before ovulation. Stage IV oocytes are characterized by the migration of the nucleus to the animal pole, and fusion of the yolk globules and oil droplets. Finally, the yolk appears as a homogeneous mass filling the interior of the oocyte.

Stage V: Atresia
Visible degeneration of the oocytes, mostly of stage III. The yolk globule contracts irregularly, beginning from the edge of the zona radiata and moving towards the center. The zona radiata begins to disintegrate and its outer surface becomes irregular. It eventually ruptures, invasion of the interior of the oocyte begins, and the yolk is phagocytosed by granulosa cells which undergo hypertrophy.

The slides of the oocytes prepared after each sampling period were examined under the microscope. The oocytes were then classified into one of the stages (I-V) described above. A total of 350-500 oocytes were classified each time to avoid bias in the frequency distribution. The percentage composition of the oocytes at various stages of development in the gonads was therefore calculated for each fish at frequent intervals throughout the experiment.

For the purposes of this text, the proliferation of primary oocytes from oogonia will be considered as the primary growth phase (stage I), and growth of primary oocytes toward yolk-laden oocytes as a secondary growth phase (stages II and III). Natural oocyte development has been observed in captive females only to the tertiary yolk globule stage, and no spawning has been recorded for the species in captivity without further inducement. Functional maturity, therefore, refers to the late tertiary yolk globule stage (III), when the mean oocyte diameter is greater than 600 μ. This is regarded as the proper stage at which to induce spawning artificially by the technique of hypophysation (Shehadeh et al., 1973a).

EXPERIMENTAL DESIGN

1. Effect of photoperiod and temperature

Eight individual experiments were designed to permute the conditions for deducing the effects of photoperiod and temperature on ovarian development.

#1 Accelerated photoperiod regime at $21 \pm 1^{\circ}$ C. Commencing March 15 the initial photoperiod of 12L/12D was increased to 14L/10D by equal increments each week until April 5th. That regime was maintained for two weeks and then decreased by equal increments to 9L/15D until May 27. That regime was maintained until June 3 and then fixed at 6L/18D until the end of the experiment on November 16.

#2 Accelerated photoperiod regime at $26 \pm 1^{\circ}$ C, with identical operational procedure as experiment #1 above.

#3 Retarded photoperiod regime from ambient to $21 \pm 1^{\circ}$ C. Commencing March 15 the natural photoperiod and ambient water temperature conditions were used until May 27. The fish were then exposed suddenly and maintained at a regime of 9L/15D for a week at constant temperature of 21° C. The photoperiod was adjusted to 6L/18D from June 3 until November 16.

#4 Retarded photoperiod regime from ambient to 26 ± 1^{o} C, with identical operational procedure as experiment #3 above.

#5 Control experiment under natural conditions. Commencing March 15 the natural photoperiod and ambient water temperature conditions ($24 - 26^{o}$ C) were maintained until November 16.

#6 Retarded photoperiod regime from ambient temperature to 17 ± 1^{o} C. Commencing March 15 a regime of 6L/18D was maintained until November 1.

#7 Retarded photoperiod regime from ambient to 17 ± 1^{o} C. Commencing March 15 the natural photoperiod and ambient water temperature conditions were used until April 18. The natural photoperiod was maintained but the temperature was lowered and maintained at 21^{o}C from that date until May 16 when the temperature was lowered further to 17^{o} C and a photoperiod of 6L/18D established. These conditions then remained constant until November 1.

#8 Control experiment under natural conditions. Commencing March 15 the natural photoperiod and ambient water temperature conditions ($24 - 26^{o}$ C) were maintained until November 1.

2. Effect of temperature alone

Four individual experiments were designed to provide data on the effect of temperature on ovarian development. Under a constant photoperiod of 6L/18D between June 3 and November 16, fish with gonads still in the refractory period were maintained in respective groups in the Environmental Control laboratory at temperatures of:

#9 17 ± 1^{o} C

#10 21 ± 1^{o} C

#11 26 ± 1^{o} C, and data compared with fish in

#12 natural conditions acting as controls ($24 - 26^{o}$ C).

The experiment was begun in June to utilise conditions established in the Environmental Control laboratory for other work.

3. Effect of exogenous gonadotropin injections

Four experiments were conducted on the effect of injecting pregnant mare's serum gonadotropin (PMSG) on fish under controlled and natural conditions. Briefly, the experimental conditions were as follows:

#13 Commencing March 15, a photoperiod regime of 6L/18D was maintained at $17 \pm 1^{\circ}$ C until November 1. In addition, each fish was injected with PMSG at the rate of 1 IU /g body weight three times per week commencing four weeks after the start of the experiment.

#14 As in #13 above, without hormone treatment.

#15 Commencing March 15, the natural photoperiod and ambient water temperature conditions ($24 - 26^{\circ}$ C) were maintained until November 1. In addition, fish were injected with PMSG at the rate of 1 IU/g body weight three times per week commencing four weeks after the start of the experiment.

#16 As in #15 above, without hormone treatment.

RESULTS

1. Photoperiod and temperature on ovarian development.

The mean percentage compositions of the oocytes during the varying stages of vitellogenesis, as sampled throughout the experimental period, are given in Tables 2.a (for #1-5) and 2.b (for #6-8).

Fish subjected to the accelerated photoperiod regime at both 21° (#1) and 26° C (#2) showed onset of stage II development by day 129 (July) and within 49 days following the regulated conditions of 6L/18D. Similarly, fish subjected to a retarded regime of natural conditions in March and April and then followed by a constant 6L/18D conditions (# 3 and 4) also showed identical development within the same time following the changeover.

The patterns of the photoperiod before the constant regime of 6L/18D differed significantly in both #1 and #2 compared with #3 and #4. In the former the fish were preconditioned in a cyclic change of increasing and decreasing photoperiod by equal increments. In the latter (#3 and #4) the fish were being exposed to an increasing daylength regime when the

Table 2.a Mean percentage composition (± SD) of oocytes by stages of vitellogenesis

Days (weeks) of experiment		0	34 (5)	74 (11)	100 (15)	129 (19)	154 (22)	185 (27)	205 (30)	217 (31)	235 (34)	246 (36)
Days (weeks) at 6L/18D					20 (3)	49 (7)	74 (11)	105 (15)	125 (18)	137 (20)	155 (23)	166 (24)
	I	100	100	100	100	80.3 (2.9)	69.2	61.0 (5.8)	49.4 (8.5)	64.8 (10.9)	65.6 (7.9)	75.4 (3.7)
#1	II					15.9 (1.0)	12.2	7.0 (0.4)	10.3 (1.3)	5.9 (1.7)	5.0 (0.9)	5.8 (1.9)
(21°C)	III					3.8 (3.8)	18.6	31.9 (5.9)	34.9 (9.9)	27.0 (14.9)	29.1 (7.9)	16.6 (3.6)
	V								5.4 (4.6)	2.3 (2.3)	0.3 (0.3)	2.2 (1.7)
	n	3	3	3	3	2	1	3	3	2	2	2
	I	100	100	100	100	93.6 (2.3)	93.5 (2.1)	83.0 (9.1)	88.9 (1.3)	80.9 (15.7)	92.9 (6.8)	95.0
#2	II					6.4 (2.3)	6.5 (2.1)	16.7 (8.8)	11.1 (1.3)	6.3 (3.5)	7.1 (6.8)	2.8
(26°C)	III							0.3 (0.3)		11.7 (11.7)		1.4
	V									1.1 (0.5)		0.9
	n	3	3	3	3	2	2	2	2	2	2	1
	I	100	100	100	100	88.0 (6.5)	66.8 (8.4)	51.7 (6.6)	47.7 (7.7)	59.6 (3.6)	87.5 (4.4)	89.0 (3.0)
#3	II					8.5 (3.3)	10.7 (1.3)	4.9 (1.0)	7.4 (0.9)	5.9 (2.6)	5.4 (1.8)	3.1 (1.1)
(21°C)	III					3.5 (3.5)	22.5 (9.1)	43.4 (6.3)	42.2 (6.2)	32.4 (1.2)	5.8 (2.7)	5.9 (1.9)
	V								2.6 (1.7)	2.1 (2.1)	1.4 (0.9)	1.9 (1.9)
	n	6	6	6	6	4	6	6	5	2	3	3
	I	100	100	100	100	93.1 (1.8)	87.4 (2.7)	81.3 (4.9)		93.5 (2.8)	92.0	94.6 (2.1)
#4	II					6.9 (1.8)	11.5 (2.0)	16.5 (4.2)		5.5 (2.7)	5.8	4.2 (1.5)
(26°C)	III						1.1 (0.7)	2.1 (0.8)		0.8 (0.4)	1.9	1.0 (0.5)
	V									0.2 (0.2)	0.3	0.2 (0.2)
	n	5	5	5	5	4	4	4		4	1	3
#5	I	100	100	100	100	100	100	100	100	100	98.7 (1.3)	96.9 (1.0)
Control	II										1.3 (1.3)	3.1 (1.0)
(24-26°C)												
	n	5	5	5	5	5	5	5	5	5	5	5

Table 2.b Mean percentage composition (± SD) of oocytes by stages of vitellogenesis.

Days (weeks) at 6L/18D		0	33 (5)	62 (9)	93 (14)	125 (18)	154 (22)	169 (25)
#6 (17 °C)	I	100	100	96.7 (3.3)	87.4 (8.2)	64.7 (12.9)	72.5 (2.4)	67.4 (1.5)
	II			3.1 (3.1)	3.7 (1.9)	7.6 (3.1)	9.3 (2.9)	7.7 (0.1)
	III			0.2 (0.2)	8.9 (7.2)	27.7 (15.6)	18.2 (0.7)	23.6 (3.0)
	V							1.3 (1.3)
	n	3	3	3	3	3	2	2
#7 (17 °C)	I	100	100	94.7 (4.2)	91.1 (1.8)	81.0 (9.2)	95.5 (1.4)	97.7 (1.9)
	II			2.1 (1.1)	3.4 (2.0)	7.3 (3.8)	2.3 (1.1)	0.5 (0.4)
	III			3.2 (3.2)	5.5 (5.5)	11.6 (9.1)	0.3 (0.3)	0.0
	V					0.1 (0.1)	1.8 (0.8)	1.8 (1.5)
	n	6	6	6	6	6	5	5
#8 Control (24-26 °C)	I	100	100	100	100	100	100	100
	II							
	III							
	V							
	n	2	2	2	2	2	2	2

photoperiod was suddenly adjusted and shortened. The changing photoperiod cycle in #1 and #2 was an attempt to simulate and condense a natural preseasonal cycle. In nature, vitellogenesis in the ovaries of the grey mullet begins shortly before the length of daylight is minimal. Neither sets of preconditions stimulated development.

The control fish (#5) subjected to natural photoperiod regimes did not attain the same stage of development as those under constant photoperiod of 6L/18D at any time, although some had begun stage II vitellogenesis by day 235.

The accumulated photoperiod for the entire experimental group in Table 2.a was 947L hr for #1 and #2, compared with 1009L hr for #3 and #4. The minor difference in the accumulated total L hr was not judged to be significant or to influence the results before the light regime of 6L/18D was established.

The responses of individual fish subjected to the constant photoperiod and differing experimental temperatures varied in the time of appearance and proportion of stage III oocytes. The later were observed between

four and eight weeks earlier in the gonads of fish maintained at 21° C (35-43%) than those maintained at 26° C (2-12%). Atresia (stage V) of vitellogenic oocytes commenced more rapidly at 26° C before the fish reached the stage of functional maturity.

During the first year, with # 1 - 5, the constant photoperiod regime of 6L/18D proved to be effective in stimulating development of vitellogenesis irrespective of any preconditioning adjustments. In the following year (# 6 - 8) the most successful procedure was repeated but at 17° C and with controls.

As in the first year, the time of onset of vitellogenesis was observed within 62 days for both #6 and #7 following constant photoperiod of 6L/18D, and irrespective of preconditioning. Fish in experiment #7 had been subjected to an advanced photoperiod at 21° C before the experiment began, with an accumulated 784L hr. The difference in percentage composition of stage III oocytes (28% compared with 12%) was pronounced. Atresia (stage V) was observed earlier in #7 although the reason was not apparent.

The photoperiod regimes and their duration for eight individual experiments are illustrated in Fig. 1. The conclusions from the experiments are readily discerned from the figure, namely, that vitellogenesis of the oocytes was stimulated effectively by a short and constant photoperiod regime of 6L/18D and that it was not related to any previous photoperiod condition. Temperature appeared to regulate maturation.

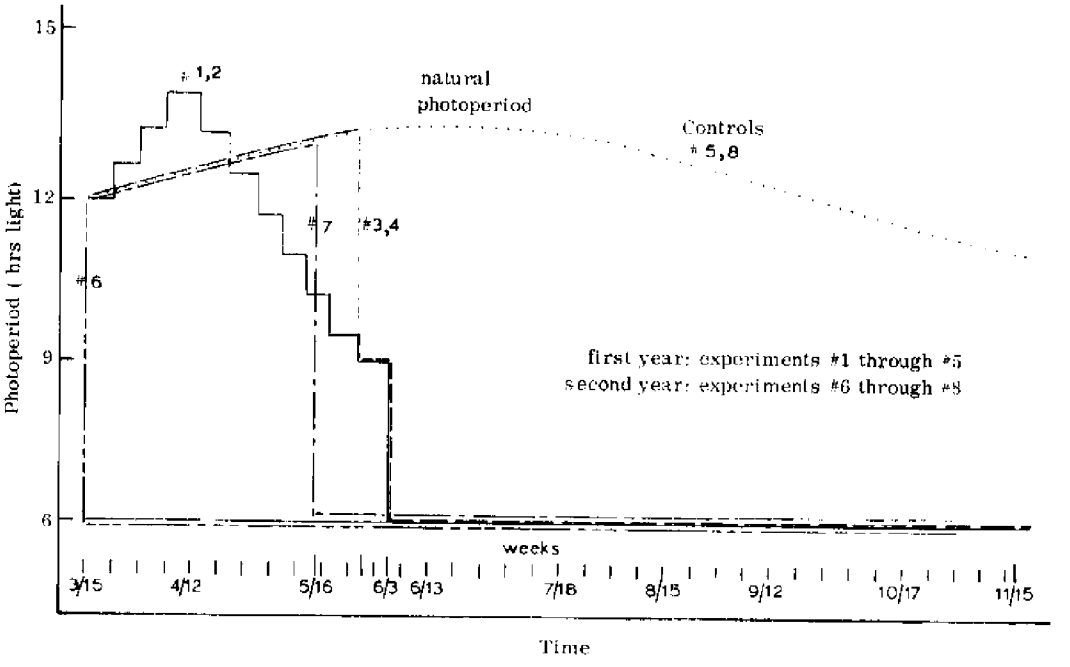


Fig. 1 Experimental light regimes and the natural light cycle in Hawaii.

2. Temperature alone and ovarian development

Data on the mean percentage composition of oocytes by stages of vitellogenesis related to temperature (with photoperiod constant at 6L/18D) are given in Table 3 and summarized by Fig. 2. Experimental temperatures were 17°, 21° and 26° C, for #9 - #11 respectively, with #12 acting as a control with ambient temperature conditions (24 - 26° C) and natural light cycle.

At 17° C, the onset of stage II occurred before day 56. Oocytes at the yolk globule stage (III) were also observed at that time. By day 115 the percentage of primary oocytes (I) had decreased substantially to 65%, whilst oocytes at the yolk globule stage (III) had increased rapidly (30.0%). Atretic ova (V) were first observed by day 141, and the percentage of stage I oocytes had increased once more.

At 21° and 26° C, the onset of stage II oocytes was also before day 56, although yolk-laden oocytes (III) were not apparent until day 84 in the fish maintained at the higher temperature (26° C). In general, the percentage composition and time of appearance of the vitellogenic oocytes of fish held at 21° C were similar to those of the fish at 17° C. The percentage of stage III oocytes was high, 41.6% by day 115. Fish held at 26° C showed less advanced development. Only 4.2% yolk-laden oocytes were observed at day 141. Atresia was again noted by day 141.

Towards the end of the experimental period, several fish held at 17° and 21° C had developed through to the stage of functional maturity (III, and over 600 μ mean oocyte diameter). A total of eight fish were selected and induced to spawn successfully by injections of partially purified salmon gonadotropin (1 mg equivalent to 2250 IU HCG). The injection dose and sequence are presented in Table 4. None of the fish held at 26° C completed vitellogenesis and no attempt was made to induce spawning.

For induced spawning of fish with oocytes in the later period of stage III (mean diameter below 650 μ), Kuo et al. (Chapter I) describe procedure options. Although the oocytes in fish maintained at 17° C were in the range 614 - 643 μ in diameter, the fish received either small increasing doses of hormone with an accumulated total of between 18.12 - 35.09 μg/g body weight from 7 to 10 injections, or between 27.8 - 32.90 μg/g body weight from two injections increasing in a 1:2 ratio. Spawning of fish held at 21° C was induced with total doses of between 21.5 - 41.5 μg/g body weight from two to four injections (Table 4).

Table 3. Mean percentage composition (± SD) of oocytes by stages of vitellogenesis

Days (weeks) at 6L/18D		0	28 (4)	56 (8)	84 (12)	115 (17)	141 (21)	162 (23)	176 (25)
#9 (17 °C)	I	100	100	95.2 (3.3)	82.0 (7.6)	65.0 (9.6)	68.9 (13.8)	75.8 (8.5)	80.0 (5.4)
	II			2.2 (1.6)	2.9 (1.0)	5.0 (1.9)	5.2 (2.6)	6.7 (1.0)	7.1 (2.4)
	III			2.7 (2.6)	15.1 (7.1)	30.0 (10.5)	25.8 (14.4)	15.7 (8.1)	8.0 (6.3)
	V						0.1 (0.1)	1.8 (0.9)	4.1 (2.1)
	n	6	6	6	6	6	4	3	3
#10 (21 °C)	I	100	100	85.4 (4.5)	63.9 (9.1)	51.9 (7.2)	62.2 (4.9)	77.1 (9.3)	87.2 (4.5)
	II			11.0 (2.6)	11.8 (1.5)	6.5 (0.6)	5.9 (1.3)	3.9 (0.5)	2.6 (0.7)
	III			3.6 (2.4)	24.9 (9.8)	41.6 (7.2)	29.7 (6.3)	17.9 (9.2)	7.0 (3.1)
	V						2.2 (1.3)	1.1 (0.7)	3.2 (0.7)
	n	6	6	6	5	6	4	4	3
#11 (26 °C)	I	100	100	93.2 (1.3)	89.4 (2.2)	81.9 (3.9)	89.3 (5.2)	92.9 (6.8)	94.7 (1.5)
	II			6.8 (1.3)	9.9 (1.7)	16.6 (3.5)	5.8 (1.9)	7.1 (6.8)	3.8 (1.1)
	III				0.7 (0.5)	1.5 (0.7)	4.4 (3.8)		1.1 (0.4)
	V						0.5 (0.3)		0.4 (0.2)
	n	6	6	6	6	6	6	2	4
#12 Control (24-26 °C)	I	100	100	100	100	100	100	100	100
	II								
	III								
	V								
	n	5	5	5	5	5	5	5	5

Table 4 Injection sequence and dose of salmon pituitary gonadotropin (1 mg equivalent to 2250 IU HCG) and spawning results with fish from experiments #9 and #10.

Fish No.	Date	Injection dose (mg)	Cumulative dose (µg/g body wt)	Mean egg diameter (µ)	Time to spawn from last injection	Spawning	Fertilization rate (%)	Experimental conditions: temperature	photoperiod
1	8/16	1	2.2	643					
	8/17	1	4.4						
	8/18	1	6.6						
	8/19	1	8.8						
	8/21	1	11.0	654					
	8/22	1	13.2						
	8/23	2.5	18.6						
	8/24	2.5	24.1	682					
	8/25	2.5	29.6						
	8/26	2.5	35.1						
	8/28	5.0	46.1	699					
	8/29	10.0	68.0						
	8/30			930	12 hr	natural	75	17°C	retarded
2	8/09	1	1.8	614					
	8/10	1	3.6	631					
	8/11	1	5.4						
	8/12	1	7.2						
	8/13	1	9.1						
	8/14	2.5	13.6						
	8/15	2.5	18.1						
	8/16	5.0	27.2	664					
	8/17	10.0	45.3						
	8/18			926	12 hr 30 min	natural	53	17°C	retarded

3	7/18	2.5	6.0	673					
	7/19	5.0	18.0						
	7/20	10.0	42.0	921	10 hr 30 min	artificial	0	17°C	retarded
4	9/20	3.0	5.0	617					
	9/21	10.0	21.5	617					
	9/22			931	15 hr 10 min	artificial	0	21°C	retarded
5	10/12	3.0	5.4	643					
	10/13	5.0	14.4	650					
	10/14	10.0	32.4	667	19 hr 30 min	natural	87	21°C	retarded
6	10/18	5.0	9.1	670					
	10/19	10.0	27.4	681					
	10/20			966	16 hr 10 min	natural	94	21°C	retarded
7	10/31	5.0	4.2	654					
	11/01	10.0	12.5	663					
	11/02	5.0	16.7	798					
	11/03	10.0	25.0	996	19 hr 50 min	natural	90	21°C	retarded
8	12/13	10.0	11.9	671					
	12/14	5.0	17.8	684					
	12/15	10.0	30.0						
	12/16	10.0	41.5	689					
	12/17			933	9 hr	natural	90	21°C	retarded

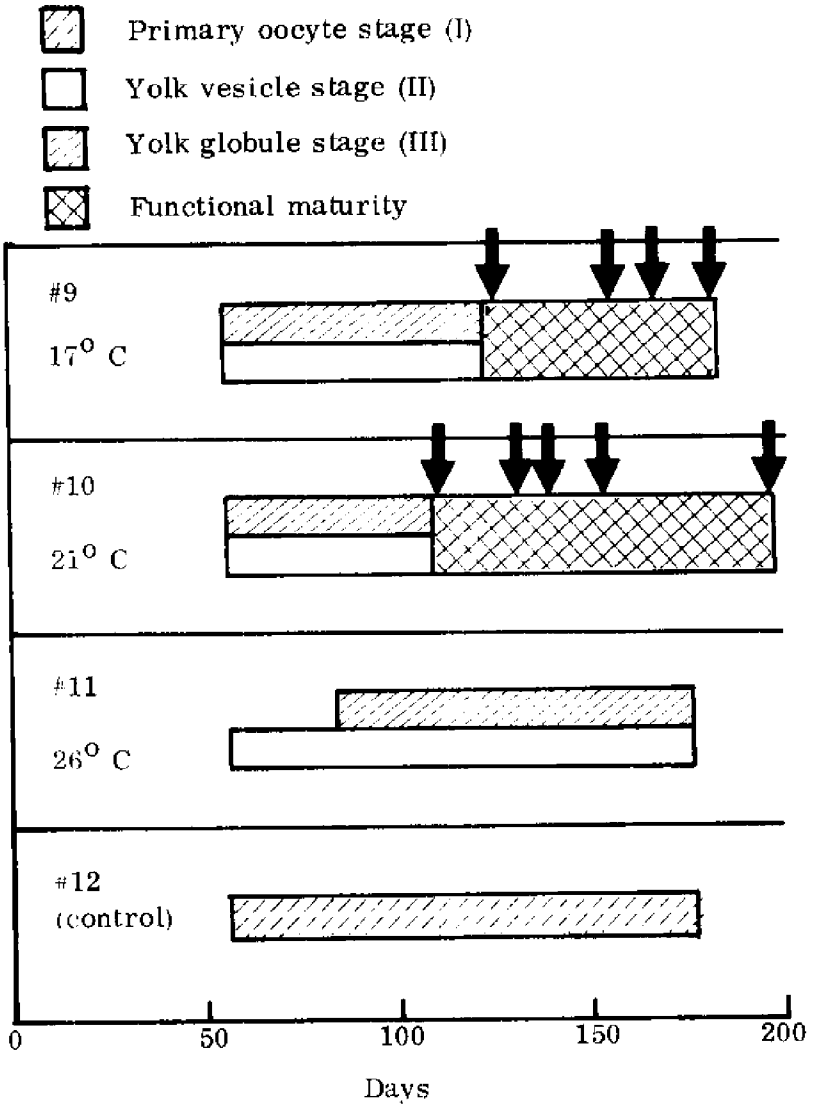


Fig. 2 Summary of ovarian development at photoperiod of 6L/18D with time (days). Induced spawning of individual fish indicated by arrows.

Some differences were observed in the morphology of stage III oocytes from fish maintained at 17° C compared with those found in fish during the natural breeding season or in other experimental conditions (#10). The oocytes were lighter in appearance and probably the yolk was less dense. The oocytes darkened as the accumulated hormone dose reached 20 μg/g body weight, and more yolk was deposited.

Examination of the oocytes of fish in the control conditions (#12) showed that no stage II oocytes developed within the experimental period.

3. Exogenous gonadotropin injections on ovarian development

The possible use of exogenous mammalian gonadotropin, pregnant mare's serum gonadotropin (PMSG) was tested for initiating ovarian development in association with photoperiod and temperature. Data on the effects on vitellogenesis by a series of injections (three each week) is presented in Table 5. Injections were begun 28 days after the start of the experiment.

For fish under conditions of a constant 6L/18D regime maintained at 17° C, both stage II and stage III oocytes were observed for both injected (#13) and non-injected (#14) individuals after 15 injections (day 62 of experiment). Thereafter, development was more rapid in the fish receiving injection (#13), though the difference in percentage composition of vitellogenic oocytes between the two groups was not significant. Vitellogenesis reached a peak by day 120 (33.5%) for #13 and by day 90 (27.7%) for #14. Atresia was more advanced for the injected fish. The duration of ovarian development was 138 days for #13, and shorter than that of #14 (over 168 days). The PMSG injections therefore accelerated the ovarian development.

Data from the control experiments (#15 and #16) indicated that, under natural photoperiod and ambient temperature conditions, vitellogenesis was initiated after 29 injections (day 94). The peak of oocyte development (28.9% stage III) was reached after 67 injections, thereafter followed by some atresia (stage V). No vitellogenic oocytes were observed in the control experiment without injection (#16). It was apparent that ovarian development was advanced by the injections of PMSG, by which vitellogenesis was initiated. No fish was observed to reach the stage of functional maturity at the ambient temperatures of 24 - 26° C with injections (#15) or without (#5, #12, and #16).

DISCUSSION

Many experiments with a variety of fish species have indicated that photoperiod and temperature are the two most significant external regulators of the reproductive cycle. The relative importance of these factors, however, varies with species.

Burger (1939) hypothesized that the time of avian gonad maturity could be predicted on the basis of a summation of photoperiod and thermal effects. The results of this study demonstrated that the onset of vitellogenesis in the grey mullet was not determined by a cumulative effect of day length but directly to the effective photoperiod of the time. Similar results have been reported for the brook trout (Henderson, 1963).

Table 5 Mean percentage composition (± SD) of oocytes by stages of vitellogenesis for females with and without injections of PMSG for ambient and 17° C temperatures with controls.

Days (weeks) of experiment		0	35 (5)	62 (9)	94 (14)	124 (18)	154 (22)	169 (25)	186 (27)	200 (29)	214 (31)	230 (33)
Number of injections				15	29	41	53	60	67	73	79	86
	I	100	100	93.6 (6.4)	76.6 (13.5)	65.3 (17.2)	65.3 (33.4)	92.7	90.8	100	100	100
#13	II			1.2 (1.2)	2.1 (1.0)	2.3 (0.4)	1.2 (0.1)	4.6	2.9			
(17 °C)	III			5.2 (5.2)	21.4 (12.7)	32.4 (17.5)	33.5 (33.5)	0.0	0.0			
plus PMSG	V							2.7	6.3			
	n	3	3	3	3	3	2	1	1	1	1	1
	I	100	100	96.7 (3.3)	87.4 (8.2)	64.7 (12.9)	72.4 (2.4)	67.4 (1.5)	75.8 (3.6)	87.0 (5.0)	88.9 (5.1)	92.3 (4.4)
#14	II			3.1 (3.1)	3.7 (1.9)	7.6 (3.1)	9.3 (2.9)	7.7 (0.1)	9.3 (1.9)	4.8 (3.0)	9.7 (4.0)	4.4 (2.7)
(17 °C)	III			0.2 (0.2)	8.9 (7.2)	27.7 (15.5)	18.2 (0.7)	13.6 (2.2)	11.9 (8.5)	6.3 (6.3)	0.3 (0.3)	0.0
without PMSG	V							1.3 (1.3)	3.0 (3.0)	2.0 (1.7)	1.2 (0.8)	3.3 (1.7)
	n	3	3	3	3	3	2	2	2	2	2	2
	I	100	100	100	99.7 (0.3)	99.3 (0.7)	94.1 (5.6)	69.2 (28.4)	61.5 (21.5)	69.4 (13.3)	92.3 (7.7)	
#15	II				0.3 (0.3)	0.7 (0.7)	5.9 (5.6)	3.1 (0.7)	9.4 (6.9)	10.5 (6.5)	7.3 (7.3)	
Ambient	III							20.0 (20.0)	28.9 (28.5)	19.9 (19.9)	0.4 (0.4)	
(24-26 °C)	V							7.8 (7.8)	0.1 (0.1)	0.2 (0.2)		
plus PMSG	n	2	2	2	2	2	2	2	2	2	2	2
	I	100	100	100	100	100	100	100	100	100	100	100
#16	II											
Control	III											
Ambient	V											
(24-26 °C)												
without PMSG	n	2	2	2	2	2	2	2	2	2	2	2

For the grey mullet, the time of onset of vitellogenesis was determined to be about eight weeks after exposure to a short photoperiod regime (6L/18D) at temperatures ranging between 17 - 26° C. The response of oocyte development to such a retarded photoperiod was consistent and not related to any preconditioning photoperiod regime, including a simulated natural photoperiod cycle. It was certain that the retarded photoperiod was more effective initiating development than temperature. The fact that vitellogenesis is induced naturally prior to the breeding season demonstrates that endogenous rhythm of reproductive activity is initiated by photoperiod and temperature, but is not toally controlled by either. The controlling mechanism of vitellogenesis in the grey mullet was the marked endogenous activity of the pituitary gland, which was itself initiated by the external environmental factor of daylight.

Once the secondary growth phase of oocyte development had begun, the rate of development was directly influenced by and inversely proportional to temperature for the experimental temperatures used, namely ambient, 17°, 21° and 26° C. A long daily photoperiod appeared to reinforce the effect of the higher temperatures (24 - 26° C) inhibiting vitellogenesis. A retarded photoperiod reinforced development at 17° and 21° C. A constant temperature of 21° C and a photoperiod of 6L/18D produced the most significant result in advancing the development of vitellogenesis. In summary, it was demonstrated that the short photoperiod of 6L/18D had a stimulatory effect initiating oocyte development, whilst temperature appeared to regulate vitellogenesis toward functional maturity.

The data indicated that a constant exposure to a temperature of 17° C hastened completion of any preceding phase of maturation and produced rapid final development with a 6L/18D photoperiod. It was believed that, at that relatively low temperature compared with ambient (24 - 26° C), development of oocyte maturation was incomplete and limited yolk deposition in stage III oocytes. Subsequent induced spawning and egg incubation showed that embryonic development failed to pass the blastula stage.

The spawning of grey mullet can be induced by injections of partially purified salmon pituitary gonadotropin SG-G100 (Shehadeh et al., 1973a) if hypophysation is begun when the fish are functionally mature. The injection schedule described by them proved to be unsuitable for the experimental fish maintained at 17° C, as the oocytes were functionally mature but the mean oocyte diameter was between 600 - 650 μ. Spawning of these fish was successfully induced by a series of injections using procedures described by Kuo et al., (Chapter I). Their schedule had been developed to induce spawning of fish that were still undergoing prolonged and slow final development at the end of

the natural breeding season. For the fish maintained at 17° C, microscopic examination of stage III oocytes revealed that they were lighter in appearance than normal. This was probably due to a reduced yolk deposition, as the oocytes became normal in appearance following an accumulation of SG-G100.

The reproductive cycle of many, if not most, vertebrates is under the dual control of an internal physiological rhythm and an external seasonal rhythm. The refractory period in the reproductive cycle is considered to be the time during which these two rhythms coincide and reinforce each other. As fish are exposed to changing environmental conditions, such as photoperiod and temperature, the external rhythm begins to dominate and its influences on the reproductive processes are transmitted by changes in the quantity of gonadotropin released from the pituitary gland. The results from this study demonstrated that vitellogenesis was initiated eight weeks after fish were exposed to a retarded photoperiod. This delay in the response to external stimuli implied that the changeover from the primary to secondary growth phase was a result of cumulative hormone influence (Barr, 1968).

Many experiments have demonstrated the effect of gonadotropins on stimulation of vitellogenesis. Ahsan and Hoar (1963) reported that luteinizing hormone (LH), alone or in combination with follicle stimulating hormone (FSH), stimulated vitellogenesis and growth of oocytes in Gasterosteus aculeatus. Neither human chorionic gonadotropin (HCG) nor PMSG was as effective as LH, although both produce some stimulation of oocyte growth. A negative effect of PMSG, alone or combined with other mammalian gonadotropins, on vitellogenesis was reported in hypophysectomized Carassius auratus (Yamasaki, 1965). By contrast, Tchechovitch (1952) found an appreciable degree of stimulation of the ovary of three teleost species injected with mammalian hormone.

The response of fish ovaries to HCG and PMSG is inconsistent. In this study, injections of PMSG were found to be effective initiating vitellogenesis, and the completion of oocyte maturation was then dependent upon water temperature. Functional maturity was noted only for those fish which were maintained at a low temperature. Atresia (stage V) of oocytes was observed earlier in the injected fish than those untreated but held under identical environmental conditions. The reason for the early appearance of atretic oocytes after injections of PMSG was not clear. The literature indicates that once vitellogenesis has begun, a continuing supply of gonadotropin is essential for maintenance, and the amount of gonadotropin secreted by the pituitary increases during this period. The result is probably due to the low injection dose (1 IU/g body wt.) throughout the experiment. In summary, the stimulatory effect of PMSG on vitellogenesis in grey mullet is clear.

VII

DESIGN OF AN ENVIRONMENTAL CONTROL LABORATORY FOR FISH

Nathaniel Bacon, B.S.

Abstract

The design of an environmental laboratory is described and illustrated. The purpose of the laboratory is to induce maturation and out-of-season spawning of fish by manipulation of photoperiod and water temperature.

Tank features and water systems are included, together with descriptions of mechanical and electrical components.

Operational problems are described, together with recent alterations which have been made to rectify them and improve the design of the laboratory.

INTRODUCTION

This paper describes and discusses the facility known as the Environmental Laboratory, which is located at the Oceanic Institute. The description takes into account the existing seawater system which supplies the Environmental Laboratory with heated, chilled, and ambient seawater.

The facility has been in use since September 1970. Therefore, operational evaluations and comments are included about some of the features incorporated in the overall design.

THE ENVIRONMENTAL LABORATORY

1. Design and superstructure

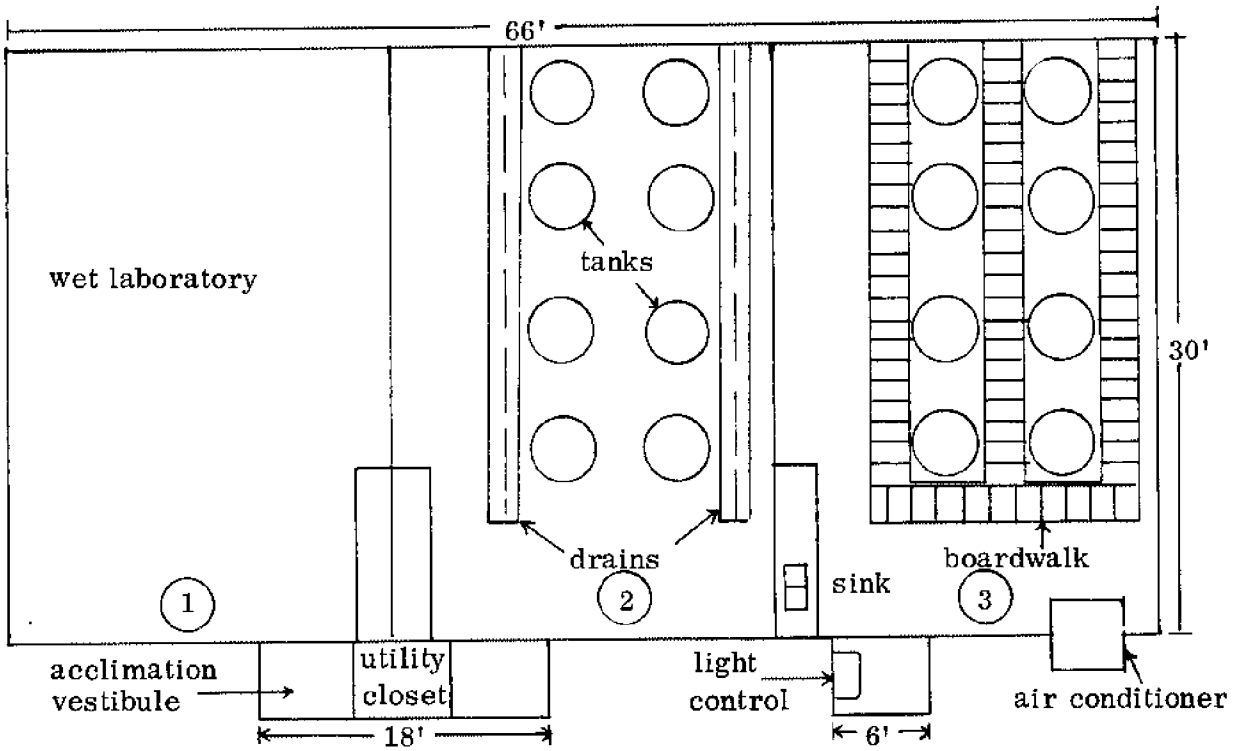
The 2440-ft^2 Environmental Laboratory is a wood-frame structure comprising three identical wet laboratories, separate vestibules, and utility closets (Fig. 1). The exterior walls are 1-inch thick fir; the partitions between the wet laboratories are double-walled, 3/8-inch exterior plywood separated by 2 x 4-inch studding. All walls in the wet laboratories have a 4-ft high splashboard (Weyerhauser 5/16-in. PL 15 Wainscot) running their entire length. The flat roof slopes toward the rear of the building, has a 1-in. layer of fiberglass insulation board and is topped with white coral chips.

Each wet laboratory has a separate 5 x 6-ft vestibule which acts as an acclimation chamber to prevent disruption of the controlled environment when operators enter or leave. The 5 x 8-ft utility closet houses a 20-gallon electric water heater, which supplies hot water for the sinks in each of the wet laboratories, and also serves as a storage room.

2. The wet laboratories

Each 22 x 30 x 9-ft high laboratory is fitted with a sink counter, two rows of holding tanks, two drainage troughs, an elevated boardwalk, a water supply system, numerous electrical outlets, and photoperiod and temperature controls (Fig. 2).

The circular holding tanks are reinforced concrete. Overall dimensions are 4-1/2 x 4-2/3 ft in diameter, and internal dimensions are 3-1/2 x 4 ft in diameter. The polyvinyl chloride (PVC) surface overflow and drain



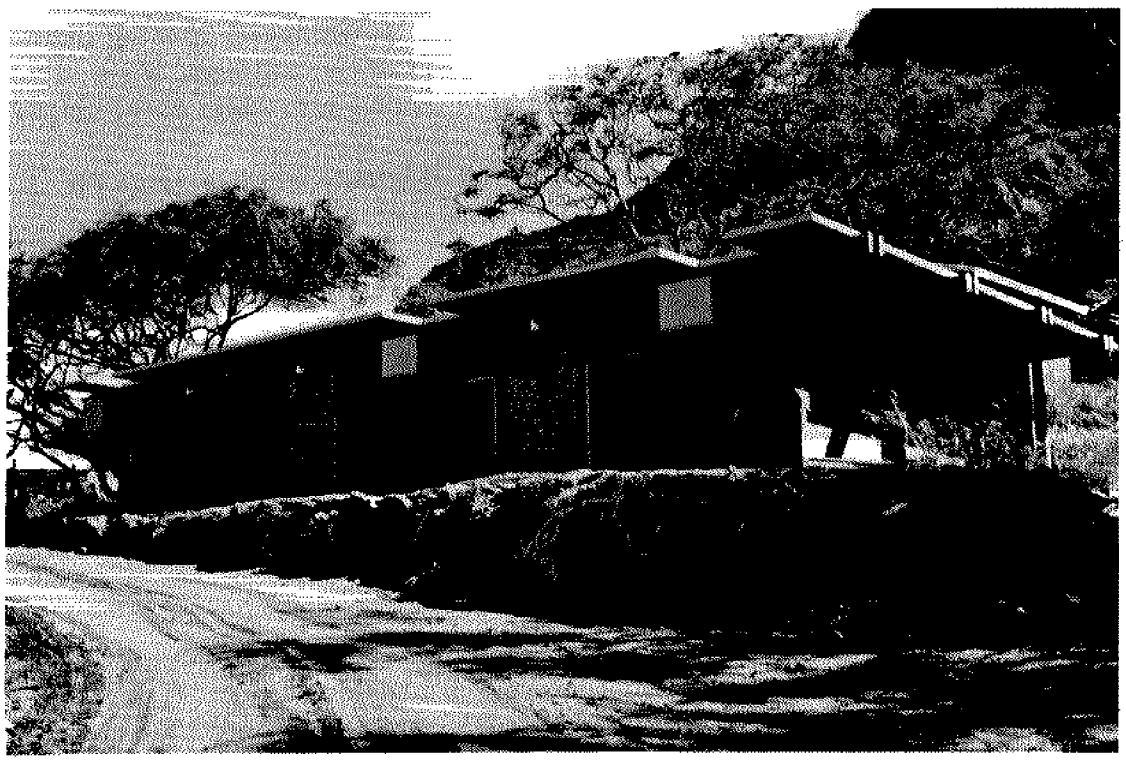


Fig. 1 Environmental Laboratory floor plan and exterior view.

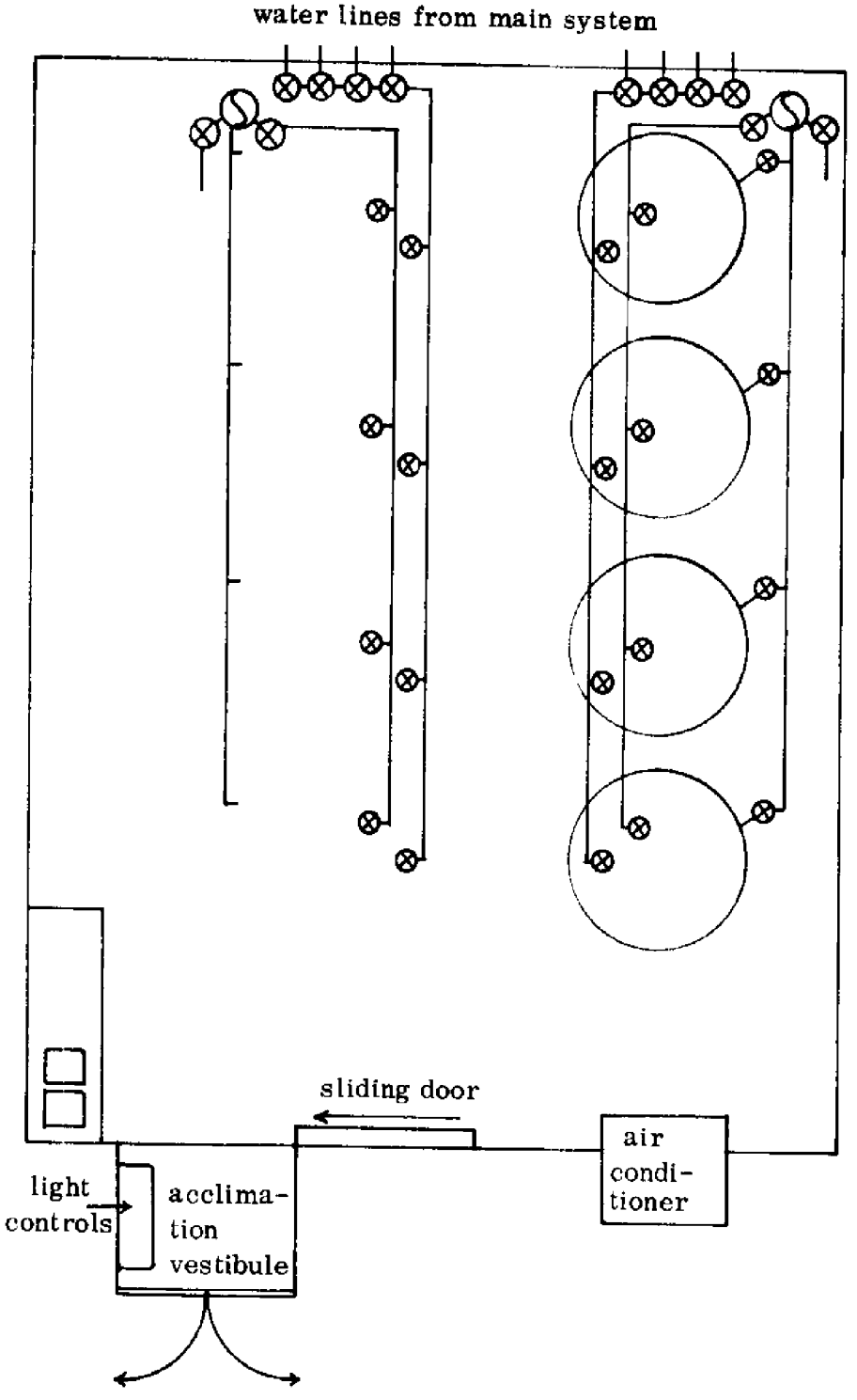


Fig. 2 Wet laboratory floor plan

assemblies fitted to each tank (Fig. 3) were cast in place during fabrication. The overflow consists of a 3-in. coupling centered 7 in. below the rim, and the drain is a 2 x 4-in. coupling, centered in the base, and attached to a 2-in. drain line.

The 2 x 4-in. coupling in the drain assembly has the 2-in. end bored all the way through, allowing an inch of threaded 2-in. drain pipe to protrude up through into the 4-in. end of the coupling to act as a threaded nipple (see inset, Fig. 3). The assembly is designed to act as a self-cleaning unit for the tanks and uses two concentric standpipes. Waste water passes through a series of 1/2-in. holes drilled at the base of a 4-in. outer standpipe, moves up between the two standpipes and overflows into the center 2-in. inner standpipe. The 2-in. center standpipe is fitted with a 2-in. threaded connector at the base to screw onto the 2-in. threaded nipple protruding through the base of the 4-in. coupling. The outer 4-in. standpipe, which is about 2-in. longer, is not threaded but pushes into the 4-in. socket. The length of the inner standpipe regulates the water depth in the tank and it can be sectioned. The design of the assembly permits good circulation of water through the tank and removes bottom sediments.

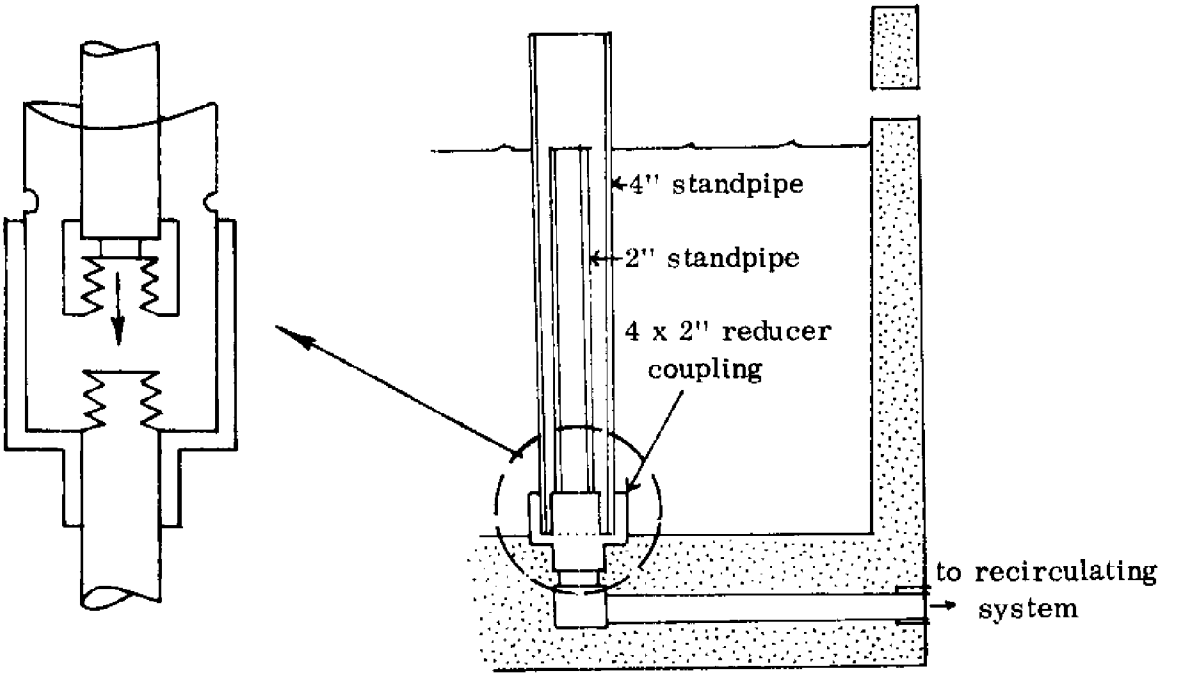


Fig. 3 Holding tank drain assembly schematic and reducer coupling detail.

The eight holding tanks in each wet laboratory are arranged in two rows of four with their drain holes positioned adjacent to the drainage troughs running parallel to each row (see floor plan, Fig. 1). The 1-1/2 x 1-1/2 x 27-ft long drainage troughs are recessed and flush with the gravel floor. They slope toward the front of the laboratory where they connect to the main drain by a 3-in. line.

An elevated boardwalk runs between and down both sides of the two rows of holding tanks. The outer arms of the 18-in. high boardwalk are 24 in. wide and have removable sections for access to the drainage troughs, valves and piping below; the center arm is 30 in. wide. The boardwalk facilitates observation into the holding tanks and access to the overhead water and lighting systems.

A 2 x 6-ft sink counter, fitted with a stainless steel double sink unit, is located near the door in each wet laboratory. The sinks are provided with hot and cold freshwater.

3. Internal water system

The water system is fully exposed and readily accessible. All pipes and fittings are schedule 80 PVC. Valves are Cabot Chemtrol ball or check valves. All PVC connections are cemented. Each row of tanks has its own manifold supply line (Fig. 4). An additional manifold, located to one side of the room,

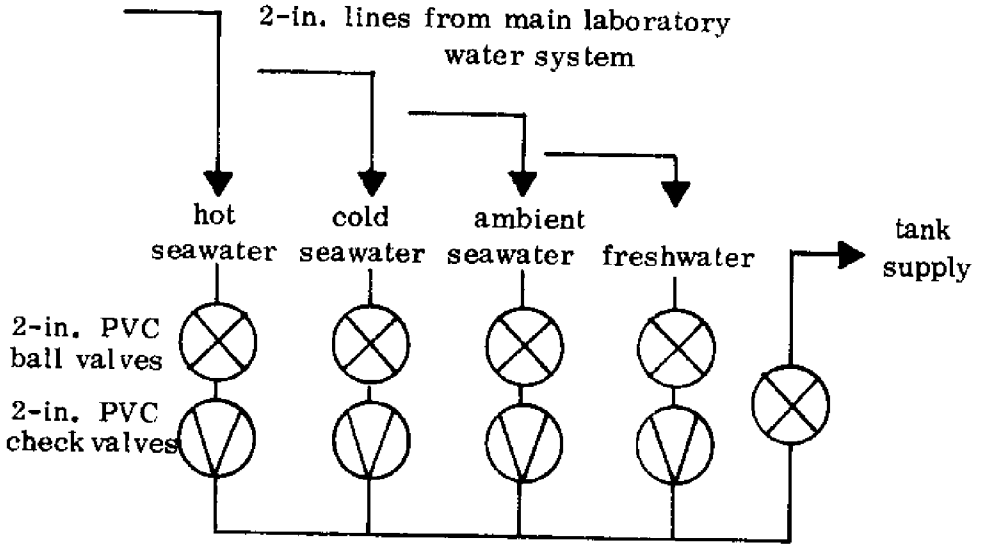


Fig. 4 Wet laboratory water distribution manifold

is available for more tanks. Four 2-in. trunk lines, which run overhead across the back wall of each laboratory, supply heated, chilled and ambient seawater and freshwater to all three wet laboratories. Each trunk line is routed through a 2-in. ball valve and 2-in. check valve to the tank supply line. The valves are arranged to permit mixing of saltwater and freshwater in the supply line without permitting it to back-up into the trunk lines through pressure imbalances. Manipulation of the valves regulates the salinity and temperature of the water flowing into the tanks. Two sets of different conditions are therefore available in each laboratory. From the valve manifold, water flows through a 2-in. supply line suspended over the length of the center boardwalk. Besides each holding tank, the supply line is tapped by a 1-in. line which extends over the tank and down to a 1-in. ball valve which controls the flow rate. Figure 5 shows one row of holding tanks, the boardwalk, the general plan of overhead piping, and at the rear of the room the manifold for incoming water lines.

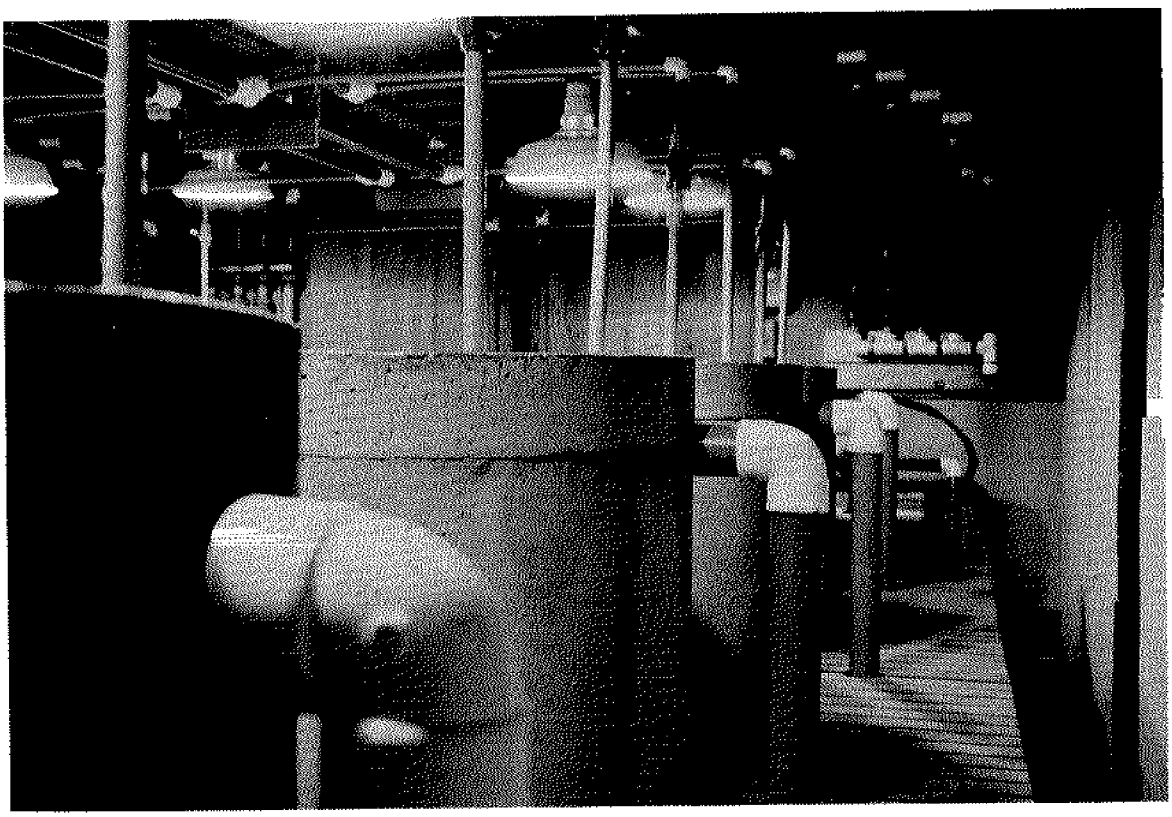


Fig. 5 Interior view of wet laboratory, showing piping.

Water leaves each holding tank either through the overflow port or through the standpipe-drain assembly. Water exiting through the former is directed into a drainage trough by 3-in. piping. Water leaving via the standpipe assembly passes along the 2-in. bottom drain line, through a ball-valve, and then connects to a 2-in. recirculation-drainage line fixed to a self-priming 3/4-hp pump (Jabsco Model PE 25-P4-37). The line is reduced to fit the 1-in. inlet and outlet of the pump. On the outlet side of the pump there is a 2-in. equal tee. Both lines leading from the tee connect to 2-in. ball valves which control the direction of flow from the pump. One of these discharges directly into the drainage trough, the other connects to an overhead supply line identical to the main delivery line described. The recirculation-drainage pump operates off a 220V, 3 phase circuit provided in each wet laboratory; each pump is wired through an overload fuse and starter. Figure 6 is a schematic of the recirculation-drainage system.

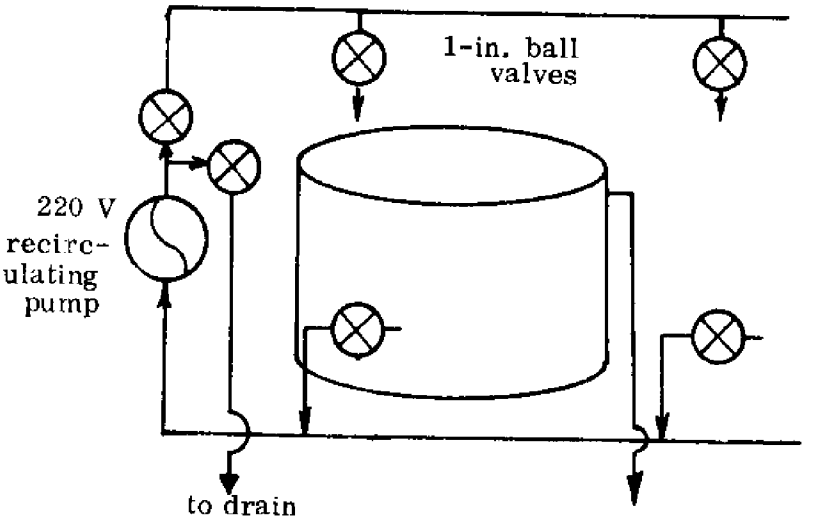


Fig. 6 Wet laboratory water recirculation and drainage piping.

4. Photoperiod and temperature controls

Eight grounded electrical outlets (110V) are conveniently located around the perimeter of the room above the splash board. Lights and light controls are also on a 110V circuit. An incandescent lamp (500 W) in a porcelain fixture is suspended over each holding tank. The lamps are controlled by a 24-hr timer (Paragon Model 4005-05) and dimmer (Power Stat Model AM136ST-2) mounted on the wall in the vestibule. With this control unit it is possible to schedule any photoperiod in a 24-hr day. Dawn and dusk periods are simulated by the dimmer unit which gradually increases and decreases the intensity of light emitted by the lamps at the beginning and end of each daylight period.

Room temperature is maintained by the wall mounted air conditioner (Carrier Model 50 DA006400) at the front of the room. Desired room temperatures between 50 and 100° F (10.0 and 37.8° C) are selected on a remote thermostat located below the air conditioner.

THE MAIN SEAWATER SYSTEM

An 80-hp pump (Floway Model 10 DOL) fitted with a stainless steel impellor, draws seawater from a 70-ft well located on the beach fronting the Oceanic Institute. Seawater is delivered through a 1000-ft length of 16-in. concrete pipe at the rate of 3000 gpm (11,356 l./min.) to an elevation of 39 ft. The line then divides into several feeder lines supplying seawater to other facilities at the Oceanic Center.

At a point before the 16-in. trunk line divides into the feeder lines, two 4-in. PVC branch lines are attached and water flow is controlled by cast iron gate valves. One line connects to a 15 hp in-line booster pump (Floway Model 18 MKH); the other is a pump by-pass loop. The booster pump supplies a 4-in. PVC line, passing through the machine room of Brittingham Laboratory, and an elevated vinyl-lined redwood settling tank. It is approximately 400 ft between the booster pump and the settling tank located 80 ft above sea level. The water level in the tank is maintained by a float switch which controls the booster pump. A 4-in. safety overflow line carries excess water in the tank to waste.

The circular settling tank is constructed of redwood staves, held together by steel cables, and a peaked shake roof fitted with a hinged access panel. The tank is 12 x 17-2/3 ft in diameter, providing a reserve capacity of approximately 16,000 gallons (60,565 l.) when depth is maintained between 9-10 ft. The outlet from the tank is a 4-ft high 4-in. standpipe located in the center of the floor.

The 4-in. outlet line delivers seawater back to the maching room in the Brittingham Laboratory at 20 psi and 72° F (22.2° C); the salinity is 32°/oo. A series of valves and tees redirect the ambient seawater to various laboratories located at the Institute. In particular, a 2-in. line supplies ambient seawater direct to the Environmental Laboratory, and a 3-in. line delivers seawater to the heater/chiller unit in the machine room. The 3-in. line running to the heater/chiller unit divides into two 2-in. lines which supply the separate components of the unit. A 2-in. ball valve controls the flow into each component.

The heater component consists of a gas-fired, 420,000 Btu./hr water heater (Ajax Water Heating Boiler Model WG 523) and a series of four heat exchangers (Model 135 GNB Pyrex brand Shell and Tube heat Exchangers (Fig. 7). It is designed to provide 100° F (37.8° C) seawater at a rate of 20 gpm (75.7 l./min.). The chiller component consists of six similar exchangers connected in series and a refrigeration unit (Carrier Model 5H60, 40 hp compressor and Carrier Model 10 FD 08227 Ethylene Glycol Cooler). It is designed to provide 40° F (4.4° C) seawater at a rate of 20 gpm.

Together with the 2-in. ambient seawater line and a 2-in. freshwater line, the two styrofoam insulated 2-in. heated and chilled seawater lines run from the Brittingham Laboratory to the Environmental Laboratory about 300 ft away.

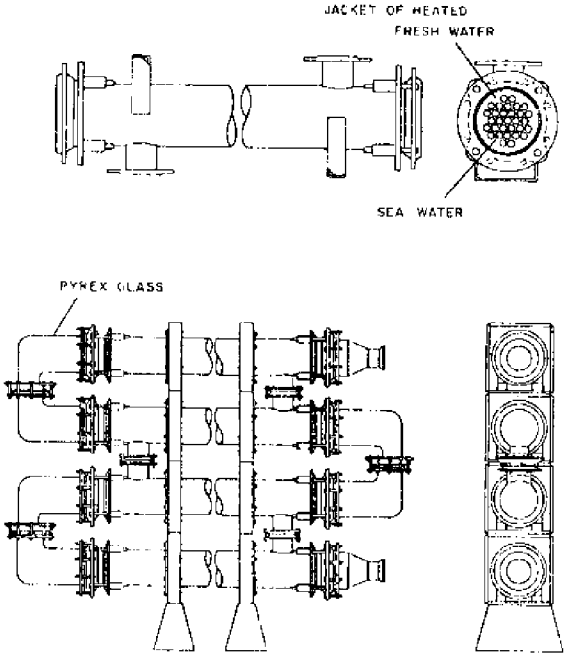


Illustration from
Lasker, R. and L. L. Vlymen, Experimental Sea-Water Aquarium. Bureau of Commercial Fisheries Circular 334, Washington, D. C. November 1969

Fig. 7 Schematic of heater unit heat exchanger assembly.

OPERATION AND DISCUSSION

Operating the Environmental Laboratory (as it was originally designed and described here) for extended periods at full capacity has not been without difficulty. Seawater pressure and volume flow to the Environmental Laboratory was insufficient to support continuous use of all the facilities at maximum. Reliable temperature and salinity adjustment could not be achieved for long periods for more than two of the wet laboratories at any one time. To overcome the problems, experimental work was restricted to two laboratories only.

Operation of the chiller unit at maximum, for long long periods, proved impossible, and was troubled by occasional shutdowns. The specified water output of water at 40^{o} F was unattainable. A continuous output of seawater at 50^{o} F (10.0^{o} C) was maintained, but the lowest temperature attainable in the wet laboratories was 55^{o} F (12.8^{o} C).

For low temperature work (below 13^{o} C), the chiller unit outlet temperature was regulated by maintaining the cooling media at a constant temperature and a constant flow rate of seawater through the heat exchanger. Since there was no flow metering device incorporated into the chiller unit, the flow was regulated by manipulating the 2-in. inlet valve and maintaining a constant back pressure in the chilled sea water line. A constant back pressure was difficult to maintain because of use of other outlets connected to the main water system. If the back pressure increased to 18 psi, a pressure sensing safety device shut the unit down, preventing the possibility of freezing in the heat exchanger. In the event of a shutdown, a battery operated horn sounded. The unit then had to be restarted manually, regardless of the cause of the shutdown.

For temperature control between 13-23^{o} C, few problems were experienced.

The drainage system designed for recirculating water in the wet laboratories had several design disadvantages. The recirculating or partially open water system could have been improved by the inclusion of a good filtration system into the circuit. Draining each tank with the pump and operating the manual valves below the boardwalk were unnecessary inconveniences.

Present improvements have replaced the circular reinforced holding tanks and boardwalks with rectangular fiberglass tanks freestanding on concrete floors. Wet tables are installed over open floor space made available by a more compact tank arrangement.

Results with the photoperiod and temperature controlled experiments have produced optimum specifications for future environmental conditions in the laboratory which will not require the extremes provided by the heater/chiller unit. The heater/chiller unit is therefore being replaced by smaller and more specifically controllable units in each wet laboratory.

Note: Use of a trade name does not imply endorsement by the Oceanic Institute.

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