Egg-quality of turbot (Scophthalmus maximus L.) kept in captive conditions.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy.

by

Lesley Ann Barton, B.Sc.

Dept. of Marine Biology
(University of Liverpool),
Port Erin,
Isle of Man.

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To mum and dad.

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SUMMARY:

The aim of this study was to investigate some of the factors affecting egg-quality of captive turbot (Scophthalmus maximus L.).

All females showed an obvious seasonal decline in their egg-size, with large fish tending to produce larger eggs. When the egg-size decline was taken into consideration, fecundity estimates of hatchery turbot were increased considerably. There was a positive correlation between egg-size and larval length (LL), and between egg-size and the yolk-sac index (YSI) of the emergent larva, but none between LL and YSI. The ratio, LL : YSI, was not proportional to egg-size; nor were the lengths and YSI's of emergent larvae affected by crossing eggs from one particular batch with different males. When monitored to Day 10, small larvae performed just as well in the rearing-system as larger larvae.

There was no obvious variations in egg water content or in the percentage concentrations of total, neutral or phospholipids in early or late season turbot eggs. The percentage concentrations of long-chained, polyunsaturated fatty acids in egg neutral lipid appeared reasonably constant between eggs of different females and throughout the spawning season, but the percentage concentrations of these fatty acids decreased in the phospholipid fraction of late season eggs. However, these observations must be heavily qualified since minimal sample numbers were analysed.

Studies into the over-ripening of ovulated turbot eggs inside the lumen of the ovary prior to hand-stripping

revealed that freshly ovulated eggs showed greater than 90% fertilisation and up to 92% hatch, but this dropped to 0% hatch in eggs retained inside the female for 1 day after ovulation. Precise ovulatory rhythms emerged from the results which were apparently not synchronised by "sunrise/sunset" stimuli. The time period between ovulations was the same for any 1 female but varied between individuals. Attempts to synchronise the ovulations of females with similar ovulatory periods by injecting with a primer of salmon pituitary extract followed by varying doses of 17αhydroxyprogesterone or 17αhydroxy, 20βdihydroprogesterone proved unsuccessful. A few turbot females had alternating long and short ovulatory cycles. Investigations revealed this was not caused by the females' paired ovaries ovulating alternately. An attempt to determine whether naturally-spawning turbot females show a spawning rhythm was unsuccessful.

Investigations into the ageing of hand-stripped gametes demonstrated that "stripped" turbot eggs could be stored in their ovarian fluid for at least 2 hours without losing their fertility. The rate at which eggs became "water-activated" varied between females but was apparently unaffected by ambient temperature or oocyte age. The percentage fertilisation produced by seawater-activated turbot milt declined rapidly approximately 15 minutes after stripping, whilst unactivated milt had a slower, constant rate of ageing. However, different males showed slight variations in their milt ageing-rates.

GENERAL INTRODUCTION

In order to make cultivation of a fish species economically sound, techniques must be devised which permit its life-cycle to be completed under artificial conditions. Attempting to stock farms with "wild-caught" eggs and larvae is extremely unreliable, very costly and poses a threat to wild fish stocks. A guaranteed supply of good-quality, site-produced eggs and yolk-sac larvae is therefore a tremendous asset to any commercial fish-farm. For this reason, Shearwater Fish Farming Ltd. suggested this investigation into factors affecting the egg-quality of broodstock turbot (*Scophthalmus maximus* L.) at their Port Erin hatchery on the Isle of Man.

The much used term,"egg-quality", is defined throughout this thesis in terms of egg viability and hatchability, and the survival and growth of the emerging larvae. Large variations in these parameters had been noted at the Shearwater hatchery, not only in eggs from different females which were held under identical conditions and fed the same diet, but also between different egg-batches of individual females.

Sperm-quality was standardised as far as possible by "pooling" semen from several males when inseminating eggs, so this was not considered to be an important limiting factor and work was chiefly focussed on egg-quality alone.

Considering the great importance of good egg-production and egg-quality in fish culture, surprisingly little previous work has been carried out to investigate these topics. Perusal of the relevant literature indicated at least four

broad areas which could account for variability in egg-quality and performance of the newly-hatched larvae:

I. Differences in physical parameters - for example, egg-diameters, lengths of emerging larvae, etcetera. (Bagenal, 1969b & 1971; Brown, 1946; Dahl, 1912; Ware, 1975).

II. Biochemical differences between eggs and, in particular, differences in their long-chained polyunsaturated fatty acid content. (Cowey et. al., 1976; Owen et. al., 1975).

III. Over-ripening of ovulated oocytes within the lumen of the ovary prior to hand-stripping. (Jones, 1970; Hirose et. al., 1977; Nomura et. al., 1974; Sakai et. al., 1975).

IV. Water-activation and ageing of hand-stripped gametes prior to fertilisation. (Rothschild, 1958; Yamamoto, 1961).

The importance of each of these factors in determining egg-quality in turbot is examined in turn in Sections I to IV of this thesis.

It is hoped that this study will not only contribute to a greater understanding of marine egg-production and -quality and suggest methods by which these parameters might be improved in a fish-farm environment, but that it will also encourage further investigations into this broad and complex field.

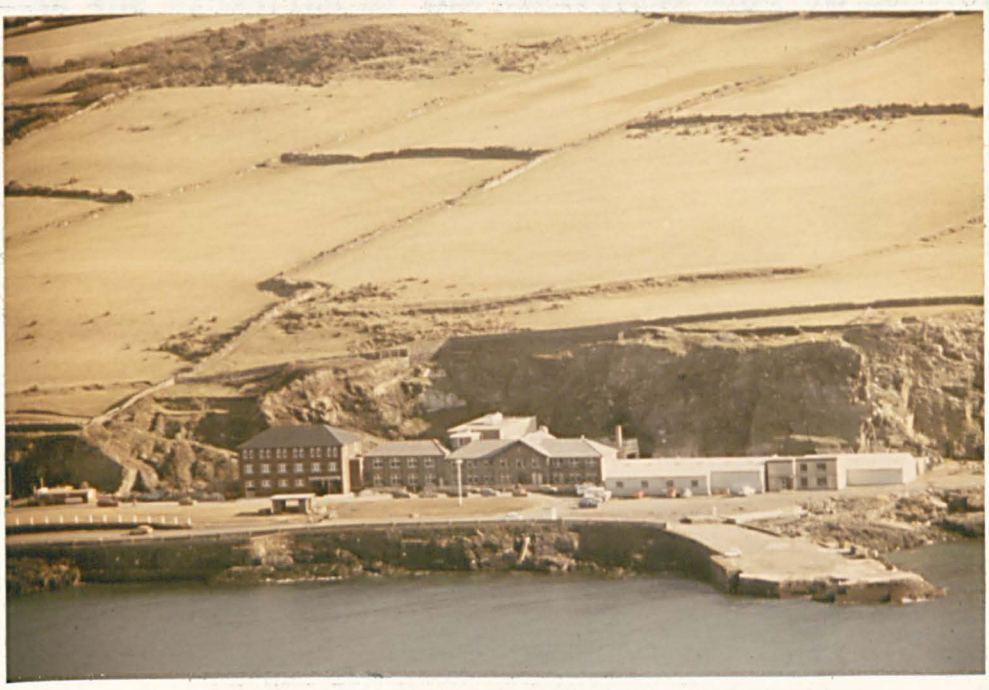


Plate I. The Marine Biological Station at Port Erin, Isle of Man, with Shearwater Fish Farming Ltd.'s hatchery buildings on the far right of the picture.

GENERAL MATERIALS & METHODS

All investigations were carried out at Shearwater Fish Farming Ltd.'s hatchery at Port Erin on the Isle of Man (with the exception of the analytical work in Section II which was performed at the NERC Unit at Menai Bridge by kind permission of Prof. Crisp and Dr.D.Holland).

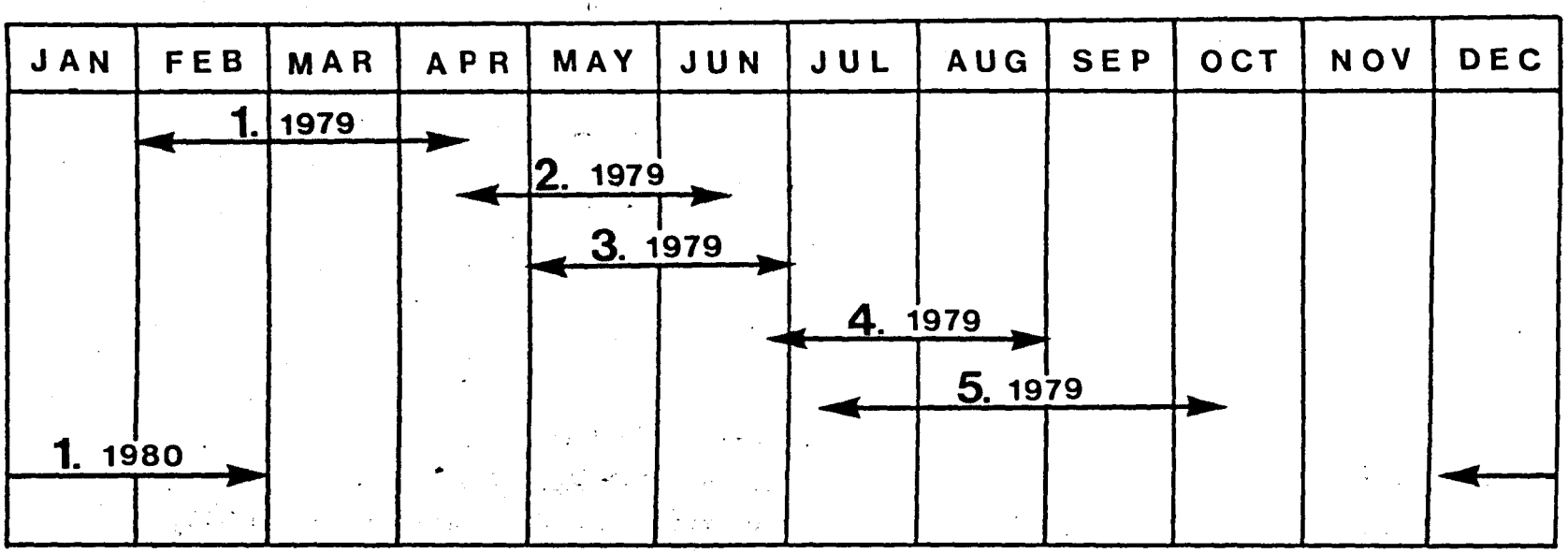
The Port Erin site (see Plate I) has a hatchery tradition dating back to 1902 when a Marine Biological Station containing a plaice hatchery was built there. It was partly financed by the Manx government but chiefly organised by the Liverpool Marine Biology Committee. In 1960-1963, Dr.J.E.Shelbourne chose the Marine Biological Station at Port Erin as the site for large-scale plaice larval rearing experiments - the quality of Port Erin's seawater playing an important part in this decision (Shelbourne, 1964). When the White Fish Authority, and later the Ministry of Agriculture, Fisheries and Food, decided to establish a marine fish-culture unit adjacent to the Marine Biological Station, seawater quality was again a major consideration (Naylor, 1981).

Shearwater have leased the fish-culture unit since 1978, concentrating chiefly on turbot-rearing but also working with Dover Sole, bass, bream and halibut.

1. BROODSTOCKS:

The hatchery contains five turbot broodstocks, each consisting of 15-20 females with approximately 6 males, held in square tanks (3.80m x 3.80m x 1.24m depth) at 14.0°C, 1m water-depth and a water-flow of approximately 13 l/minute.

Fig. 1. The different spawning periods of Shearwater Fish Farming Ltd.'s 5 turbot broodstocks in the year 1979-80. The different spawning times are caused by holding the separate broodstocks under different light/dark regimes.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4. 1979
5. 1979
1. 1980

Fig. 1.

Wild turbot spawn once a year, from May to August (Jones, 1974). However, at Port Erin each captive broodstock was kept in a different light-regime so that they spawned at different times of the year. This meant that, usually, ripe eggs were available 10 months of each year (see Fig.1).

The broodstocks were fed to satiation three times a week on a diet of chopped whiting and whole sprats. (These fish were chosen because they closely resembled turbot's natural diet, and because sprat are rich in essential long-chained fatty-acids.) Each individual fish was freeze-branded for identification purposes using a dry ice/methanol mixture and brass branding-irons.

2. DETERMINATION OF MATURITY STAGES AND COLLECTION OF GAMETES:

Turbot did not spawn and fertilise eggs naturally in these captive conditions and so gametes had to be hand-stripped from ripe fish and artificial insemination carried out.

Jones (1974) has given a detailed account of the maturation cycles of turbot. At the Port Erin hatchery, the maturity stages of female turbot were monitored by removing the fish from the tank, assessing the degree of swelling of her ovaries, and attempting to handstrip hyaline eggs by applying gentle pressure along the ovaries (see PlateII). The following simplified maturity scale was employed:

0 : no swelling of the ovaries.

1 : slight swelling.

2 : ovaries swollen; reddening of vent.

Plate II. Testing the "ripeness" of a female turbot by applying pressure over her ovaries.

Plate III. "Stripping" milt from a male turbot.

Plate IV. "Stripping" eggs from a female turbot.

Plate V. Artificially inseminating eggs with a few millilitres of milt suspension.

3 : ovaries very swollen and quite soft; spawning imminent.

4 : "running" with hyaline eggs.

5 : spent; ovaries regressing.

The ripeness of male turbot was assessed by removing them from the tank and attempting to express white semen from their vent by a circular, squeezing action and collecting it with a 1 ml glass pipette (see Plate III).

Running females were routinely stripped twice a week. The general procedure for hand-stripping and artificial insemination was as follows. Firstly, a "pool" of sperm was made by stripping milt from three or four males into approximately 25ml of seawater (see Plate III). This "pool" was used to fertilised subsequent eggs in an attempt to eliminate male variability and reduce the possibility of using inviable sperm.

Eggs were then stripped from each female into netted 33cm x 23cm x 21cm plastic tanks containing seawater (see Plate IV) and fertilised with a few ml of milt suspension (see Plate V). Separate tanks were used for eggs from each female.

The eggs were allowed to stand for approximately 30 minutes and were then lifted out inside the net, blotted dry with tissue paper, and weighed on a top-pan balance. The weight of the wet net was subtracted from the gross weight to give an approximation of total egg-weight. The eggs were tipped out of the nets back into the plastic tanks of seawater where dead eggs sank to the bottom of the tank whilst viable eggs floated at the water-surface. (However, it must be noted that not all floating eggs were viable).

LINCOLN'S (1976) QUALITATIVE SCALE AS APPLIED TO TURBOT EGGS.

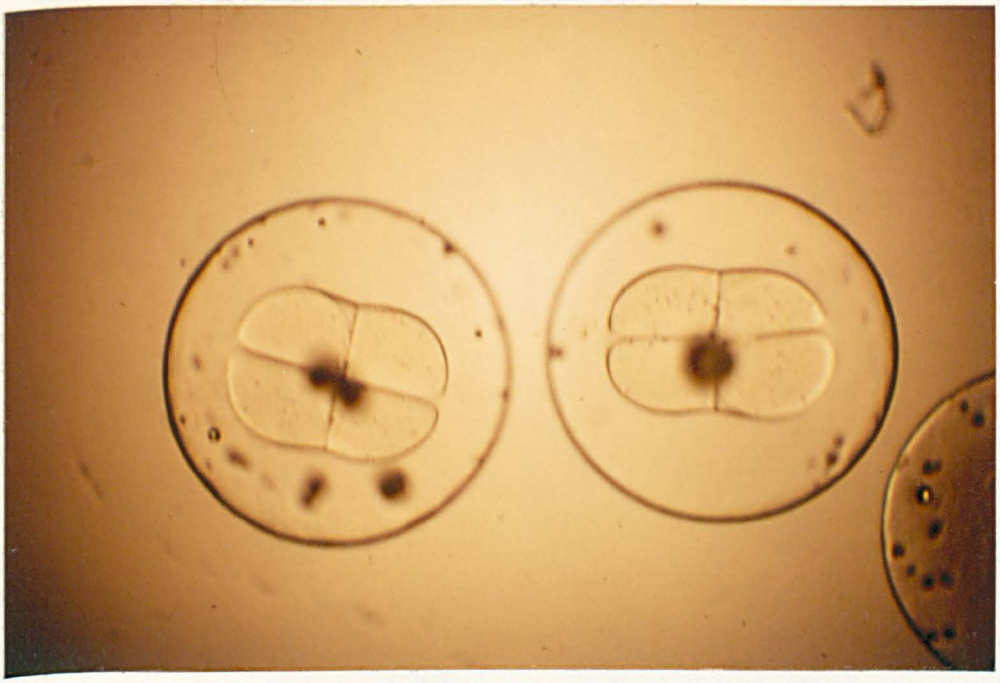


Plate VI. Good quality eggs - clear membranes, symmetric divisions.

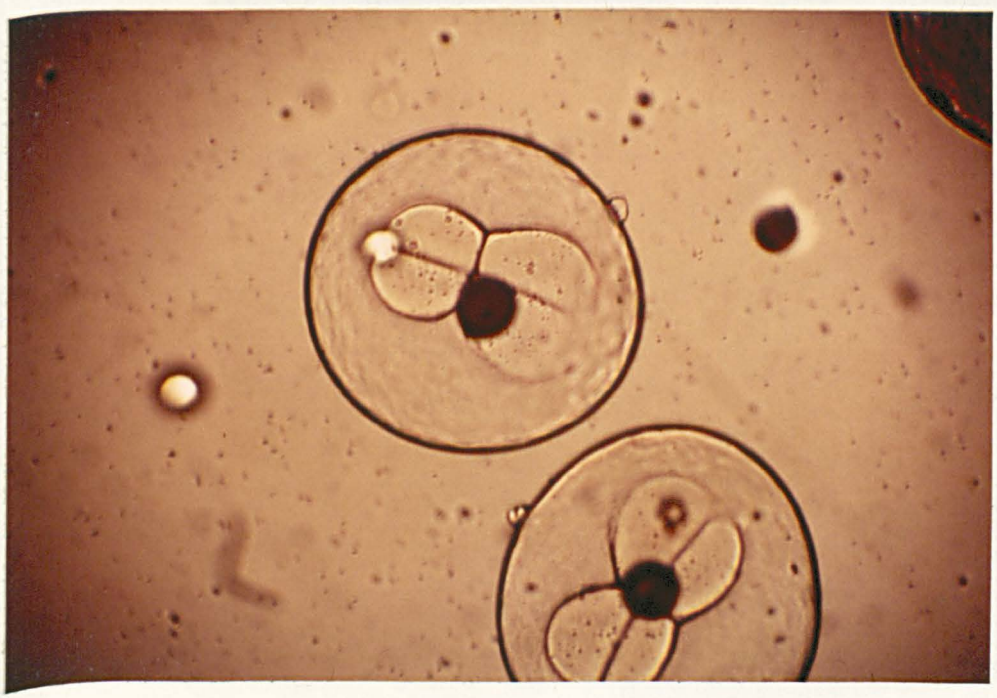


Plate VII. Fair quality eggs - divisions slightly asymmetric, smaller areas of contact between cells.

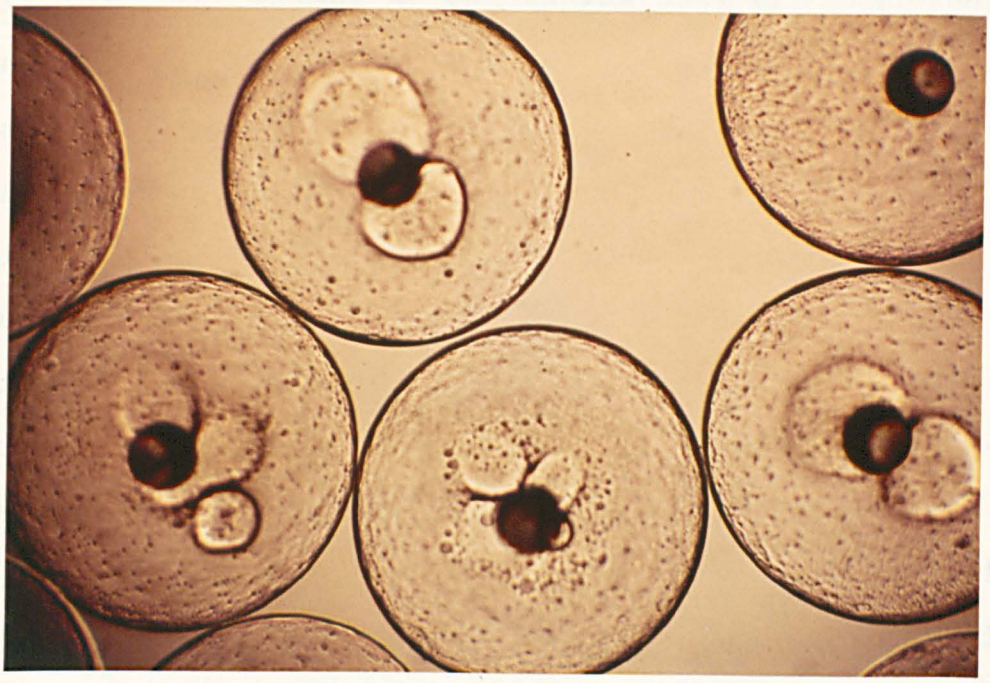


Plate VIII. Poor quality eggs - irregular divisions, cells loosely connected, pitted membranes.

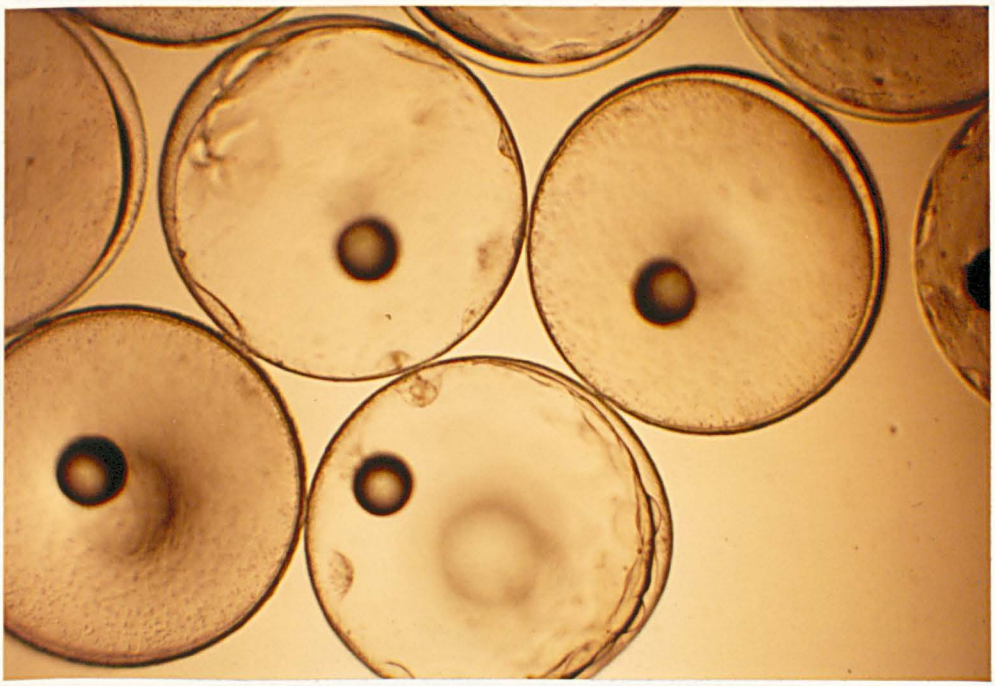


Plate IX. Dead, "golfballed" eggs - infertile, pitted membranes, "dimpled" cytoplasm.

The sunken eggs were removed by siphoning, collected in a net and weighed. When the floating layer consisted chiefly of viable eggs, the weight of the sunken eggs was deducted from the total egg-weight to give the approximate weight of viable eggs. These weights were used to estimate egg-production for general hatchery records.

Finally, the eggs were allowed to stand for three hours and then the quality of the first and second divisions were assessed by visual examination using an Olympus BHB microscope at 40x magnification. Lincoln's (1976) "qualitative-scale" for distinguishing good and poor-quality plaice eggs was used to determine which egg-batches were of sufficient viability to justify incubation. Plates VI-IX demonstrate this qualitative-scale as applied to turbot eggs. (At first, all batches of eggs were put into the incubation system regardless of the quality of their first divisions. However, it was later decided to discard eggs showing "poor quality" first divisions, as well as infertile eggs, prior to incubation).

When eggs were fertile and showed "good" or "fair-quality" first divisions, the relevant stripping-tanks were floated in 180 l bins in an egg-incubation room until they equilibrated to the same temperature as the incubation-tanks. The contents of the stripping-tank were then tipped into the larger bins. Egg-batches from different females were incubated in separate bins.

3. EGG-INCUBATION:

Fertile eggs were incubated at 12 - 13°C in a constantly-illuminated, temperature-controlled room supplied with

Plate X. Shearwater's egg-incubation room.

fresh, heated seawater which had been previously filtered through a 50μ mesh (see Plate X). This particular incubation temperature was chosen because it induced a conveniently short incubation period of approximately 150 hours (6 days) whilst still remaining close to the range of mean sea-surface temperatures encountered by naturally-spawned turbot eggs in the North Sea (Jones, 1972).

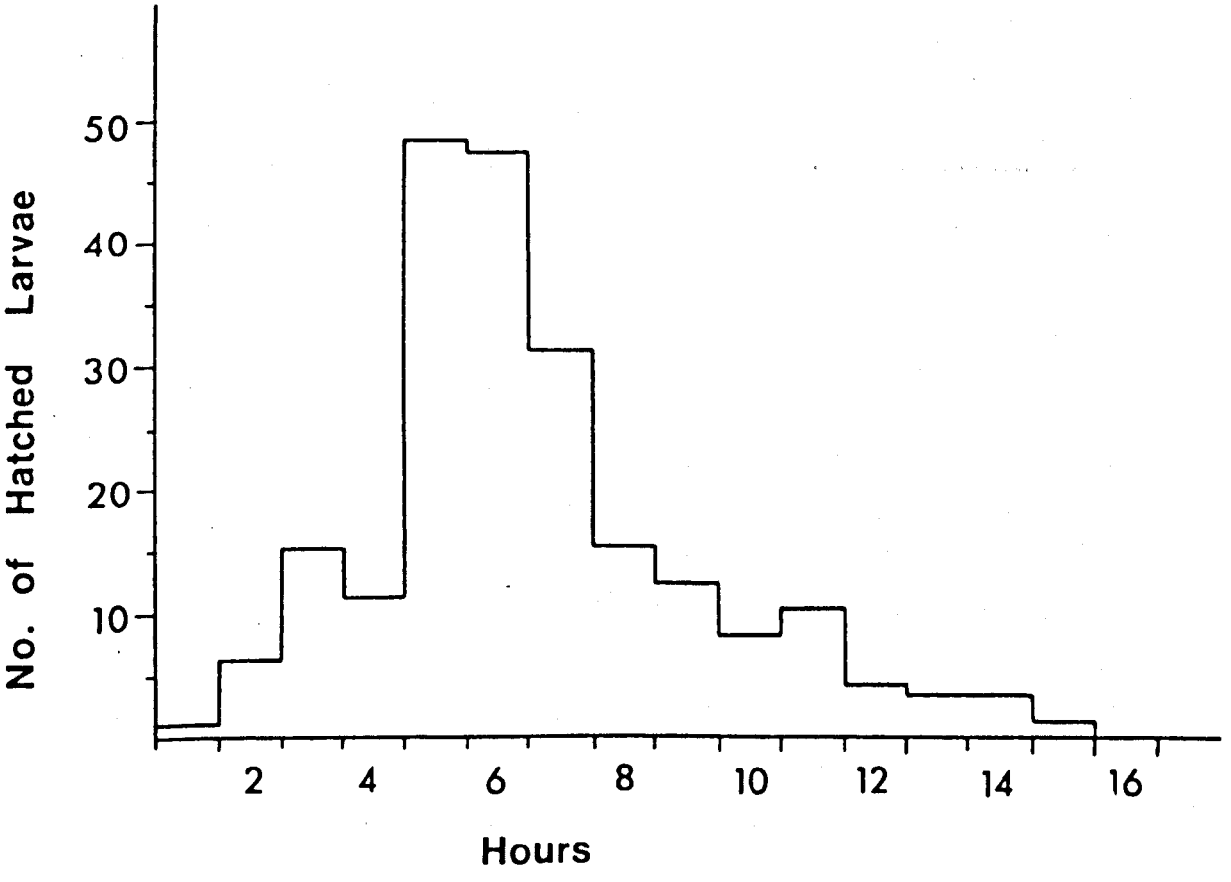
For the first three days, the eggs were kept in 180 l bins which had a slow inflow of fresh seawater (approximately 1.0 l/min) and any dead eggs were siphoned out each day, weighed and subtracted from the weight of viable eggs. On day 3, the remaining eggs were transferred to 90 l bins which contained static seawater with streptomycin sulphate and sodium penicillin each at a concentration of 50ppm. These antibiotics were necessary because the eggs were very prone to bacterial infections. Oppenheimer (1955) had clearly shown streptomycin and penicillin, used at concentrations of 50ppm., to be efficient bactericides without being toxic to fish eggs and larvae.

The eggs remained in these conditions until hatching, which occurred on Day 6 at 12.5°C incubation temperature. (Jones (1972) has already given a detailed account of the embryological stages of turbot eggs and larvae, and so this will not be reiterated in this thesis). Once hatching was completed (this usually took 15-17 hours; see Fig. 2 which shows a typical hatching-frequency distribution), a slow water flow (0.5 l/min.) into the 90 l bins was resumed in order to flush away hatching debris and enzymes. A record was kept of the incubation success of each egg-batch.

The yolk-sac larvae were stocked in larval rearing

Fig. 2. A typical hatching frequency distribution for turbot larvae. This was determined by placing duplicate samples of 200 post-gastrulation eggs into plastic beakers containing 1 litre of sea-water and counting the number of larvae which hatched in each 1 hour time period after the onset of hatching. Hatched larvae were removed from the beaker at each counting.

Fig. 2.



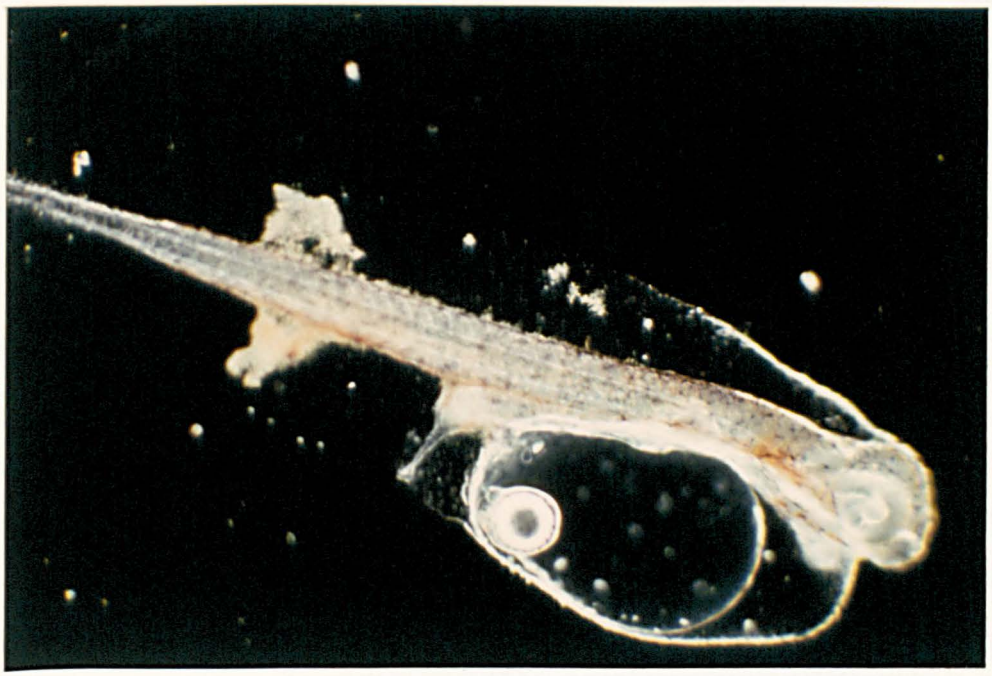


Plate XI. A newly-hatched turbot larva.

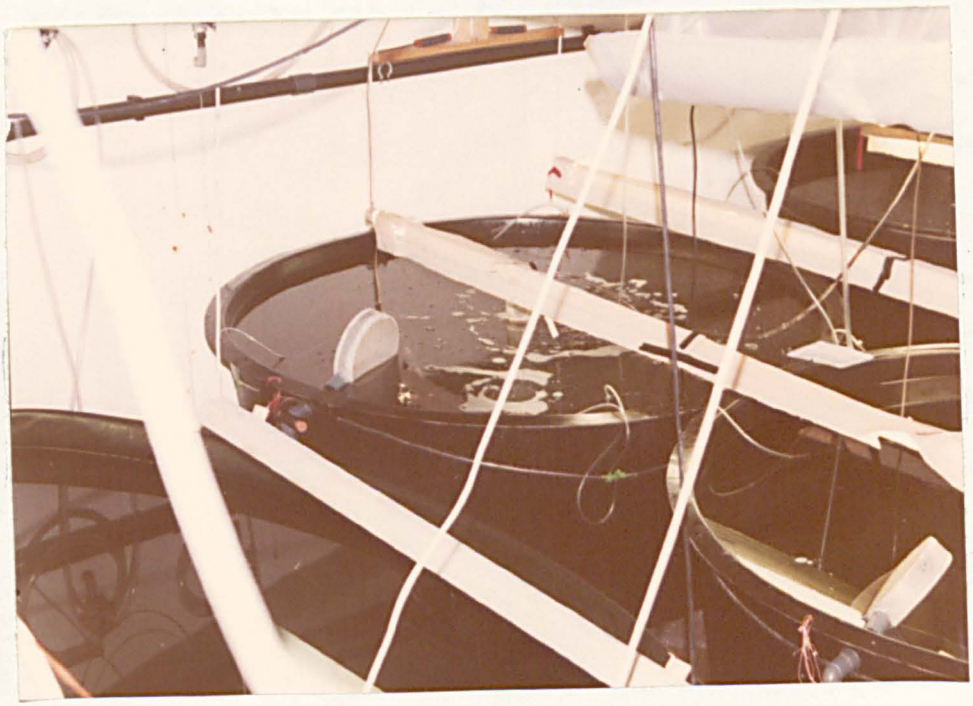


Plate XII. Shearwater's larval rearing tanks.

tanks ranging from 200 to 3,000 l capacity at densities of 5 to 20 larvae per litre (see Plates XI & XII). The tanks were constantly illuminated and gently aerated. 2 to 3 days after hatching and prior to complete orientation of the larvae, the temperature was gradually raised to 20°C and food added. The larvae were fed initially on marine rotifers (Brachionus plicatilis. Muller.) cultured in the hatchery on a variety of algae including Isochrysis galbana, Monochrysis lutheri and Phaeodactylum carna. (Isochrysis and Monochrysis algae were used because they are rich in poly-unsaturated, long-chained fatty acids which are essential to the diet of turbot. See Section II for further details). During the first 10 days, the rotifers were maintained at a density of 5 to 10 per ml and fresh algae was added to the rearing tanks three times a day. Freshly hatched Artemia salina nauplii were presented for the first time at Day 6 to 10 and continued to be fed to the larvae until Day 20 to 25, the algal/rotifer regime being discontinued. At this point, the larvae were approximately 20mm long and could be weaned on to artificial diets. The larvae completed eye-migration and metamorphosed at Day 70 by which time they had reached a length of 30mm. They continued to be "on-grown" until they reached marketable size, which usually took a little over one year (Jones, 1973). However, since this thesis is concerned only with egg-quality and -viability, no detailed account will be given of larval rearing and on-growing.

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SECTION I

INTRODUCTION

Egg-size is relatively constant for species of marine, pelagic egg-laying fish from a given geographical area (Bagenal, 1966; Ware, 1975) and is, in fact, one of the criteria used in egg-identification (e.g. Ehrenbaum, 1911; Hiemstra, 1962). However, there are marked variations in egg-size between populations in addition to a well-documented seasonal decline in egg-diameter.

One would expect quite complex intra-specific relationships between egg-size and fecundity, food-availability and population density; and for these, in turn, to be related to the length of the larvae on hatching and the size of their yolk-sacs. Apart from a few exceptions (e.g. Smyly (1957) working with bullheads, and Bagenal (1966) with plaice) most of the literature points to a negative correlation between the number and size of fish eggs of a given species (Baxter, 1959 & 1963; Baxter & Hall, 1960; Blaxter & Hempel, 1963; Hempel & Blaxter, 1967; Kändler & Dutt, 1958; Lyamin,1956; and Ware, 1975). This would appear to be a logical relationship from a consideration of space within the adult ovary and conservation of reproductive effort, but does not explain why a mutation for high fecundity has not spread through all fish populations by natural selection. One might expect it to be advantageous for an individual to release as many eggs as possible. Obviously, something must be opposing the trend towards high fecundity and small egg-size.

Dahl (1912 - cited by Svärdson, 1949) was the first to suggest that large eggs produced large larvae with better

growth rates. Brown (1946) proved this to be true for brown trout. She also showed that the mortalities which occurred were generally among the smallest fish in each of her experimental groups. Svärdson (1949) incorporated these observations in his paper on the effect of natural selection on egg-number in fish, where he suggested that the advantages of high fecundity are counterbalanced by the greater survival value of large larvae derived from large eggs, thus limiting the number of eggs produced.

Lack (1947, 1948a & b) has discussed selection pressures in relation to birds and mammals and suggested that the parents' limited feeding resources provided the pressure for reducing the number of eggs or young. He was able to test this hypothesis by calculating the survival rate in different clutch-sizes of the starling (Lack, 1948c) and found that there was indeed an optimal clutch-size above which the chances of survival to the adult stage decreased.

Similarly, there is now considerable evidence in the literature verifying Svärdson's "limited fecundity" hypothesis for fish. For example, Bagenal (1969b) held brown trout under natural conditions in the Lake District and found that survival was significantly higher in fry derived from large eggs than in those from small eggs. Blaxter and Hempel (1963) compared herring larvae from eggs of different size and origin and again found, when comparing different egg-groups, that smaller eggs gave rise to small larvae with a lower yolk-sac to body-size ratio. Egg-size and larval size was less variable within a given group and they found that the size of the larvae on hatching was not significantly correlated with egg-size. However, the volume and weight

of the yolk-sacs at hatching were directly proportional to egg-size within most of the groups. This would suggest that larger larvae have a longer period after hatching in which to initiate active feeding. This is particularly important since in many fish species, the time of first feeding represents the most critical period in the fish's life-history - that is, the point at which most mortalities occur. (Jones, 1974; Marr, 1956; Riley, 1966; Shelbourne, 1957.)

Further evidence for Svärdson's hypothesis comes from Baxter (1959) who found that summer/autumn spawning herrings in the north-west North Sea have many, small eggs while the spring-spawning fish of both the Clyde and Norway have fewer, larger eggs - thus confirming work on Irish herrings by Farran (1938).

Bagenal (1966) acknowledges these results but does not believe the inverse relationship between fecundity and egg-weight holds true for marine species with planktonic eggs since egg-size is virtually constant for given populations of these species. He also points out that Svärdson's hypothesis was only offered to explain egg-size/fecundity variations within populations (where fish are directly competing) and not really between different populations.

One might also expect food-availability to directly limit egg-size. However, perusal of the literature indicates that this is not true. Scott (1962) working with rainbow trout (Salmo gairdneri) related a restricted diet with lower fecundity at maturation due to follicular atresia, but not to the size of the eggs. Hester (1964) found a similar situation in the viviparous guppy where a restricted diet reduced the number of offspring but not their size.

Wootton (1973) showed that, in the three-spined stickleback (Gasterosteus aculeatus), high food levels increased the percentage of fish which matured, the number and frequency of spawnings, and the mean number and total weight of eggs produced per spawning. Food levels had no effect on the size of the eggs. In contrast, when comparing the faster growing bullheads(Cottus gobio) from Lake Windermere with the slower growing ones from River Brathay, Smyly (1957) found that the former were more fecund and had larger eggs within their ovaries.

However, Bagenal (1969a &1973) points out that the results observed in the food-ration experiments of Scott (1962) and Bagenal (1969a) could have been produced indirectly by stress due to increased competition for food amongst the fish.

Superimposed on all these inter-relationships affecting egg-size is a seasonal decline in the size of fish eggs which is quite well-documented for both pelagic and demersal marine species, and some freshwater species (Bagenal, 1971; Cushing, 1967; Hempel & Blaxter, 1967; Hiemstra, 1962). Simpson (1959) found that, in the North Sea, older plaice tend to spawn earlier than younger fish whilst Määr (1950) and Nikolsky (1950) have both shown that in a given population of fish, larger females tend to produce larger eggs. It is therefore possible that this seasonal decline in egg-diameter might be caused by the age-structure of the spawning population changing as the spawning season progresses. However, Hislop (1975) showed that individual captive whiting gave smaller eggs later in their spawning season.

Bagenal (1971) points out that monthly mean egg-sizes

of fish with planktonic eggs cannot be correlated with density changes within eggs brought about by temperature and/or salinity fluctuations. However, Simpson's work with plaice (1959) suggests an association between egg-size, spawning-place and time, which in turn is related to water temperature and the production cycle. Southward and Demir (1974) have also shown that egg-size decreases during the spawning season in the cornish pilchard and that this size reduction is negatively correlated with temperature. Hislop (1975) observed the same relationship with whiting egg-size and temperature.

Demersal eggs, which are usually larger than planktonic ones, also show a seasonal variation in egg-size. (Bagenal, 1971). For example, in herring there is a large size reduction from February to May and then egg-size increases again from June to December. Here too then, the size is negatively related to water temperature. Data on freshwater eggs is too limited to make a generalisation about seasonal variations in size.

The functional significance of the seasonal change in egg-diameters is probably due to a combination of factors. For example, Bagenal (1971), Baxter (1959 & 1963), Cushing (1967), and Simpson (1959) suggest that egg-size variation is related to the site of the spawning ground and the initiation of the production cycle in that particular location. They suggest that the earlier, larger eggs give rise to large larvae with greater yolk-sac reserves which enable them to survive longer without food and so make more attempts at food-capture. There is less need for large yolk-supplies in the later, summer batches of eggs because by then the

production cycle is more advanced and plankton is abundant.

Hempel and Blaxter (1967) point out that predation on eggs is probably higher in the summer, so it may be more advantageous to release numerous small eggs in that respect. In addition, Jones (1973) and Jones and Hall (1974) suggest that egg-size variation might be an adaptation so that the hatched larvae are better suited to capture the size of food particles available to them at that particular time - either large overwintering populations of copepods, or the nauplii and small copepodites which abound in the summer plankton.

Ware (1975) devised a mathematical model to describe the relationship between egg-size, growth and natural mortality of larval fish. He predicted that fish spawning when incubation periods are long and/or variable (that is, during periods of low water temperature) ought to produce large eggs with large yolk reserves. However, the model predicts a shift of selective advantage towards small eggs as the incubation period shortens (that is, as the temperature increases) since the number of eggs that hatch will more than compensate for the greater mortality rate experienced by these larvae due to their small initial size and high abundance.

Finally, different species probably have different controlling factors affecting their egg-size due to peculiarities in their life-cycles. For example, Moffatt and Thompson (1975) postulate the evolution of different egg-sizes in the beach-spawning grunion as an adaptation to different tidal cycles.

In brief, the existing literature concerning physical parameters of fish eggs can be summarised as follows:

a) There are complex intra-specific relationships between

egg-size, fecundity, poulation density, and food availability.

b) There is usually a negative correlation between the number and size of eggs produced.

c) Low food-rations do not usually affect egg-size directly, but sometimes reduce fecundity via follicular atresia. However, it is difficult to isolate the effects of low food availability from stress due to competition for food.

d) Superimposed on these inter-relationships is a seasonal decline in the egg-size of marine fish populations. This may be (i) an "apparent" decrease caused by older, larger fish spawning first, since these tend to produce larger eggs, and/or (ii) an"actual" decrease of egg-size in each individual fish.

e) The functional significance of this seasonal decrease in egg-size might be to allow the emerging larvae to be of a suitable size and have sufficient yolk-reserves to permit them to start feeding on the extant zooplankton populations. In addition, it may well be an adaptation to changing ambient water-temperature, with larger eggs containing greater yolk-reserves being produced when water temperatures are low and causing long incubation periods. It could also be a means of reducing predation on the eggs.

f) Larger eggs usually give rise to larger larvae with bigger yolk-reserves. In some species (e.g. herring) this has been proved to be an advantage at the onset of active feeding and causes larvae to have better growth rates.

g) Peculiarities in life-cycles can cause anomolous trends in egg-diameters (e.g. beach-spawning grunion).

Apparently, then, variations in egg-size can play an

important role in determining egg-quality and the subsequent survival and growth-rate of the emerging larvae. Jones (1972) stated that considerable variation in egg-size is encountered between turbot females (though all eggs tend to lie within the range 0.9 - 1.2mm in diameter). It was therefore decided to examine this parameter more closely and, in particular, to determine:

1. Whether a seasonal decline in egg-diameter occurred in female turbot and, if so, was this an "actual" or "apparent" decrease.
2. The relationship between egg-diameter, length of the emerging larva and its yolk-sac index (yolk-sac length x yolk-sac depth).
3. Whether using different males to fertilise a particular batch of eggs affected the length and yolk-sac indices of larvae emerging from the different "crosses".
4. The relationship between egg-size and the subsequent growth and survival of the emerging larvae.
5. Whether larger females tend to produce larger eggs.
6. Whether force-feeding female turbot during their spawning season affected the egg-diameters of their later batches of eggs.

It was also decided to compare the absolute fecundity of turbot computed by analysis of ovaries of wild-caught fish (Jones,1974) and the actual egg-yield of captive turbot females subjected to hand-stripping methods of egg-collection.

MATERIALS & METHODS

All adult fish at the Shearwater Fish Hatchery were freeze-branded for identification purposes and egg-batches from individual females were stripped and incubated separately. This system therefore provided an excellent means of collecting data on the physical characteristics of eggs and their emerging larvae, and relating them back to the particular female from which they had been stripped, as well as the male(s) used in their fertilisation.

1. SEASONAL DECLINE IN EGG-DIAMETER:

After a female had been stripped, and her eggs fertilised and put into a separate incubation-tank, a sample of 20 viable eggs was taken out after gastrulation (Day 3) and egg-diameters measured on an Olympus BHB microscope using an eyepiece graticule (40 grats. ≡ 1mm). Two diameters at right angles to each other were measured per egg and an average taken. The mean egg-diameter was then computed for the 20 egg sample. This was repeated for successive egg-batches of individual females throughout the spawning season.

2. RELATIONSHIP BETWEEN EGG-SIZE, LENGTH OF THE EMERGING LARVA AND ITS YOLK-SAC INDEX:

It was decided that measuring larvae 3 hours after the first appearance of hatched larvae in the incubation-tank adequately standardised the age of larvae used in these ex-

periments.

Egg-batches that were about to hatch (Day 6) were frequently checked for the onset of hatching. Larval length and the length and depth of yolk-sacs were measured for a sample of 20 larvae (using the same apparatus as in egg-diameter determination) 3 hours after the onset of hatching. The larvae were anaesthetised with MS222 prior to measurement. Larval length was measured from the lower jaw to the tip of the tail (inclusive of the primordial fin). The length and depth of the yolk-sac were multiplied together to give a "yolk-sac index" (Douglas, 1977). This was assumed to be proportional to the yolk-sac volume. Damaged or "crooked" larvae were not included in the sample. The larvae lengths and yolk-sac indices were then related to the respective egg-diameters measured on Day 3.

3. EFFECT OF DIFFERENT MALE-CROSSES ON THE LENGTHS AND YOLK-SAC INDICES OF LARVAE HATCHING FROM A PARTICULAR BATCH OF EGGS:

A female which had recently ovulated (see Section III for details of ovulation-prediction) was chosen and her eggs stripped into a 33cm x 23cm x 21cm plastic tank half-filled with seawater. Three small, glass vials were taken and labelled. Approximately 1ml of milt was stripped from three males and put into separate vials. Different pipettes were used in the stripping of each male to prevent cross-contamination of milt.

Approximately 15g of eggs were netted out of the plastic tank and blotted dry of excess seawater. 1g samples of the eggs were then quickly weighed into 9 crystallising

glasses containing equal amounts of seawater. 1ml of seawater was added to each of the vials of milt and these were shaken. 0.5ml of each milt suspension was added to three of the crystallising glasses - giving triplicates of each of the male-crosses. The 9 crystallising glasses were taken to an egg-incubation room where they were floated in separate 33cm x 23cm x 21cm plastic tanks, containing equal amounts of seawater with streptomycin sulphate and sodium penicillin at 50ppm, until the water temperatures had equilibrated. The contents of the crystallising glasses were then tipped into the larger plastic tanks. The eggs were incubated at 12.5°C in these static water conditions until they hatched.

When the larvae in all the tanks had completed hatching, their numbers in each tank were counted and the lengths and yolk-sac dimensions of 20 larvae taken from each tank were measured and compared. MS222 was again used to anaesthetise the larvae just prior to measurement. The triplicates of each cross were not measured consecutively in order to reduce the error caused by larvae growing and yolk-sacs diminishing during the time taken to measure larvae from other tanks.

The experiment was repeated using eggs from a different female and milt from three different males.

4. RELATIONSHIP BETWEEN EGG-SIZE AND THE SUBSEQUENT GROWTH AND SURVIVAL OF EMERGING LARVAE:

A comparison of the growth-rate and survival of large larvae (emerging from eggs stripped from a female early in her spawning season) and small larvae (from eggs stripped

from the same female at the end of her spawning season) was carried out until the larvae were 10 days old.

Eggs were stripped from a female that was at the beginning of her spawning season and incubated as usual. Their mean egg-diameter was measured on Day 3. On the day of hatching (i.e. Day6 eggs; Day 0 larvae) three black, plastic tanks (90cm x 30cm x 30cm) were put into a constant temperature room and filled with 30 l of seawater that had been passed through a 50μ mesh. The constant temperature room was kept at 19.5($\pm$ 0.5)°C. The seawater was inoculated with a mixture of Pseudoisochrysis and Phaeodactylum algae at a concentration of approximately 120 cells/μl and 300,000 rotifers per tank. Three overhead fluorescent strip-lights gave at least 400 lux of light to all the tanks. Gentle aeration was set up in each tank and they were left to "mature" for 2 days. 400-600ml of algae mixture were added twice a day to replenish algal levels.

A sample of 20 larvae was taken from the egg-incubation room each day and their lengths and yolk-sac dimensions measured. Larvae were not returned to the experimental incubation tank after measurement in case MS222 or the measurement procedure affected their growth-rate.

On Day 2, three lots of 300 larvae were counted into small, plastic beakers (400ml capacity) and these were floated in the black plastic tanks of algae and rotifers until the water temperatures equilibrated. The larvae were then tipped into the rearing-tanks and the aeration increased slightly. Daily measurements of larval lengths and yolk-sac dimensions were continued - a sample of 10 larvae being taken from each larval-tank. Again, larvae were not return-

ed to these tanks after measurement. Algal and rotifer densities were kept at approximately 120 cells/µl and 3-5 rots./ml respectively.

On Day 10, the experiment was ended and the number of surviving larvae in each tank was counted. A second set of larval tanks were set up in exactly the same manner when the female adult gave small eggs and larvae at the end of her spawning season. Larval lengths and yolk-sac indices were measured as before . The growth-rates and survival figures of the two types of larvae were then compared.

This comparison was performed three times, each time using larvae from a different female.

5. RELATIONSHIP BETWEEN A FEMALE'S MAXIMUM MEAN EGG-DIAMETER IN ANY SPAWNING SEASON AND HER LENGTH, WEIGHT AND CONDITION INDEX:

Length from the tip of the lower jaw to the end of the caudal fin was measured to the nearest mm for each fish. Weight was determined to the nearest 10g by suspending the live fish in a net from a spring balance and then subtracting the weight of the empty net. All measurements were taken 2 months before each broodstock was expected to spawn (i.e. before the females' ovaries had started to swell) with the exception of Broodstock 1, which was measured 2 months after the completion of spawning and the resumption of normal feeding.

The condition index (K) was computed using the following equation:

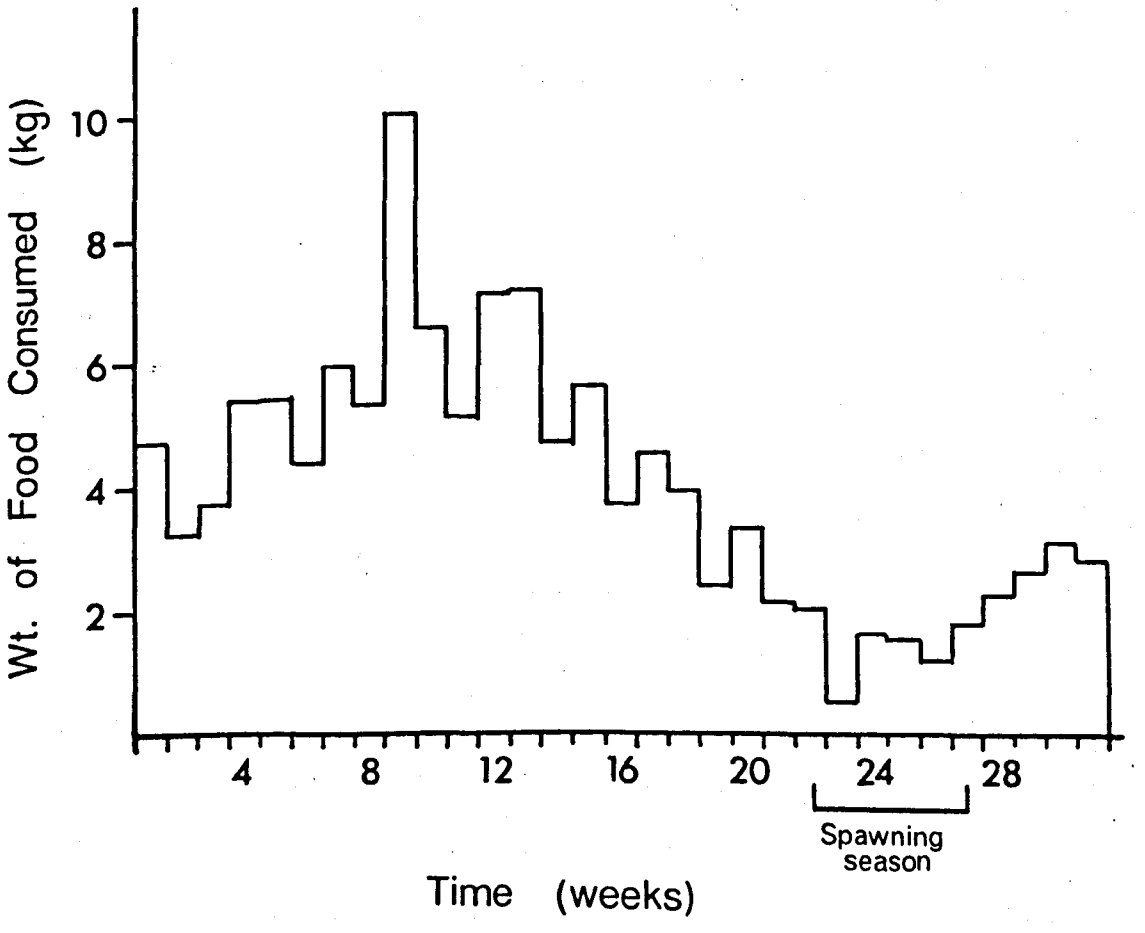
$$K = \frac{W \times 100}{L^3}$$

where: W = weight in g

L = length in cm

Fig. 3. Weekly food consumption of a turbot broodstock consisting of 18 females and 11 males. Food consumption was computed by weighing food thrown into the broodstock tank and then subtracting the weight of uneaten food removed from the tank the next day. The small amount of food consumed during the spawning season was eaten almost entirely by males and non-spawning females.

Fig. 3.


Wt. of Food Consumed (kg)
10
8
6
4
2
4
8
12
16
20
24
28
Spawning season
Time (weeks)

Mean egg-diameters were measured for all egg-batches from each female when eggs were 3 days old. Variation in mean egg-diameter throughout the spawning season could therefore be assessed for each fish. Each female's maximum mean egg-diameter of that particular spawning season was chosen to relate to the female's size rather than the minimum value or overall mean because the latter two parameters depend on the duration of a fish's spawning season. Those fish which spawned for the longest period of time gave the smallest eggs at the end of their spawning season.

6. EFFECT OF FORCE-FEEDING FEMALE TURBOT DURING THEIR SPAWNING-SEASON ON THE EGG-DIAMETERS OF THEIR LATER BATCHES OF EGGS:

Turbot females hardly eat during their spawning season (see Fig. 3). It was thought that this might be a contributory factor to the seasonal decrease in egg-diameter and so attempts were made to force-feed three females throughout their spawning season.

A sprat "purée" was made by mincing whole sprat and then homogenising 250g of this with approximately 40ml water until the resulting slurry could be easily drawn up and down the barrel of a 50ml disposable syringe. The barrel was then connected to a 30cm long plastic tube of 1cm internal diameter (I.D.) by means of a small, elastic connecting tube. The purée was squeezed to the far end of the plastic tube so that no air would be forced into the fish's gut. The end of the tube was then inserted into the female's mouth, passed as far down the oesophagus as possible and 10ml of purée per kg fish body weight was squeezed into the fem-

ale's gut.

The three fish were force-fed three times a week commencing as soon as their voluntary food-consumption declined drastically (approximately 1-2 weeks before spawning). This was, initially, rather a quick and easy procedure. However, after about a fortnight of this treatment, the fish began to look very stressed. They struggled considerably as soon as they were lifted out of the water and this made force-feeding increasingly difficult. Anaesthetic could not be used due to the high frequency of treatments. In addition, the oocyte maturation of the three females had slowed down considerably since the treatment had begun and it was feared that additional stress might block spawning altogether. It was therefore decided to abandon the experiment.

All three fish spawned a few weeks later.

7. COMPARISON OF THE ABSOLUTE FECUNDITY OF "WILD" TURBOT AND THE EGG-YIELD OF CAPTIVE TURBOT SUBJECTED TO HAND-STRIPPING:

Although turbot do not spawn naturally in the holding conditions at "Shearwater", Port Erin, some passive shedding of over-ripe eggs occurred if the fish were left for too long between strippings. This was thought to be caused by fresh ovulations displacing older eggs from the lumen of the ovary and could be monitored by passing a fine net through the broodstock tank-water each day. No freshly ovulated eggs were ever found in the water.

However, during the first two spawning seasons, all fish were stripped routinely twice a week. It can therefore be assumed that almost no eggs were lost to the exter-

ior by passive shedding since it was later found that most females ovulate every 3.5 days (see Section III) and most females seemed to be capable of retaining two ovulated batches of eggs in her ovary.

The weight of eggs obtained from each stripping of each female in these first two broodstocks was measured and a 1g sample of seawater-washed eggs taken from each batch and preserved in 10% formalin. The number of eggs in each of these samples were later counted using a Bogorov tray and a "Kyowa Optical" binocular microscope. These figures were multiplied by their respective egg-batch weights to give the number of eggs in each batch. Egg-numbers for successive batches of each female were then totalled to give an approximate egg-yield for each female.

These figures were then compared with the absolute fecundities computed by analysis of wild-caught turbot ovaries by Jones (1974).

FEMALE & SPAWNING SEASON		MEAN EGG-DIAMETER (mm) OF FIRST BATCH		MEAN EGG-DIAMETER (mm) OF FINAL BATCH	
A.	1979	1.053	(0.010)	1.002	(0.010)
B.	1979	1.028	(0.006)	0.989	(0.007)
C.	1979	1.035	(0.013)	0.979	(0.017)
D.	1979	1.059	(0.023)	0.993	(0.010)
E.	1979	1.028	(0.011)	0.954	(0.015)
F.	1979	1.026	(0.025)	0.931	(0.012)
G.	1979	1.020	(0.016)	0.939	(0.012)
H.	1979	1.024	(0.013)	0.989	(0.009)
I.	1979	0.988	(0.013)	0.916	(0.008)
J.	1979	0.999	(0.010)	0.907	(0.010)
K.	1979	1.018	(0.020)	0.995	(0.021)
L.	1979	1.010	(0.011)	0.979	(0.019)
M.	1979	1.039	(0.013)	1.012	(0.021)
N.	1979	1.053	(0.010)	0.970	(0.011)
O.	1979	1.024	(0.010)	0.977	(0.012)
P.	1979	0.997	(0.010)	0.944	(0.006)
Q.	1979	1.011	(0.006)	0.988	(0.008)
R.	1979	1.030	(0.012)	0.961	(0.013)
S.	1979	1.060	(0.013)	1.036	(0.012)
T.	1979	1.001	(0.015)	0.947	(0.011)
U.	1979	1.036	(0.016)	0.975	(0.008)
V.	1979	1.019	(0.010)	0.965	(0.016)
W.	1979	1.023	(0.007)	0.951	(0.013)
A.	1980	1.046	(0.007)	0.991	(0.007)
B.	1980	1.062	(0.012)	0.998	(0.007)
C.	1980	1.050	(0.012)	0.997	(0.012)

Table 1. Seasonal Decline In Egg-Size (Standard deviations in parentheses).

Figs. 4a, b, c & d. The rate of egg-diameter decline throughout the spawning season in 4 different turbot females. Each point on the graphs represents the mean of 20 egg-diameters. The standard deviations of the means are indicated by barred lines(see key). The stripping frequency was twice a week.

Seasonal Decline in Egg-Size:

Fig. 4a.

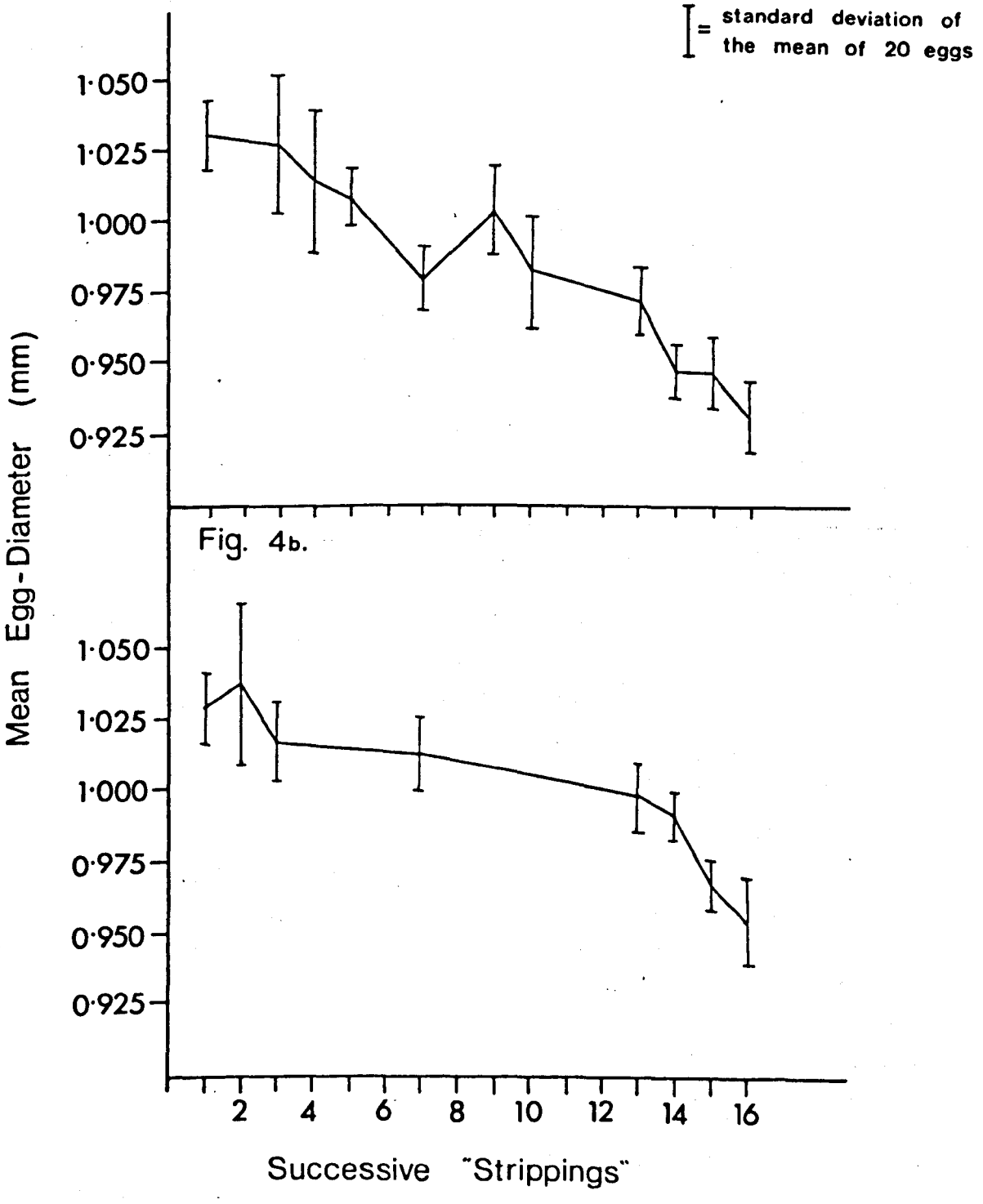


Fig. 4c.

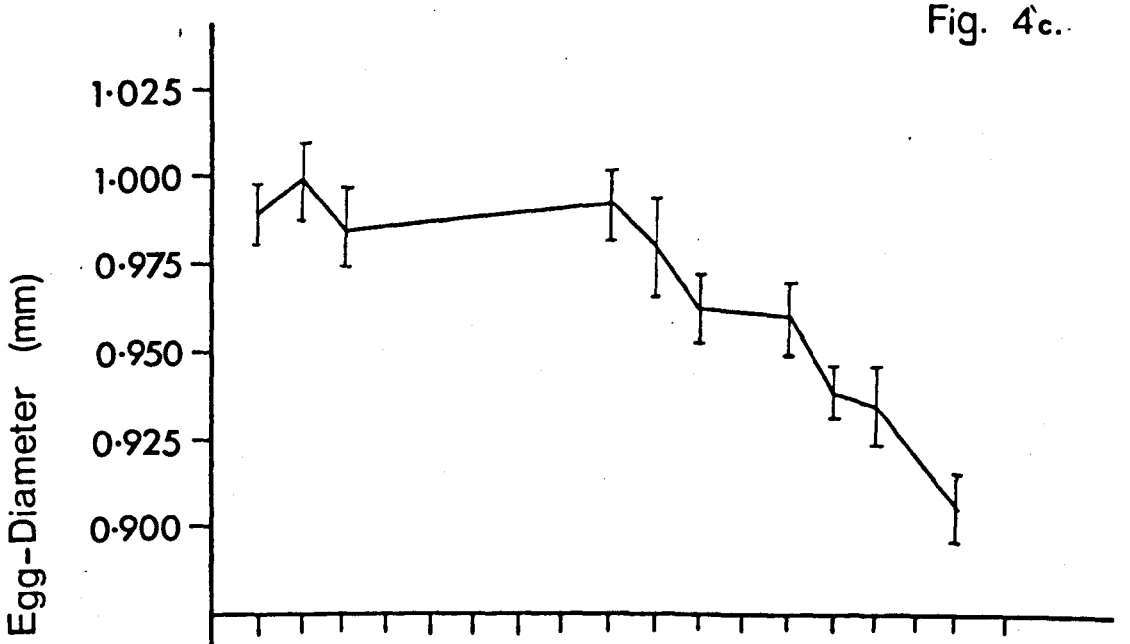

1·025
1·000
0·975
0·950
0·925
0·900

Fig. 4d.

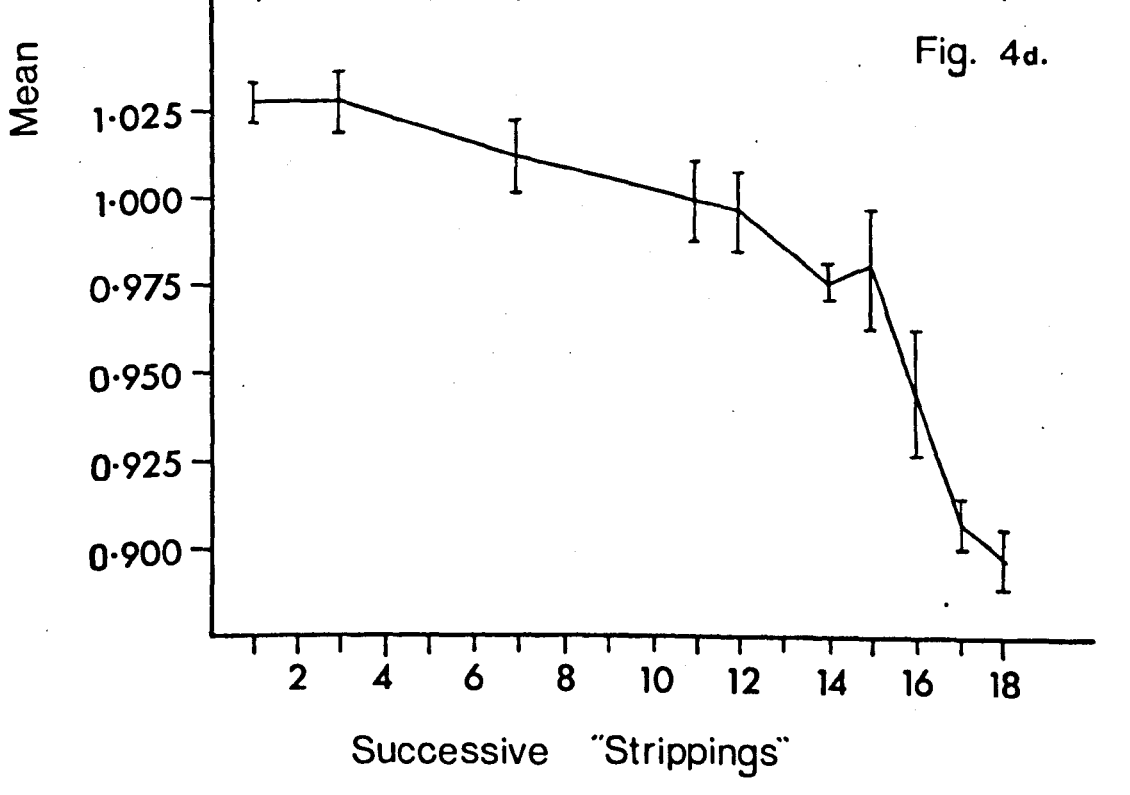

Mean Egg-Diameter (mm)
1·025
1·000
0·975
0·950
0·925
0·900
2
4
6
8
10
12
14
16
18
Successive "Strippings"

Fig. 5. The relationship between the mean length of newly-hatched turbot larvae and the mean egg-diameter of the egg batch from which they hatched. A functional regression line (Ricker, 1973) was fitted to the data.

Fig. 5.

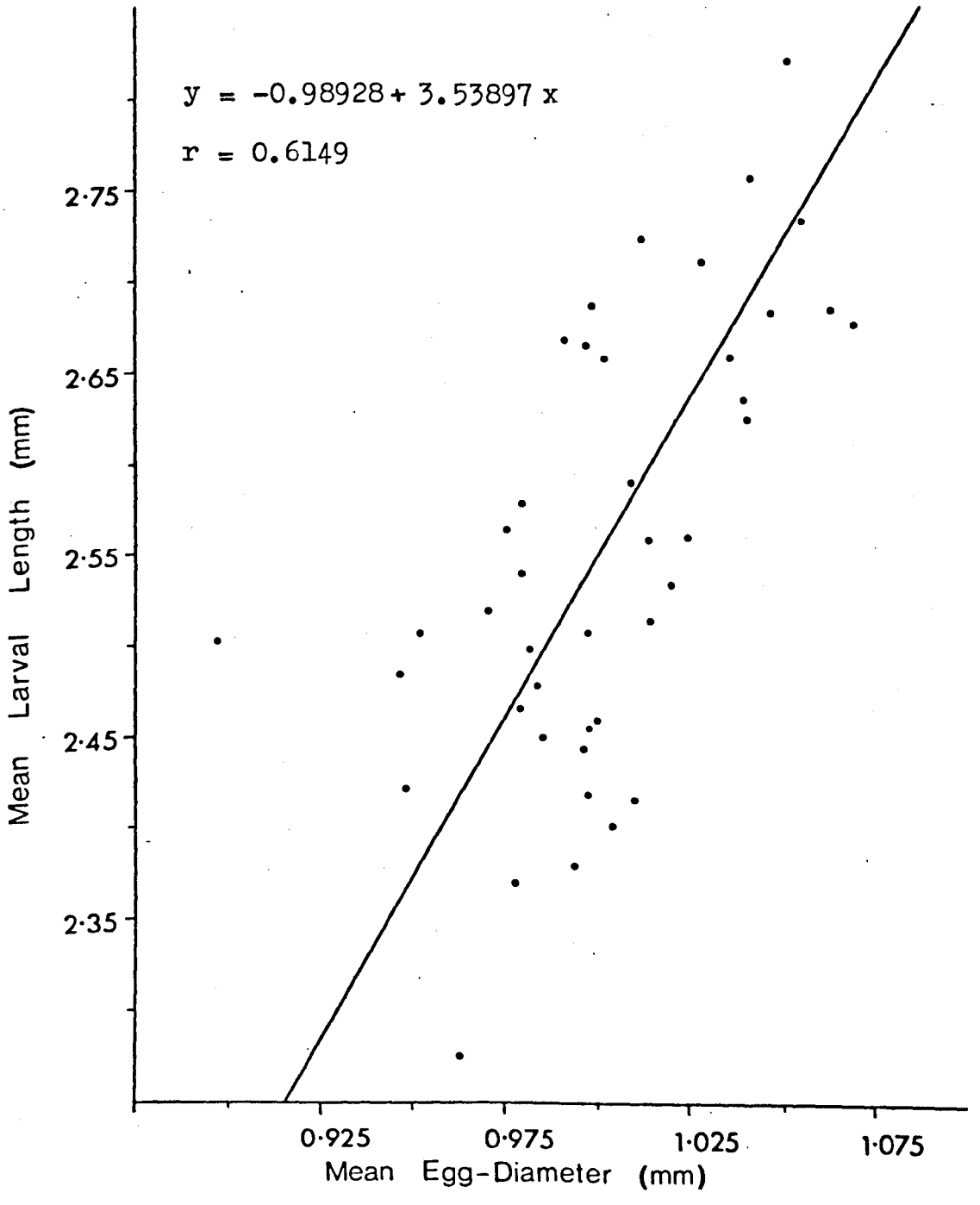
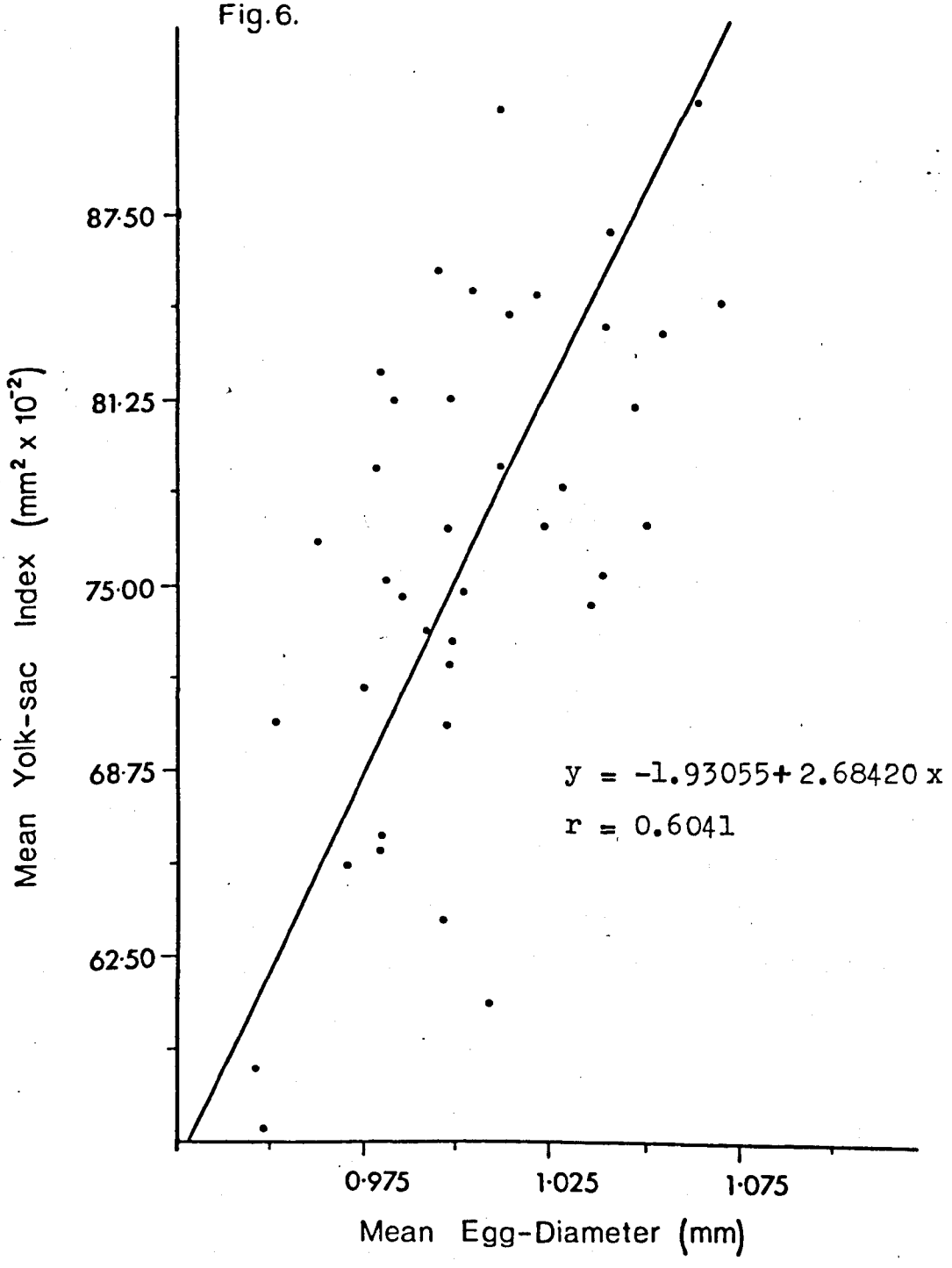

y = -0.98928 + 3.53897 x
r = 0.6149
2·75
2·65
2·55
2·45
2·35
Mean Larval Length (mm)
0·925
0·975
1·025
1·075
Mean Egg-Diameter (mm)

Fig. 6. The relationship between the mean yolk-sac index (yolk-sac length x yolk-sac depth) of newly-hatched turbot larvae and the mean egg-diameter of the egg batch from which they hatched. A functional regression line (Ricker, 1973) was fitted to the data.

Fig. 6.


87·50
81·25
75·00
68·75
62·50
Mean Yolk-sac Index (mm² x 10⁻²)
y = -1.93055+ 2.68420 x
r = 0.6041
0·975
1·025
1·075
Mean Egg-Diameter (mm)

RESULTS

1. SEASONAL DECLINE IN EGG-DIAMETER:

Each female showed a seasonal decline in her egg-size. The rate of decline varied between individuals as did the difference between the initial and final mean egg-diameters. Table 1 gives the mean egg-size for 23 females' initial and final spawnings. Figs. 4a,b,c & d give examples of the different rates of diameter-decline in four of these females.

2. RELATIONSHIP BETWEEN EGG-SIZE, LENGTH OF THE EMERGING LARVA AND ITS YOLK-SAC INDEX:

Functional regression lines (Ricker, 1973) were fitted to the plots of mean larval length versus mean egg-diameter measured 3 hours after first hatching, and mean yolk-sac index versus mean egg-diameter, also measured 3 hours after first hatching (Figs. 5 & 6 respectively). The correlation coefficients (r) were 0.6149 and 0.6041 respectively. The correlations between larval length and egg-diameter and between yolk-sac index and egg-diameter were found to be significant at the 95% level ($P < 0.05$).

The relationship between the two parameters plotted in Fig. 5 can be expressed by the equation:

Larval Length = -0.98928 + 3.53897 Egg-diameter

(The 95% confidence limits of the slope are: 4.71244 and 2.65771).

The yolk-sac index versus egg-diameter relationship is expressed by the equation:

Yolk-sac Index = -1.93055 + 2.68420 Egg-diameter

(The 95% confidence limits of the slope are: 3.62888 and

FEMALE	YOLK-SAC INDEX : LARVAL LENGHTH RATIO EARLY SEASON LARVAE	YOLK-SAC INDEX : LARVAL LENGTH RATIO LATE SEASON LARVAE
I	0.340	0.339
II	0.274	0.270
III	0.302	0.301
IV	0.308	0.293
V	0.334	0.333
VI	0.301	0.310
VII	0.297	0.305

Table 2. Body-proportions of Early & Late Season Yolk-Sac Larvae.

a) Female A.

	SAMPLE	MALE I	MALE II	MALE III
LARVAL LENGTH (mm)	A	2.533 (2.655 / 2.411)	2.687 (2.745 / 2.629)	2.666 (2.749 / 2.583)
	B*	2.715 (2.756 / 2.674)	2.740 (2.788 / 2.692)	2.825 (2.857 / 2.793)
	C	2.951 (2.986 / 2.916)	2.917 (2.972 / 2.852)	2.910 (2.955 / 2.865)
YOLK INDEX SAC (mm^2)	A	0.694 (0.744 / 0.645)	0.640 (0.688 / 0.593)	0.611 (0.671 / 0.551)
	B	0.566 (0.576 / 0.556)	0.587 (0.635 / 0.539)	0.548 (0.560 / 0.538)
	C*	0.459 (0.475 / 0.442)	0.533 (0.554 / 0.513)	0.468 (0.487 / 0.450)

b) Female B.

	SAMPLE	MALE I	MALE II	MALE III
LARVAL LENGTH (mm)	D	2.813 (2.867 / 2.759)	2.758 (2.836 / 2.680)	2.806 (2.852 / 2.758)
	E	2.814 (2.869 / 2.759)	2.742 (2.995 / 2.489)	2.780 (2.839 / 2.731)
	F	2.892 (2.933 / 2.851)	2.883 (2.917 / 2.849)	2.927 (2.964 / 2.870)
YOLK INDEX SAC (mm^2)	D	0.458 (0.477 / 0.440)	0.464 (0.482 / 0.446)	0.439 (0.468 / 0.420)
	E	0.440 (0.482 / 0.399)	0.503 (0.534 / 0.472)	0.448 (0.470 / 0.426)
	F*	0.424 (0.444 / 0.404)	0.456 (0.478 / 0.434)	0.403 (0.419 / 0.387)

Table 3. The results of two experiments comparing the mean lengths and yolk-sac indices of turbot larvae which have the same mother, but different fathers. 95% confidence limits are given in parentheses. Asterisks denote rows in which the 95% confidence limits did not overlap (see text for further details).

1.98544).

There was no significant correlation between larval length at hatching and yolk-sac index ($r = 0.0818$), or between the ratio of yolk-sac index : larval length (y.s.i./l.l.) and egg-diameter. In fact, this ratio appeared to remain reasonably constant for the larvae of any particular female, regardless of the time of spawning season to which they belonged (see Table 2).

3. EFFECT OF DIFFERENT MALE-CROSSES ON THE LENGTHS AND YOLK-SAC INDICES OF LARVAE HATCHING FROM A PARTICULAR BATCH OF EGGS:

Tables 3a & b each compare the mean lengths and yolk-sac indices of larvae hatching from a single batch of eggs which had been split into three "lots" - each egg-lot being fertilised by a different male. The figures in parentheses give the 95% confidence limits of the mean. The larvae grew as the measurements were being made (it took approximately 20 minutes to collect and measure each sample of 20 larvae) so only three consecutive treatments were compared with each other (i.e., the figures in each row in Tables 3a & b). When this was done, it was found that in all but three rows (which are asterisked in Tables 3a & b), the 95% confidence limits overlapped. It was therefore deduced that there was no significant difference between the lengths and yolk-sac indices of larvae which shared the same mother but had different fathers.

Tables 4a & b give the numbers of larvae hatching in each treatment tank. These show considerable variation, both between different males and between triplicate samples

a) Female A.

SAMPLE	MALE I	MALE II	MALE III
A	9	18	30
B	24	28	46
C	36	22	34
	TOTAL 69	TOTAL 68	TOTAL 110

b) Female B.

SAMPLE	MALE IV	MALE V	MALE VI
D	12	12	23
E	19	8	22
F	31	66	32
	TOTAL 62	TOTAL 86	TOTAL 77

Table 4. The number of hatched larvae in each male-cross experiment.

FEMALE	SURVIVAL FIGURES FOR GROWTH COMPARISON TRIALS		
	TANK NUMBER		
	1	2	3
I (Early-season eggs)	0	12	13
I (Late-season eggs)	138	0	24
II (Early-season eggs)	0	101	78
II (Late-season eggs)	0	2	4
III (Early-season eggs)	6	9	0
III (Late-season eggs)	205	0	140

Table 5. Number of larvae surviving in each rearing-tank on Day 10 after hatch in each of the growth comparison trials. (Each trial was terminated on Day 10.)

Fig. 7a. Comparison of growth rates of early and late season turbot larvae. Both types of larvae shared the same mother (Female I). Zero on the x axis indicates the onset of hatching.

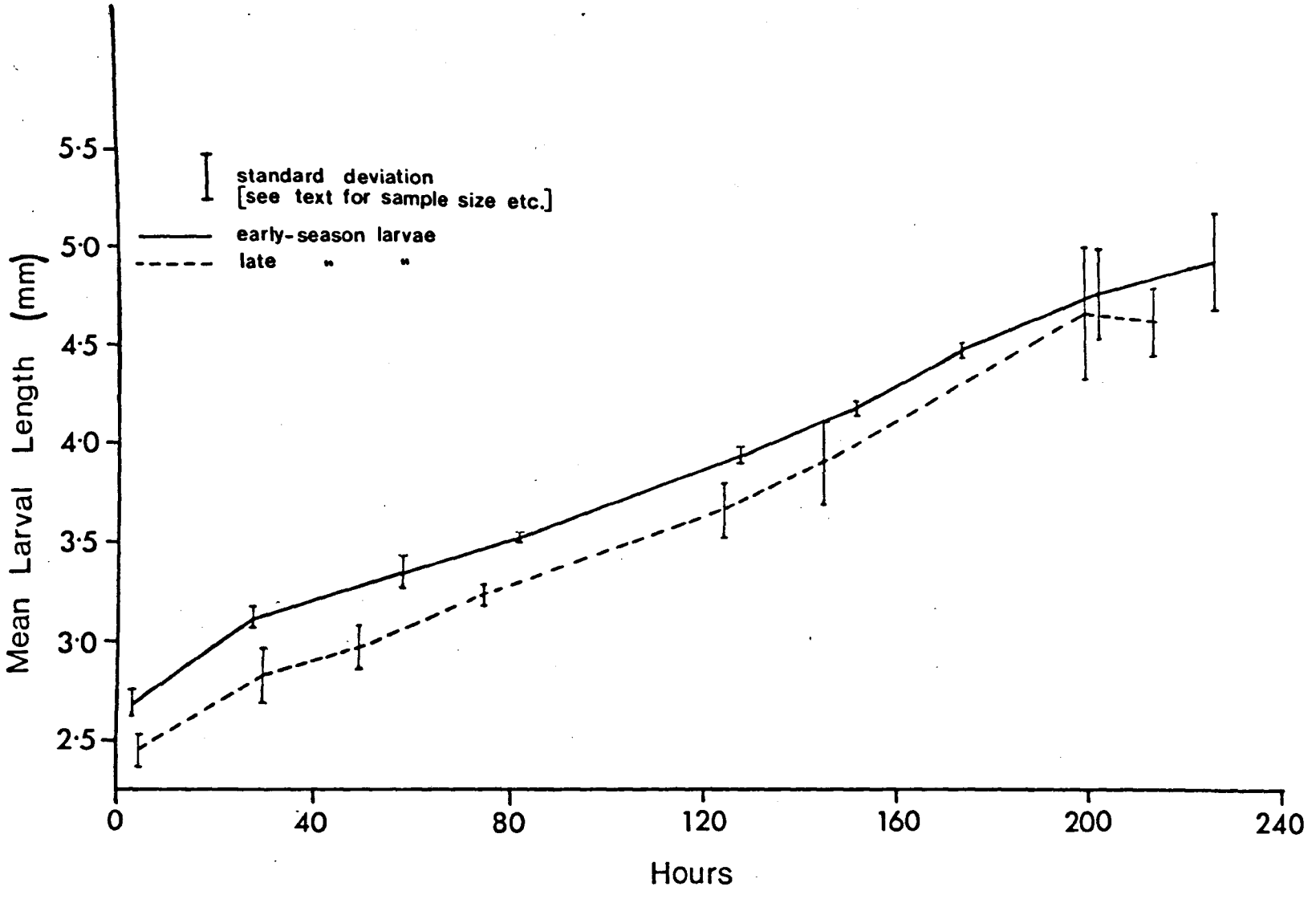

standard deviation
[see text for sample size etc.]
early-season larvae
late " "
Mean Larval Length (mm)
5·5
5·0
4·5
4·0
3·5
3·0
2·5
0
40
80
120
160
200
240
Hours

Fig. 7a

Fig. 7b. Comparison of growth rates of early and late season turbot larvae. Both types of larvae shared the same mother (Female II). Zero on the x axis indicates the onset of hatching.

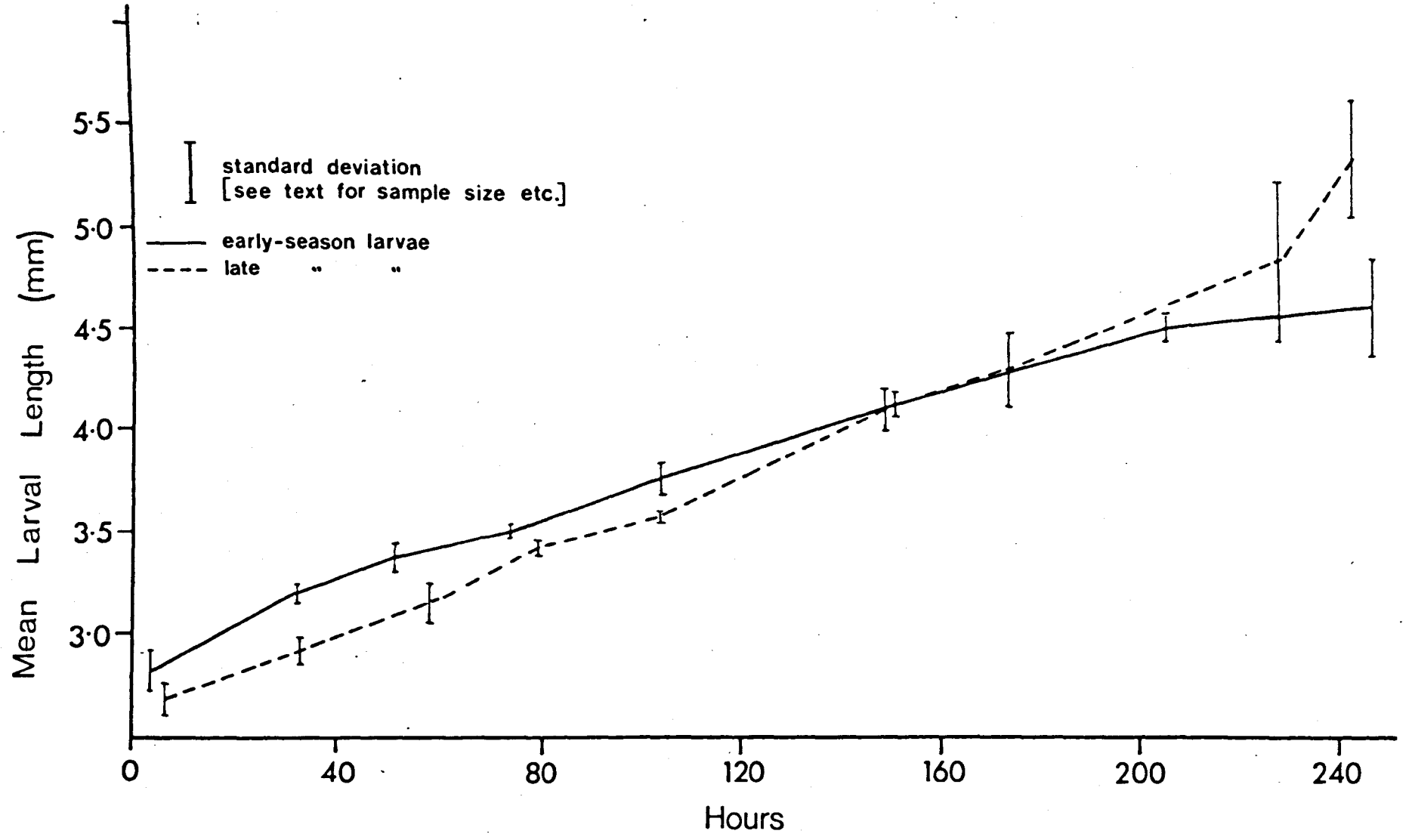

standard deviation
[see text for sample size etc.]
early-season larvae
late " "
Mean Larval Length (mm)
5·5
5·0
4·5
4·0
3·5
3·0
0
40
80
120
160
200
240
Hours

Fig. 7b

Fig. 7c. Comparison of growth rates of early and late season turbot larvae. Both types of larvae shared the same mother (Female III). Zero on the x axis indicates the onset of hatching.

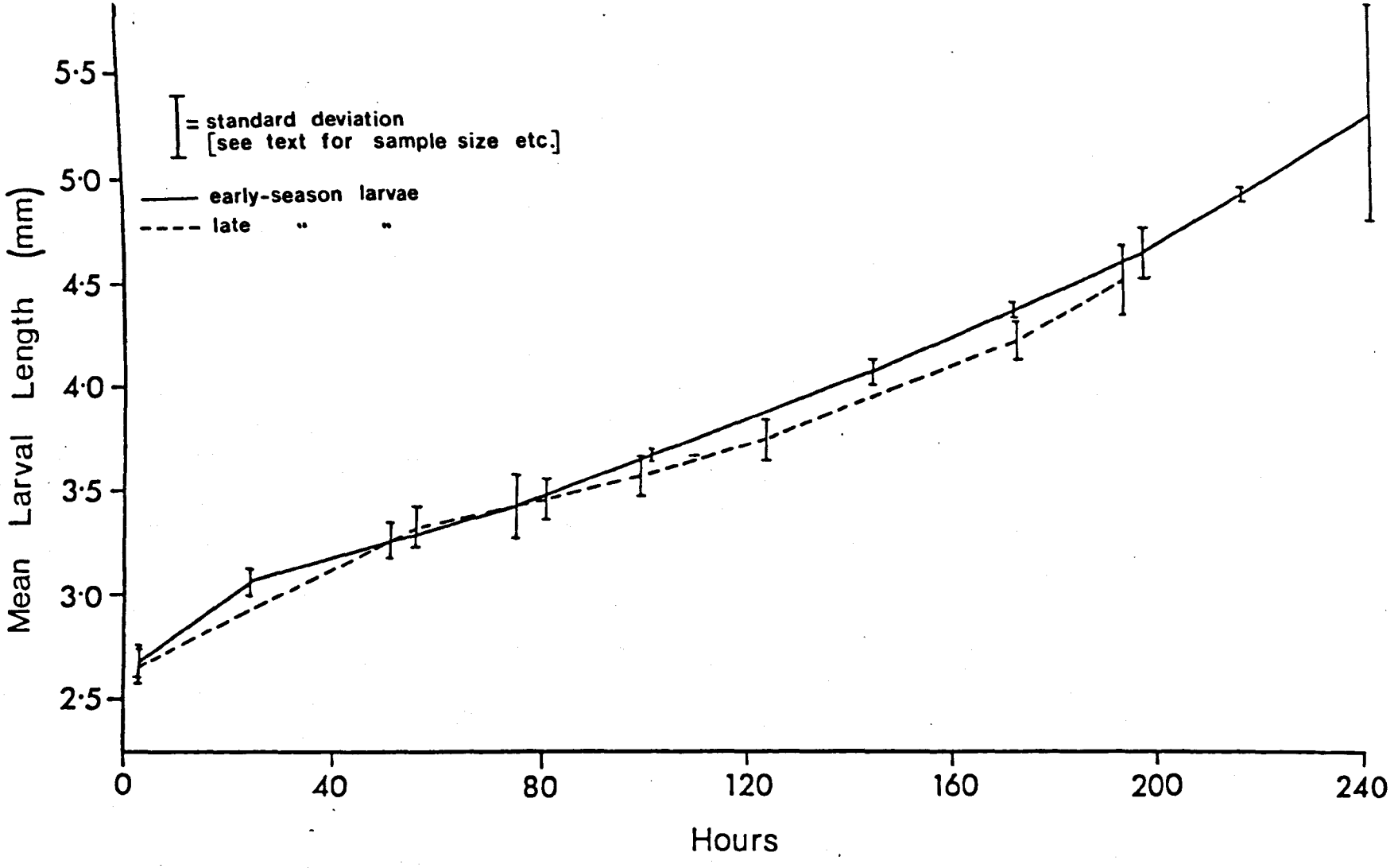

= standard deviation
[see text for sample size etc.]
early-season larvae
late " "
Mean Larval Length (mm)
5·5
5·0
4·5
4·0
3·5
3·0
2·5
0
40
80
120
160
200
240
Hours

Fig. 7c

using the same male.

4. EFFECT OF EGG-SIZE ON THE SUBSEQUENT GROWTH AND SURVIVAL OF EMERGING LARVAE:

Figs. 7a,b & c compare the growth-rates of the early- and late-season larvae of three females up to the tenth day after hatching. A mean length was taken for 10 larvae from each of the triplicate experimental tanks and then a further mean length was computed using these three mean values. Each point in Figs. 7a,b & c therefore represents a mean of three means. (The mean larval lengths and yolk-sac indices for each of the triplicate experimental tanks are given in Appendix I b). The growth-rates varied slightly, but in general the growth-rates of late-season larvae were as good as those of early-season larvae. Thus, as can be seen in Figs. 7a & c, the late-season larvae tended to remain slightly smaller. However, it is quite possible that the lengths of the two types of larvae would have converged if the experiment had been continued for longer since their standard deviations were already overlapping at Day 7. Although the lengths of the late-season larvae of Female II (Fig. 7b) became greater than those of early-season larvae around Day 6, this was probably due to a shortage of rotifers in the larval tanks containing the early-season larvae on Days 3-4. Although remedied as quickly as possible, the growth of the early-season larvae seemed to suffer for this period of insufficient food.

Table 5 gives the numbers of larvae surviving in each experimental tank on the tenth day after hatching. There is considerable variation both between early- and late-

Fig. 8. The relationship between a turbot female's maximum mean egg-diameter in a single spawning season and her weight. A functional regression line (Ricker, 1973) was fitted to the data.

Fig. 8.

■ - Fish heavily infested with tapeworm.

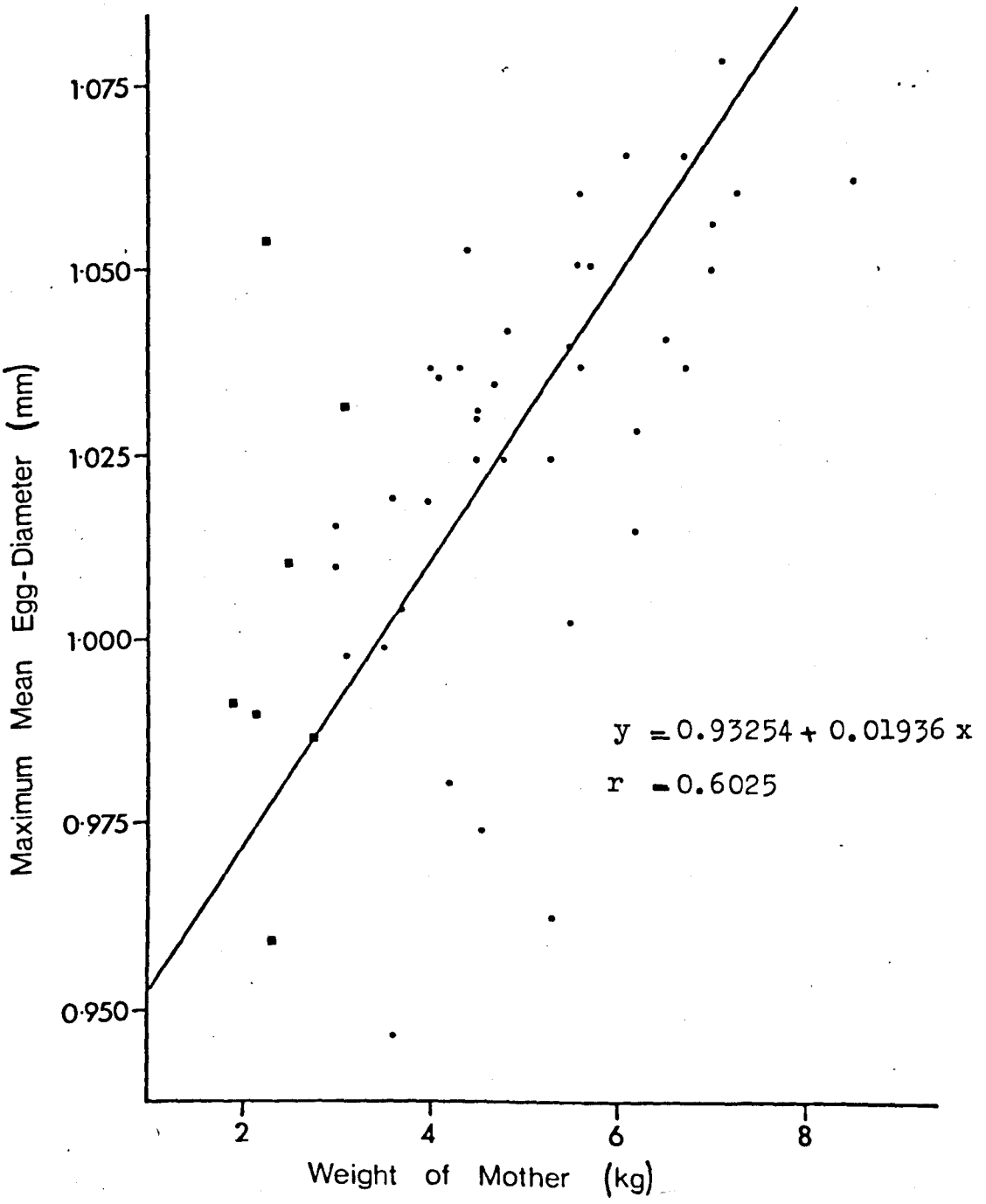
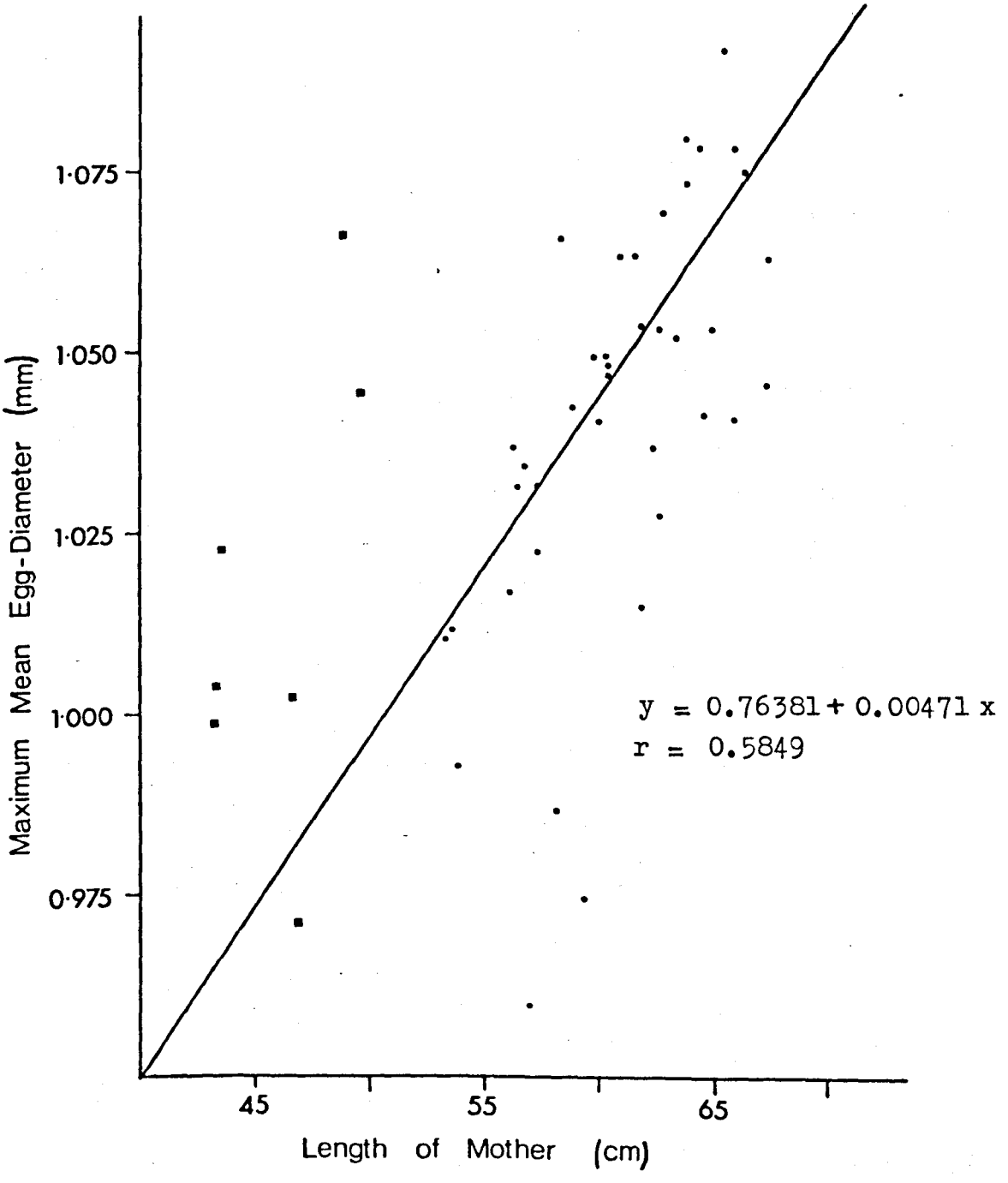


Fig. 9. The relationship between a turbot female's maximum mean egg-diameter in a single spawning season and her length. A functional regression line (Ricker, 1973) was fitted to the data.

Fig.9.

■ - Fish heavily infested with tapeworm.



season larvae, and between triplicate tanks of the same types of larvae. However, neither early nor late season larvae had persistently better survival figures, so there did not seem to be any significant change in larval performance as the spawning season progressed.

5. RELATIONSHIP BETWEEN A FEMALE'S MAXIMUM MEAN EGG-DIAMETER IN ANY SPAWNING SEASON AND HER LENGTH, WEIGHT AND CONDITION INDEX:

Figs. 8 & 9 are plots of a turbot female's maximum mean egg-diameter in a single spawning season against her weight and length respectively. Functional regression lines (Ricker, 1973) were fitted to the two sets of data, giving a correlation index (r) of 0.6025 for maximum mean egg-diameter versus weight and 0.5849 for maximum mean egg-diameter versus length. This means that each correlation is significant at the 95% level ($P < 0.05$).

The relationship between the two parameters plotted in Fig. 8 can be expressed by the equation:

Max. mean egg-diameter = 0.93254 + 0.01936 Wt. Female (The 95% confidence limits of the slope are: 0.02550 and 0.14696).

The maximum mean egg-diameter versus length of the mother relationship is expressed by the equation:

Max. mean egg-diameter = 0.76381 + 0.00471 Female length (The 95% confidence limits of the slope are: 0.00624 and 0.00356).

No relationship was found between a female's maximum mean egg-diameter and her condition index (K).

Fig. 10. Comparison of hatchery and wild turbot fecundity.

- Absolute fecundity data obtained for "wild" turbot by Jones (1974).

- Estimations of the egg-yields of Port Erin hatchery turbot females by Jones et. al. (1979).

* Recent estimations of the egg-yields of Port Erin hatchery turbot which take into consideration the seasonal increase in the number of eggs per gram weight caused by the decline in egg-size.

Fig.10. Comparison of Hatchery and Wild Turbot Fecundity

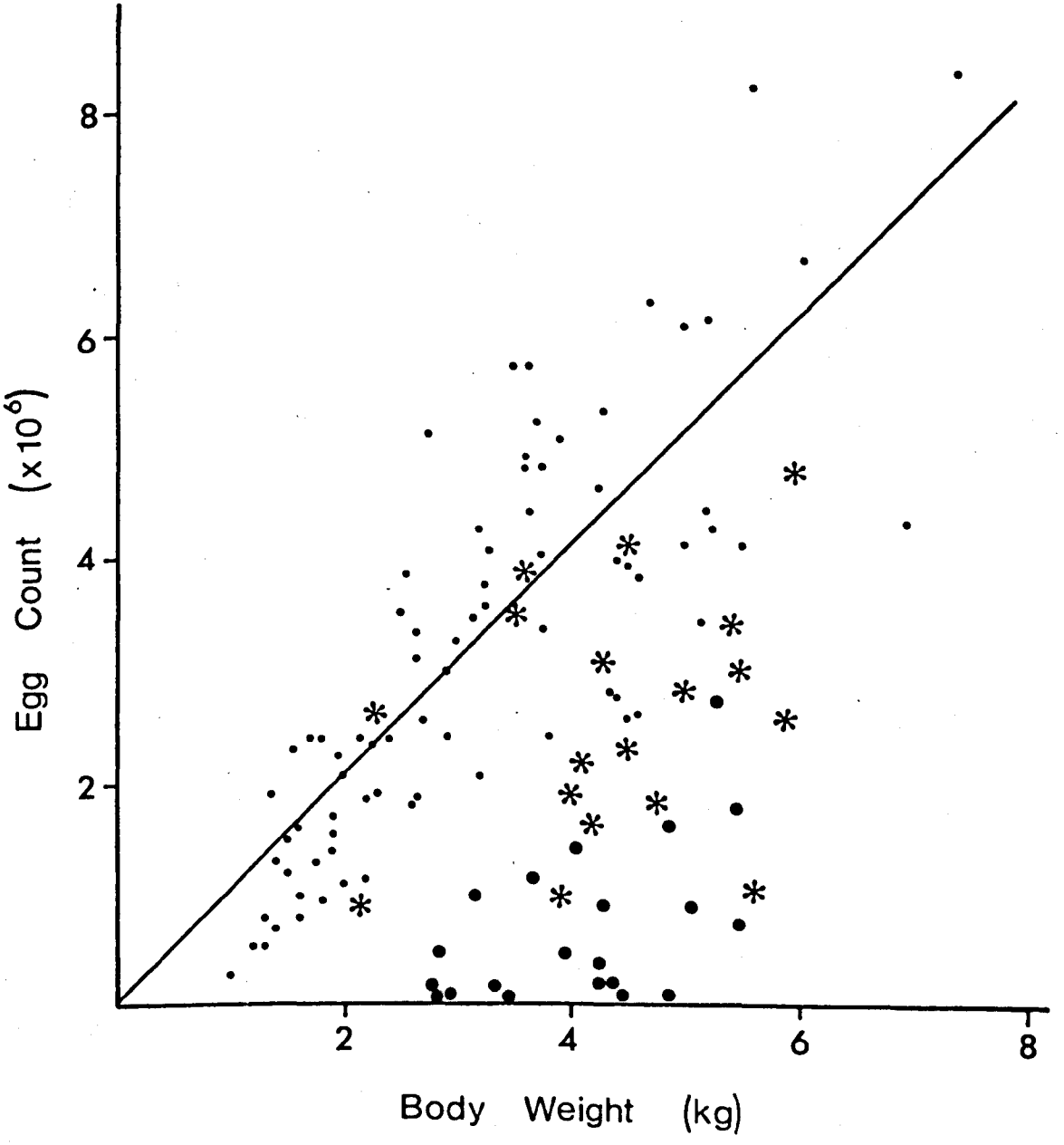
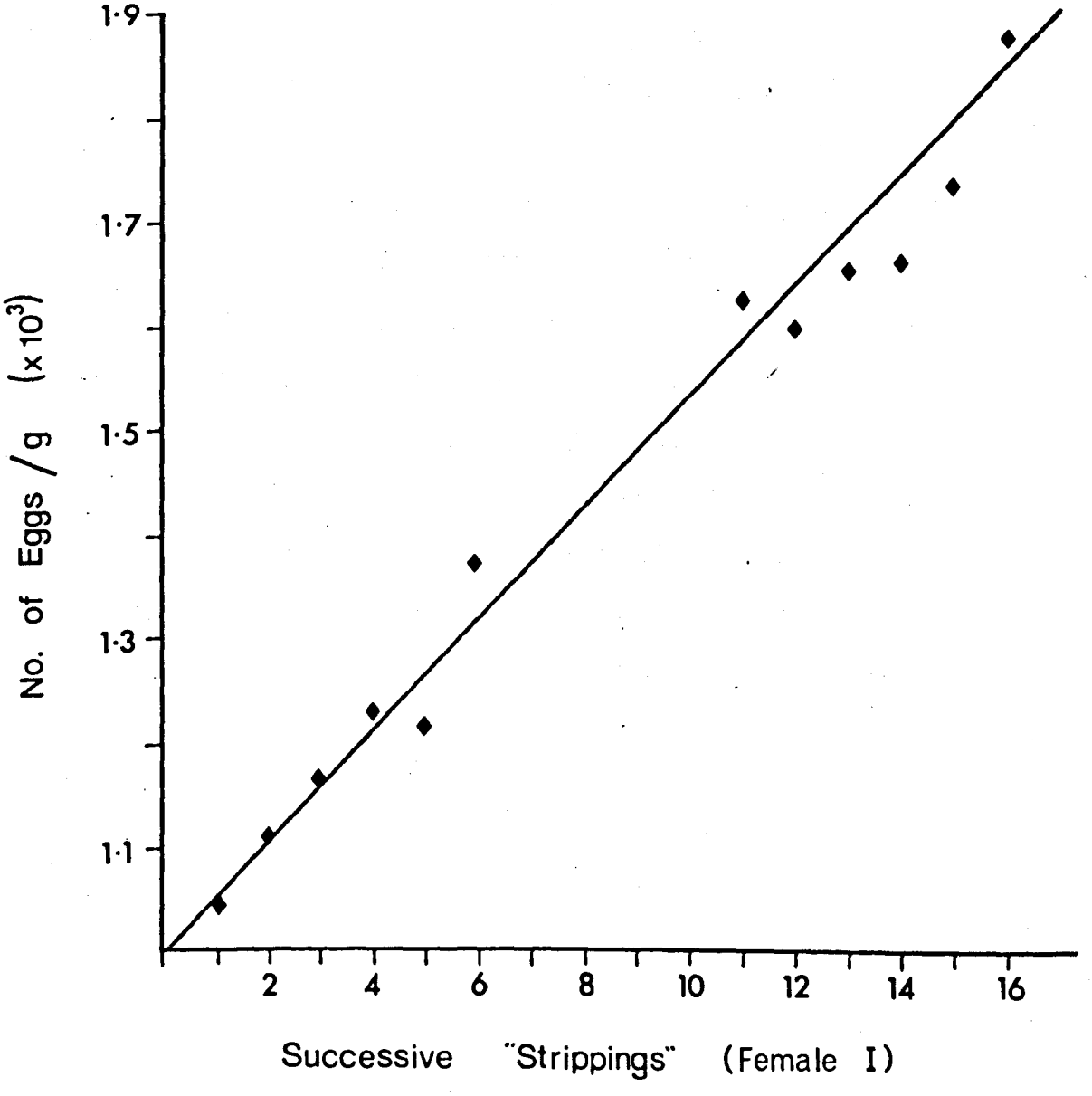


Fig. 11. Seasonal variation in the number of turbot eggs per gram. Eggs from Female I were used throughout this figure.

Fig. 11. Seasonal Variation in the Number of Eggs per Gram



6. COMPARISON OF THE ABSOLUTE FECUNDITY OF "WILD" TURBOT AND THE EGG-YIELD OF CAPTIVE TURBOT SUBJECTED TO HAND-STRIPPING:

Fig. 10 compares the absolute fecundity data obtained for "wild" turbot by Jones (1974); the estimations of the egg yields of Port Erin hatchery turbot females by Jones et. al. (1979), and recent estimations of the egg-yields of hatchery fish that take into consideration the seasonal increase in the number of eggs per gram weight caused by the decline in egg-size. These fluctuations make a significant difference to egg-yield computations because at the beginning of the spawning season, eggs are usually of such a size that there are 900-1000 eggs per gram, whereas at the end of a long spawning season a female's eggs may be so small that there are up to 1900 per gram (see Fig. 11).

It can be seen from Fig. 10 that taking the egg-size decline into consideration increases the fecundity estimates of hatchery fish considerably, although they are still somewhat lower than the fecundity estimates of "wild" fish.

DISCUSSION

1. SEASONAL DECLINE IN EGG-DIAMETER:

From the results presented here it is clear that turbot exhibit a small but obvious decrease in mean egg-diameter as the spawning season progresses. Further evidence for this phenomenon has come from Girin (1979) who found that turbot eggs collected from a naturally-spawning broodstock tended to decrease in size later in the season. However, the decline noted by Girin was statistically insignificant - probably due to the fact that the broodstock contained several females which would start and finish spawning at different times, so masking the phenomenon to a certain extent.

The reason for this size-decline is unclear. Turbot release their eggs from May until early August in the wild (Jones, 1974), and so there should be abundant supplies of zooplankton for larvae hatching throughout the spawning season. Therefore, one would not expect the egg-size decline in turbot to have evolved in response to fluctuating supplies of zooplankton, as suggested for other species by Bagenal (1971), Baxter (1959 & 1963), Cushing (1967), Jones (1973) and others. It would seem more likely that Ware's (1975) hypothesis which suggested that egg-size is related to the ambient water-temperature (and thus incubation-time) provides a plausible reason for this phenomenon in turbot.

2. RELATIONSHIP BETWEEN EGG-SIZE, LENGTH OF THE EMERGING LARVA AND ITS YOLK-SAC INDEX:

There is a significant correlation between egg-size and larval length, and between egg-size and the yolk-sac

index of the emergent larva, but none between larval length and yolk-sac index. Therefore, large eggs tend to give rise to longer larvae or larvae with bigger yolk-sacs, but large larvae do not necessarily have large yolk-sacs.

This is further illustrated by comparing yolk-sac index : larval length ratios. In contrast to herring, in which larger eggs hatch to give large larvae with an increased yolk-sac index : larval length ratio (Hempel & Blaxter, 1963), the yolk-sac index : larval length ratio in turbot is not proportional to egg-size. However, this ratio appears to remain similar for larvae emerging from eggs spawned by any one particular female - suggesting that the body proportions of newly-hatched turbot larvae might be genetically determined by the mother. However, no firm conclusions can be made about this due to insufficient data.

3. EFFECT OF DIFFERENT MALE-CROSSES ON THE LENGTHS AND YOLK-SAC INDICES OF LARVAE HATCHING FROM A PARTICULAR BATCH OF EGGS:

"Crossing" one female with several different males did not have any significant effect on the lengths or yolk-sac indices of the emerging larvae. Again, this suggests that the body proportions of newly-hatched larvae are genetically determined by the mother. It must be noted, however, that in hybrid experiments performed at Port Erin in which turbot eggs were fertilised with milt taken from male brill, the newly- hatched larvae were significantly bigger than normal turbot x turbot larvae. In this instance, the males must have affected the larval length.

The number of larvae hatching in each treatment tank

varied considerably, but this was probably as much due to egg variability as milt variability because the eggs had not been sorted for viability before fertilisation, but had been quickly weighed out to prevent "activation" occurring (see Section IV).

4. EFFECT OF EGG-SIZE ON THE SUBSEQUENT GROWTH AND SURVIVAL OF EMERGING LARVAE:

Larvae emerging from large eggs spawned at the beginning of the season did not seem to have any advantage in terms of growth or viability over small larvae from late in the spawning season, and the latter performed just as well under hatchery conditions.

There is no reason to believe that late-season larvae should be at a greater disadvantage in an "insufficient-food" regime, either, since their yolk-sac index : larval length ratios are no smaller than those of the larger early-season larvae.

5. RELATIONSHIP BETWEEN A FEMALE'S MAXIMUM MEAN EGG-DIAMETER IN ANY SPAWNING SEASON AND HER LENGTH, WEIGHT AND CONDITION INDEX:

Figs. 8 & 9 show a significant correlation between the weight and length of females and the maximum mean diameter of the eggs they produce - that is, big fish tend to produce larger eggs at the start of their spawning season. This confirms work by Määr (1950) and Nikolsky (1950) who also found that, in a given population of fish, larger females tend to produce larger eggs. Unfortunately, it could not be determined whether this correlation was a function of age

(i.e., whether older fish tended to produce larger eggs than younger fish of the same size) because the age of most of the female turbot was uncertain.

There was no correlation between a female's maximum mean egg-diameter and her condition-index (K).

6. COMPARISON OF THE ABSOLUTE FECUNDITY OF "WILD" TURBOT AND THE EGG-YIELD OF CAPTIVE TURBOT SUBJECTED TO HAND-STRIPPING:

Absolute fecundity estimates made by Jones (1974) suggested that wild turbot had a fecundity of approximately 1×10^6 eggs per kg body weight. However, egg-yield figures calculated for Port Erin hatchery turbot by Jones et. al. (1979) were well below that estimation. These workers were concerned that this might be due to artificial holding conditions, stressing females by hand-stripping, or an inadequate broodstock diet (Jones, pers. comm.).

However, the egg-yield figures presented here suggest that the fecundity of Port Erin hatchery turbot is not too far behind that of wild fish when the seasonal decline in egg-diameter is taken into account. Most of these egg-yield estimates are still below Jones' fecundity estimate (1974), but it must be remembered that Jones estimated the total number of secondary oocytes in each fish's ovary. This is likely to be a higher figure than the actual egg-yield since oocyte atresia is a common occurrence in fish's ovaries (Bowers & Holliday, 1961; Dunn & Tyler, 1969; Htun-Han, 1978; Wiebe, 1968).

It is therefore suggested that the holding conditions, stripping techniques and broodstock diet at Port Erin do not seriously limit fecundity.

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SECTION II

INTRODUCTION

Fish differ markedly from terrestrial animals in that the major polyunsaturated fatty acids in their tissues are of the ω3 rather than ω6 series. This applies to marine and freshwater fish alike, although the latter have higher concentrations of ω6 polyunsaturates than marine fish (Ackman, 1967). Biochemical evidence suggests that the greater degree of unsaturation found in the tissue fatty acids of aquatic, and especially marine, animals is an adaptation to a relatively constant, low temperature environment (Kemp & Smith, 1970; Knipprath & Mead, 1968; Smith, 1967; Smith & Kemp, 1969; Thompson et. al., 1975), and probably serves to control membrane fluidity at these lower temperatures (Kimelberg & Papahadjopoulos, 1974).

It is well-documented that 18:2ω6 and 20:4ω6 fatty acids have full essential fatty acid (EFA) activity in small terrestrial omnivores, whilst ω3 series fatty acids have partial EFA (Alfin-Slater & Aftergood, 1968; Cowey & Sargent, 1972; Guarneri & Johnson, 1970). During the last decade, there has been considerable interest concerning EFA activity in fish. Lee et. al.(1967), Castell et. al.(1972a) and Yu and Sinnhuber (1972 & 1976) all demonstrated the dietary essentiality of linolenic or ω3 series acids for rainbow trout. Castell et. al.(1972b & c) indicated that linolenic acid (18:3ω3) should comprise at least 1% of the diet (or 2.7% of the dietary calories) to prevent the occurrence of deficiency syndromes in trout. Watanabe et. al. (1974) confirmed these observations whilst Takeuchi and Watanabe (1976) showed that 22:6ω3 and possibly 20:5ω3 fatty

acids have greater EFA activity for trout than linolenic acid - that is, the latter is used as a precursor for these ω3 long-chained highly polyunsaturated fatty acids (ω3HPUFA).

In contrast, Owen et. al.(1975), Cowey et. al.(1976a & b) and Gatesoupe et. al.(1977) demonstrated the inability of turbot to further desaturate and chain-elongate linolenic acid to 20:5ω3 and 22:6ω3. Owen et. al.(1975) suggest this might be due to the absence or reduction of enzymes responsible for this transformation. Since Leger et. al. (1979) found that turbot have a limited capacity for transforming 18:3ω3 to 22:6ω3 given a high substrate concentration (3.7 % of the diet), the latter explanation seems more plausible.

18:3ω3 therefore has little EFA activity for turbot and the long-chained ω3HPUFA must be supplied preformed in the diet. Owen et. al.(1975) and Cowey and Sargent (1977 & 1979) point out that this state should not present any problems to "wild" turbot since they are extreme carnivores and 22:6ω3 is the major polyunsaturated fatty acid in their natural diet.

A similar condition has been described for red sea bream (Fujii & Yone, 1976; Kanazawa et. al., 1979; Yone & Fujii, 1975) and the work of Owen et. al.(1972) suggested that plaice could not further desaturate and chain-elongate 18:3ω3 under the dietary regimes used in their experiment.

Bearing in mind the dietary essentiality of ω3HPUFA in turbot, and the fact that mature females do not feed for 2-3 weeks prior to and throughout their spawning season (see Fig. 3), this preliminary investigation was carried

out to assess whether EFA levels in eggs decreased as the spawning season progressed.

MATERIALS & METHODS

1. COLLECTION OF SAMPLES

Three females with a history of previous spawning seasons of at least two months duration were chosen for the study. Small samples of eggs were collected at the beginning, middle and end of each fish's spawning season. Only freshly-ovulated eggs were used. (See section III for methods used to determine the time of ovulation).

a) Unfertilised eggs were stripped into filtered seawater and allowed to stand for about 20 minutes until the dead, overripe eggs sank.

b) Approximately 2g of eggs were netted from the layer of freshly-ovulated eggs floating at the surface, and these were blotted with tissue-paper until they were completely "surface-dry."

c) They were then weighed on a Mettler balance and put into screw-topped glass vials fitted with Teflon caps which were lined with metal foil.

d) Chloroform-methanol 2:1 v/v, with 2,6-di-tert-butyl-p-cresol (BHT) at 0.01% w/v to prevent oxidation of lipids, was immediately added to the vials in sufficient quantity to completely cover the eggs.

e) The vials were stored until analysis in an upright position at -20°C in a deep-freeze. The maximum storage-time of samples prior to lipid extraction was 3.5 months.

Additional eggs were collected at each sampling-time for dry-weight determinations. These eggs were blotted dry with tissue paper until "surface-dry", then triplicate sub-samples were weighed into round-bottomed glass flasks,

and freeze-dried. Their dried weights were determined and expressed as a percentage of their original wet weight.

Each sample's mean egg-diameter and number of eggs per gram were determined as in Section 1.

2.EXTRACTION OF TOTAL LIPIDS

Folch et.al's (1957) method for extracting total lipid was adapted for this study. Only glass vials which had been previously rinsed in chloroform-methanol were used throughout.

a) Each sample was homogenised in 10ml chloroform-methanol 2:1 (with BHT) in a centrifuge tube,using a top-drive homogeniser,for at least 5 minutes. (Ice was used to cool the sample-tube.)

b) Further C-M 2:1 (with BHT) was added giving a solvent :sample ratio of 15:1 v/w. This ensured a single phase.

c) The tube was covered with aluminium foil,packed in ice, and left to extract at 4° C for 20 minutes.

d) Tissue settled to the bottom of the tube and the supernatant was decanted and filtered through C-M washed glass-wool into a graduated stoppered cylinder.

e) The sediment was washed twice with 0.2 volumes of C-M 2:1 (with BHT),centrifuged at 800g for 10 minutes and the supernatants combined.

f)0.2 volumes of water were added to the combined supernatants in a stoppered measuring cylinder. This was flushed with nitrogen and shaken to form an emulsion (It was important to release pressure in the measuring cylinder after shaking.)

g) The emulsion was quickly decanted into centrifuge tubes and centrifuged for 10 minutes at 800g.

h) The upper phase was removed by aspiration and discarded.

i) A little Folch upper phase solvent system (Chloroform :methanol:water, 3:48:47 v/v/v) was carefully "layered" on top of the lower phase and swirled very gently. The epilayer was removed by aspiration and the process repeated.

j) The extract was filtered through C-M washed Whatman IPS paper(silicone treated filter paper) to remove any traces of upper phase and precipitated protein. The IPS paper was changed after each 100ml filtered.

k) The solvent was evaporated in a Bucchi Rotorvaporator at 50°C and the vacuum released under nitrogen.

l) The sample was then taken up in 5ml of chloroform in a Teflon-capped glass vial and stored in a deep-freeze.

3. SEPARATION OF NEUTRAL AND PHOSPHOLIPIDS

a) Columns were constructed as follows: the fine end of a 1cm wide glass chromatography column was blocked with a small glass-fibre plug. A slurry of chloroform-Kieselgel 40 (70-230 mesh,defined) was then carefully added to the column until it evenly filled a height of about 7cm.

b) 2ml of the 5ml total lipid extract was taken and blown down to 1ml.

c) This concentrated sample was carefully added onto the surface of the silicic-acid column and washed through with 20ml of chloroform.

d) The eluent was evaporated on a Bucchi Rotorvaporator at 50°C and shaken up in 2ml chloroform. This was the neutral lipid fraction.

e) The silicic acid column was then eluted with 20ml methanol to collect the phospholipid fraction.

f) The eluent was evaporated as before and taken up in 2ml of chloroform:methanol 2:1

g) The neutral and phospholipid fractions of one of the samples were run against a lipid standard on a TLC plate to check that there was no cross-contamination.

4 QUANTITATIVE DETERMINATION OF TOTAL, NEUTRAL AND PHOSPHOLIPIDS

The spectrophotometric method of Marsh & Weinstein (1966). which was modified by Holland & Gabbott (1971), was used to determine the percentage concentration of total lipid in the complete samples and the percentage concentration of neutral and phospholipid in the separated samples. The procedure was as follows-

a) 50µl triplicates of lipid samples, blanks and 5,10,20,28 and 40 µg of tripalmitin standard in 50µl of chloroform were dried in C-M washed glass test tubes.

b) 500µl concentrated H_2SO_4 were added to each tube. The contents were stirred and charred on a heating block at 200°C for 15 minutes.

c) The tubes were then cooled and the contents diluted to 2.0ml with distilled, deionised water and shaken vigorously.

d) The contents of each tube were read on a spectrophotometer at 375nm in a 2.0cm pathlength cell.

e) The weight of lipid in each sample was calculated from the standard plot of weight versus optical density and was then expressed as a percentage of the original wet weight of the egg sample.

5. PREPARATION OF FATTY-ACID METHYL ESTERS FOR ANALYSIS BY GAS LIQUID CHROMATOGRAPHY

The method described by Morrison & Smith (1964) was used as follows-

a) 500µl of lipid extract was placed in a 5ml Reactivial followed by 3ml of 14% boron trifluoride in 2:1 methanol: dichloromethane. This was flushed with nitrogen and mixed thoroughly.

b) The Reactivial was heated on a heating-block for 1 hour at 100° C.

c) The sample was allowed to cool and then 2 volumes of petroleum ether 30-40° C and 1 volume of water were added. It was shaken and allowed to separate. The top phase was removed via pippette and reserved, and the extraction was repeated again keeping the top phase.

d) Nitrogen was used to blow off the ether and the sample was taken up in 100µl chloroform.

e) Meanwhile, the continuous elution chromatography tank was prepared by pouring elution solvent (petroleum ether 30-40° C: diethyl ether, 95:5) into it and allowing equilibration.

f) 40µl of the sample was "spotted" onto a precoated silica gel G thin-layer chromatography plate (TLC plate) about 1.5cm above the bottom of the plate. Nitrogen was used to

evaporate the solvent.

g) 3 samples and 10µl of methyl ester standard were spotted on each TLC plate(with at least 3cm separation between the spots.)

h) The plates were chromatographed in the continuous elution tank for 1 hour 15 minutes. They were then removed and dried in the tank under a stream of nitrogen.

i). The area of the plate where the sample had run was covered with another TLC plate with silica gel facing silica gel, and the area where the standard had run was exposed to iodine vapour in an enclosed chromatography tank. The methyl ester spot stained yellow in about 10 minutes. This was used to locate the methyl ester spots of the samples. The area to be removed from the plate was marked lightly with a pencil before the standard spot faded,(the spots were approximately 3cm wide), and the silica gel corresponding to each sample was scraped off with a razor and transferred to separate Reactivials.

j) The methyl esters were extracted three times by adding a few mls. of C-M 2:1 and shaking well. The Reactivial was then centrifuged for 5 minutes at 800g and the supernatants combined.

k) The combined supernatants were evaporated under a stream of nitrogen and taken up in 100µl hexane. They were then stored under nitrogen in a deep-freeze.

l) The TLC plate was replaced in the iodine tank to check that all the methyl esters had been collected.

Samples were usually extracted and methylated three at a time.

6.GAS-LIQUID CHROMATOGRAPHY(GLC)

GLC analysis was carried out on a Pye 104 gas chromatograph fitted with a conventional glass column 1.3m long x 4mm internal diameter, packed with 5% SP1000 on Acid-Washed Chromosorb DMCS (diamethyl chlorosilane). Fatty acids were identified by comparison with known standards and by the log.retention plots of isothermal runs at 200°C.(Ackman,1972). The Pye 104 amplifier was set at an attenuation of 5×10^2. The gas pressures were as follows:-

hydrogen : 1.0 kg/cm^2

air : 0.6 kg/cm^2

argon (carrier gas) : 2.0 ml/minute

The chromatograph was linked to a Supergrator III computing integrator(Colombia Scientific, U.S.A.)which computed and printed out the percentage concentrations of the different fatty acids.

SAMPLING TIME	FEMALE I	FEMALE II	FEMALE III
a) Beginning of spawning season	8.66 (0.00)	8.76 (0.11)	8.90 (0.16)
b) Mid-spawning season	8.93 (0.04)	8.63 (0.00)	8.89 (0.02)
c) End of spawning season	8.75 (0.07)	8.80 (0.04)	8.91 (0.08)

Table 6. Weights of freeze-dried turbot eggs (expressed as a percentage of their wet weights) throughout the spawning season. (Standard deviations in parentheses).

FEMALE AND SAMPLING TIME	LIPID CONTENT OF EGGS (EXPRESSED AS % WET WEIGHT)				
	TOTAL LIPID	NEUTRAL LIPID (N)	PHOSPHO-LIPID (P)	N+P	$\frac{N}{P}$
I a	1.288 (0.069)	0.692 (0.008)	0.598 (0.001)	1.290	1.16
I b	1.342 (0.000)	0.710 (0.014)	0.583 (0.014)	1.293	1.21
I c	1.506 (0.014)	0.698 (0.025)	0.605 (0.024)	1.303	1.15
II a	1.246 (0.068)	0.618 (0.020)	0.558 (0.016)	1.176	1.11
II b	1.219 (0.000)	0.657 (0.028)	0.574 (0.011)	1.231	1.15
II c	1.420 (0.078)	0.727 (0.020)	0.504 (0.035)	1.231	1.44
III a	1.481 (0.000)	0.690 (0.036)	0.559 (0.007)	1.249	1.23
III b	1.333 (0.150)	0.714 (0.032)	0.646 (0.009)	1.360	1.10
III c	1.527 (0.000)	0.657 (0.028)	0.587 (0.012)	1.244	1.34

Table 7. Results of a Marsh and Weistein Lipid Assay performed on a) early, b) mid- and c) late season eggs (Standard deviations in parentheses).

FEMALE AND SAMPLING TIME	EGGS PER GRAM	MEAN EGG-DIAMETER (mm)	WEIGHT OF LIPID IN A SINGLE EGG (µg)		
			TOTAL LIPID	NEUTRAL LIPID	PHOSPHO-LIPID
I a	965	1.056 (0.008)	13.35 (0.72)	7.17 (0.08)	6.19 (0.09)
I b	1400	0.989 (0.010)	10.10 (0.88)	5.07 (0.10)	4.19 (0.12)
I c	1490	0.975 (0.011)	10.10 (0.09)	4.68 (0.17)	4.06 (0.16)
II a	1015	1.048 (0.009)	12.27 (0.67)	6.08 (0.20)	5.50 (0.16)
II b	1100	1.035 (0.013)	11.08 (0.00)	5.98 (0.25)	5.22 (0.10)
II c	1310	1.002 (0.009)	10.84 (0.60)	5.55 (0.15)	3.85 (0.27)
III a	1490	0.975 (0.014)	9.63 (0.54)	4.63 (0.24)	3.75 (0.05)
III b	1535	0.968 (0.012)	8.53 (0.76)	4.65 (0.21)	4.21 (0.06)
III c	1565	0.963 (0.007)	9.54 (0.37)	5.25 (0.37)	3.91 (0.28)

Table 8. Lipid content per egg throughout the spawning season for a) early, b) mid- and c) late season eggs (Standard deviations in parentheses).

Fig. 12. A typical gas-chromatogram of turbot egg neutral lipid. The fatty acid peaks of importance to this study are identified in the key below. Chromatography conditions are as described in the text. Chart speed is 213 mm/hour.

Fig. 12

a	16:0	e	18:2ω6	i	20:3ω6	m	22:5ω3
b	16:1ω9 & 7	f	18:3ω3	j	20:4ω6	n	22:6ω3
c	18:0	g	20:1ω9 & 7	k	20:5ω3 and 22:1ω11 & 9 & 7 (not resolved)		
d	18:1ω9 & 7	h	18:4ω3	l	22:4ω6		

Fig. 13. A typical gas-chromatogram of turbot egg phospholipid. The fatty acid peaks of importance to this study are identified in the key below. Chromatography conditions are as described in the text. Chart speed is 213 mm/hour.

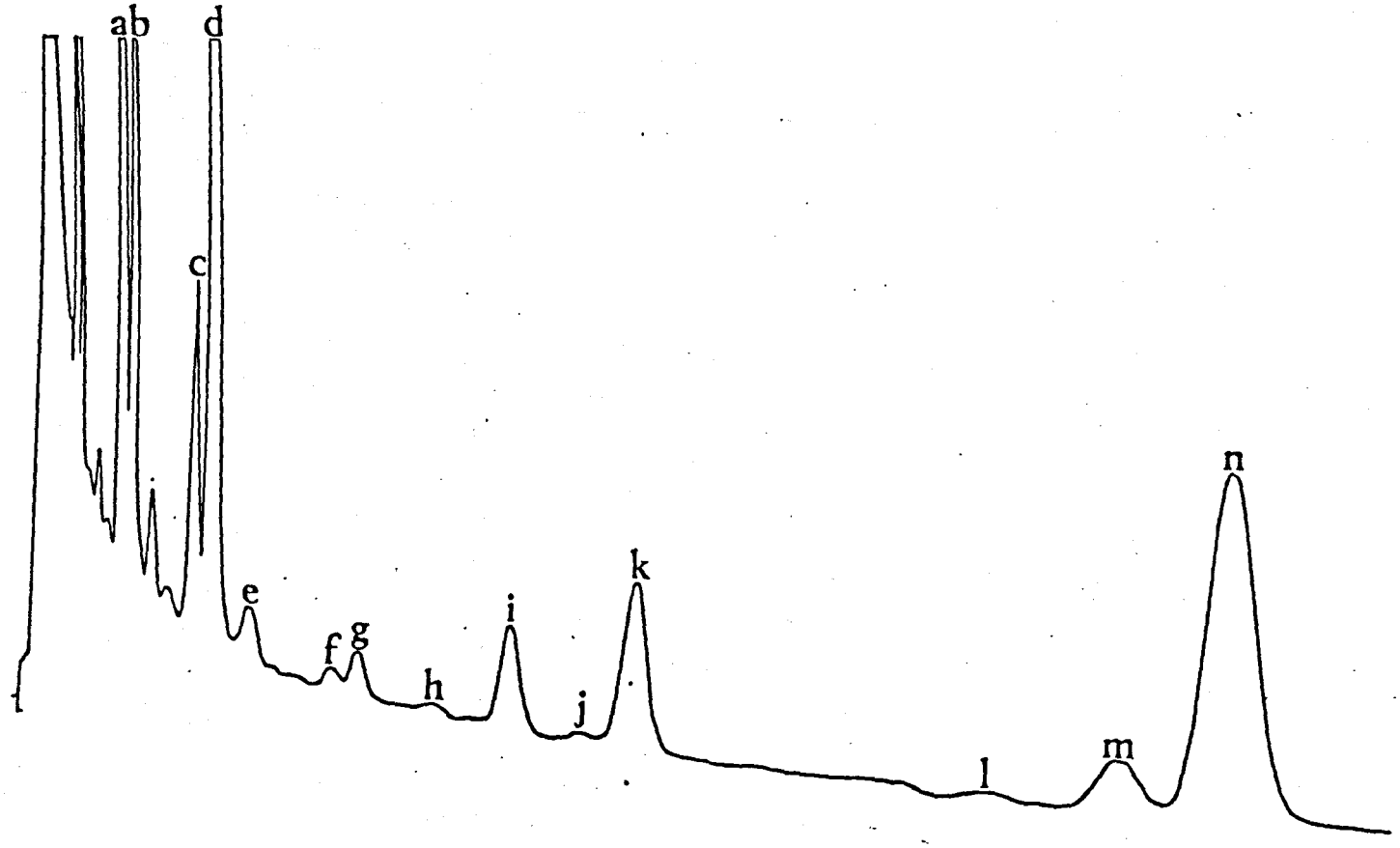

Fig. 13

a	16:0	e	18:2ω6	i	20:3ω6	m	22:5ω3
b	16:1ω9 & 7	f	18:3ω3	j	20:4ω6	n	22:6ω3
c	18:0	g	20:1ω9 & 7	k	20:5ω3 and 22:1ω11 & 9 & 7 (not resolved)		
d	18:1ω9 & 7	h	18:4ω3	l	22:4ω6		

FATTY ACID	FEMALE I			FEMALE II			FEMALE III		
	a	b	c	a	b	c	a	b	c
16:0	18.89	18.66	16.75	21.24	16.41	25.03	14.34	8.60	18.01
16:1ω9 & 7	11.24	9.78	5.28	15.50	12.91	4.14	12.46	12.45	11.52
18:0	7.23	8.12	10.31	3.55	7.28	13.24	5.19	8.44	8.01
18:1ω9 & 7	31.80	30.89	27.85	40.97	40.07	21.64	44.36	42.01	32.75
18:2ω6	4.61	4.52	6.53	1.71	3.22	7.93	1.23	5.75	3.34
18:3ω3	2.32	2.74	4.39	1.50	2.17	5.27	0.72	3.42	2.95
18:4ω3	1.42	1.41	2.72	0.61	1.01	3.19	Trace	1.70	0.88
20:1ω9 & 7	2.06	1.46	1.56	0.60	1.74	0.97	1.63	1.54	0.91
20:3ω6	2.24	0.66	1.82	0.43	0.32	2.27	3.35	Trace	0.74
20:4ω6	1.14	1.19	1.82	Trace	0.80	1.82	0.66	1.38	0.95
{20:5ω3 22:1ω11,& 9,& 7}	3.53	4.27	4.41	2.70	2.71	2.39	3.01	2.83	3.60
22:4ω6	0.44	Trace	2.85	Trace	0.75	2.59	0.84	0.57	2.42
22:5ω3	1.99	3.37	2.27	1.13	1.62	2.52	2.65	2.11	2.58
22:6ω3	11.19	12.92	11.45	9.99	9.00	7.01	9.56	9.20	11.36

Table 9. The percentage fatty acid composition of turbot egg neutral lipid throughout the spawning season. a, b, and c denote samples taken at the beginning, middle, and end of the season, respectively. Trace indicates where the fatty acid composition is ≤ 0.20 percent.

FATTY ACID	FEMALE I			FEMALE II			FEMALE III		
	a	b	c	a	b	c	a	b	c
16:0	16.80	20.14	28.73	18.23	19.17	24.97	17.27	24.32	45.67
16:1ω9 & 7	5.89	4.67	2.82	5.85	8.03	6.24	5.07	2.93	1.06
18:0	6.25	6.03	7.21	4.83	5.73	5.17	5.82	7.32	10.28
18:1ω9 & 7	17.49	15.95	17.65	17.19	25.81	16.34	15.98	16.15	8.83
18:2ω6	1.62	1.52	0.24	1.29	1.08	0.51	2.48	2.75	1.85
18:3ω3	0.34	0.57	0.43	0.29	0.49	Trace	0.74	1.14	2.45
18:4ω3	0.44	0.61	0.63	0.35	0.39	Trace	0.53	1.41	0.45
20:1ω9 & 7	1.44	1.33	0.66	1.21	0.99	1.13	1.76	1.30	0.70
20:3ω6	3.83	4.10	3.46	3.42	3.30	2.89	3.57	4.57	2.34
20:4ω6	0.61	0.51	0.72	0.43	Trace	0.86	0.72	Trace	Trace
{20:5ω3 {22:1ω11, & 9, & 7}	8.48	8.04	6.00	7.94	5.90	7.23	8.75	8.98	5.30
22:4ω6	0.43	Trace	0.49	0.48	0.43	0.58	0.26	Trace	0.45
22:5ω3	4.85	4.66	3.81	4.80	3.43	4.34	4.73	1.68	2.67
22:6ω3	31.54	31.85	27.18	33.68	25.27	29.72	32.37	27.45	17.95

Table 10. The percentage fatty acid composition of turbot egg phospholipid throughout the spawning season. a, b, and c denote samples taken at the beginning, middle, and end of the season, respectively. Trace indicates where the fatty acid composition is ≤ 0.20 percent.

RESULTS

The percentage dried-weight of eggs show no significant variation either between individual females or throughout the spawning season.(Table 6). Table 7 shows the percentage concentrations of total, neutral and phospholipids as determined by a Marsh and Weinstein Assay (1966). Although the total lipid content appears to increase slightly at the end of each female's spawning season, there is no such increase in the combined neutral and phospholipid values, suggesting the total lipid variation is caused by changing concentrations of classes such as glycolipids, lipoproteins etc.. The ratio of neutral to phospholipid shows no significant difference over the spawning season.

Although the amount of lipid per g. eggs remains constant throughout the season the eggs themselves become smaller so that there are more eggs per g. at the end of the spawning season. Therefore these later eggs have a smaller total weight of lipid per egg. The weight of total, neutral and phospholipid per egg listed in Table 8. were computed using each sample's percentage lipid concentrations, mean egg-diameter and number of eggs per gram.

Figs.12& 13 show typical gas-chromatograms of turbot eggs neutral and phospholipid fractions.

Table 9 shows the percentage concentration of different fatty acids in the neutral lipid fraction. 22:5 $\omega 3$ and 22:6 $\omega 3$ fatty acid levels appear reasonably constant between the eggs of different females and

throughout each female's spawning season. However, the levels of 18:0 tend to increase slightly as the spawning season progresses as do the percentage concentrations of 18:3 ω3 and 22:4 ω6, whilst 16:1 ω7 and 18:1 ω9 levels fall. Other fatty acid levels tend to remain fairly constant throughout the spawning season.

Table 10 shows the percentage concentration of the same fatty acids in the phospholipid fraction. In contrast with the neutral lipid, the percentage concentration of 22:6 ω3 and, to a lesser extent, 22:5 ω3 appear to decrease in the later batches of eggs whilst 16:0 levels increase as the spawning season progresses. (It was not possible to assess whether 20:5 ω3 levels fluctuated because the chromatograph did not resolve the 20:5 ω3 and 22:1 ω11,9 peaks.)

DISCUSSION

From the results of this pilot-investigation, it would appear that levels of essential fatty acids (EFA) (e.g.22:5ω3 and 22:6ω3) present in egg neutral lipid fractions do not show any significant variation as the spawning season progresses. However, percentage concentrations of EFA appear to decrease slightly in the phospholipid fraction of eggs spawned later in the season.

In addition, the levels of 16:1, 18:0, 18:1, 18:3 and 22:4 fatty acids appear to show slight seasonal fluctuations in the neutral lipid fractions whilst phospholipid 16:0 levels increased as the spawning season progressed. The reasons for these variations are unclear. Takeuchi and Watanabe (1976) found that tissue 16:1ω7 and 18:1ω9 fatty acid levels tended to increase when rainbow trout were fed EFA-deficient diets and decreased again when the fish received 18:3ω3 or 22:6ω3 acids in their food. However, since 16:1ω7 and 18:1ω9 levels present in the neutral fractions of all three turbot females used in this study and the phospholipid fraction of Female III tended to decrease as the spawning-season progressed, this data is not indicative of a late-season deficiency in EFA in the female turbot.

The percentage concentrations of other fatty acids and the percentage egg dried-weights remained relatively constant throughout the season.

Nonetheless all these observations must be heavily qualified by the fact that minimal sample numbers were used in this study. A more comprehensive sampling and

analysis programme may well prove the variations to be circumstantial. It is also worth emphasising that even if later-spawned eggs do have reduced levels of polyunsaturated, long-chained phospholipids they may not be sufficiently reduced to cause detrimental effects to the egg-membranes. Indeed, the results of experiments comparing growth and survival of larvae hatched from early- and late-spawned eggs stripped from the same females used in this GLC analysis suggest that larvae hatching from later eggs perform just as well in the laboratory as do early-season larvae (see Section I). Although female turbot stop feeding for up to 8 weeks during their spawning season, they feed quite heavily in the months prior to spawning (see Fig. 3) so it is quite probable that they have sufficient EFA reserves to prevent deficiencies in their later eggs. The 6 month old turbot used in Owen et. al.'s (1975) and Cowey et. al.'s (1976a & b) lipid deficiency experiments would have had much smaller reserves of EFA at the beginning of their 16 week deficient-diet regime than these 6-7 year old mature females at the onset of spawning.

Footnote:

It was originally planned to compare the fatty acid content of eggs from "wild-caught" females stripped at sea in order to assess the adequacy of Shearwater's broodstock diet of whiting and sprat. However, attempts to obtain "wild" eggs from Port Erin, Lowestoft, and Menai Bridge (Anglesey) failed and so this part of the study was abandoned.

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SECTION III

INTRODUCTION

Although the over-ripening of ovulated eggs inside the lumen of the ovary prior to hand-stripping has long been recognised as a possible cause of poor egg-quality in captive fish (e.g., Jones, 1970; Riley & Thacker, 1969), surprisingly few workers have investigated this phenomenon.

Nomura et. al.(1974) studied the morphological changes which occur in over-ripened eggs of the rainbow trout. They found that eggs retained in the body cavity for 10 days (or longer) after ovulation began to show signs of over-ripening. The yolk of these eggs was semi-transparent and the oil-globules aggregated into a lump. Further investigations revealed the partial collapse of cortical alveoli and formation of a "blastoderm-like structure permeated with oil-globules". This phenomenon affected the activation of the eggs by water-contact since the subsequent collapse of the cortical alveoli was restricted and bipolar differentiation was retarded.

Sakai et. al.(1975) investigated the percentage of eyed eggs, the hatching rate and the incidence of deformed and/or moribund alevins of rainbow trout in relation to the over-ripening process. They found that ova had highest viability when they were stripped 5-7 days after ovulation - that is, before the morphological changes described by Nomura et. al. (1974) occurred.

Hirose et. al.(1977) found a similar pattern with ayu eggs. Here, the percentages of eyed eggs and hatched larvae were greatest 1 day after ovulation and then decreased rapidly. They suggested that ayu eggs remained in good condition for approximately 48 hours and were completely over-rip-

ened 8 days after ovulation.

It was thought that similar over-ripening processes to those described by the Japanese authors might be causing poor egg-batches in the Shearwater turbot broodstocks and, furthermore, would explain the large variation found in the physical appearances of hand-stripped eggs. A closer investigation of this topic was therefore decided upon. However, in order to study the rate at which ovulated, unstripped eggs over-ripened inside the lumen of the ovary, the precise time at which they had been ovulated needed to be determined. This was attempted in two ways:

1. Preliminary catheterisation experiments.
2. Ovulation-prediction using plots of percentage fertilisation and hatch of eggs stripped from particular females each day.

The appearance of precise ovulatory rhythms in the results obtained from the second technique led to further investigations being made into the following:

3. The possibility of synchronising the ovulatory rhythms of several females via hormone-injections in order to facilitate hand-stripping.
4. Ovulatory rhythms of fish kept in a constant light regime.
5. Whether an individual's ovaries ovulate simultaneously or alternately.
6. Whether ovulatory rhythms continue in naturally-spawning females.

The following sub-sections deal with each of these in turn. Since the earlier investigations prompted later ones,

the results of each individual experiment have been fully discussed before describing the next part of the investigations.

Fig. 14. Catheter for intra-ovarian sampling.

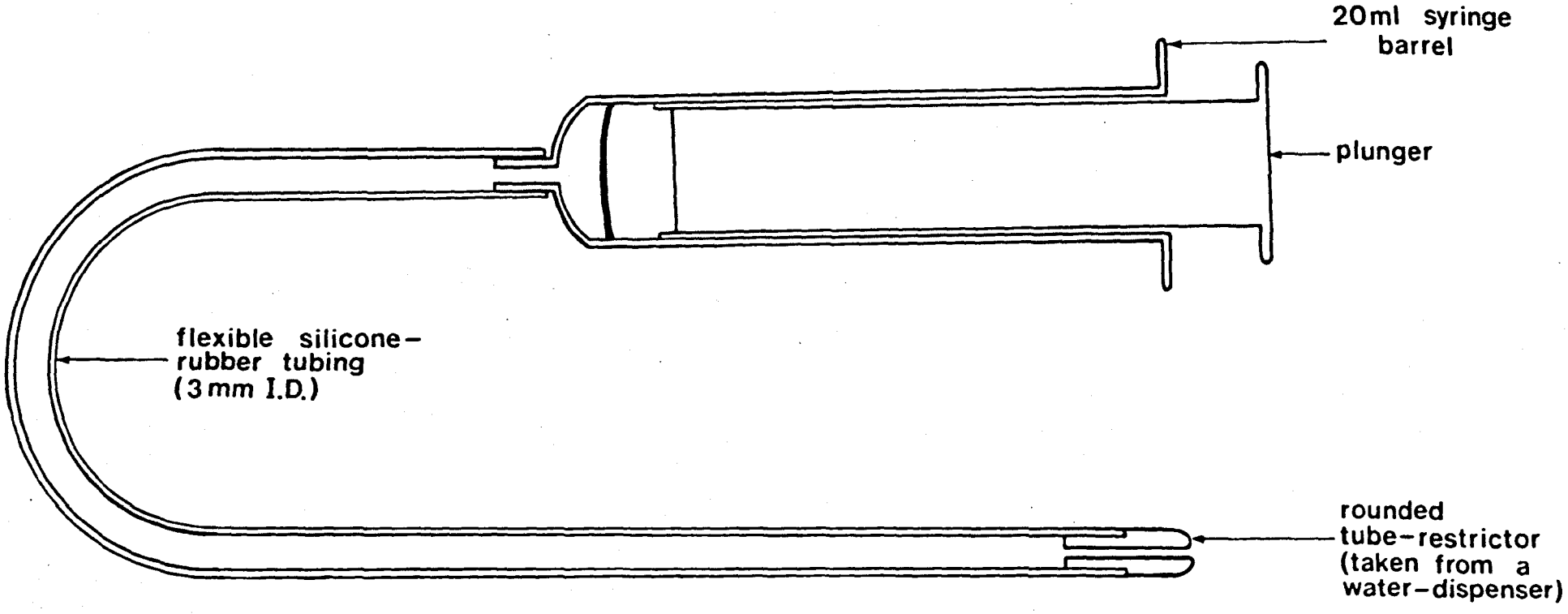

20ml syringe barrel
plunger
flexible silicone-rubber tubing (3mm I.D.)
rounded tube-restrictor (taken from a water-dispenser)

Fig. 14.

1. PRELIMINARY CATHETERISATION EXPERIMENTS:

There are no visible external signs to indicate when a fish has just ovulated. Therefore, it was decided to make *in vivo* observations of the maturation of oocytes by taking regular catheter samples from the ovaries of fish as they approached their spawning period. It was hoped that regular monitoring of oocyte development would give a good indication of when a fish's first ovulation of that season would occur. It was important to catch the first ovulation of the season in order to simplify the study of egg-ageing since, at that point, all the eggs released inside the lumen of the ovary would be of the same age. Later in the season, batches of eggs "overlap" each other and complicate the picture.

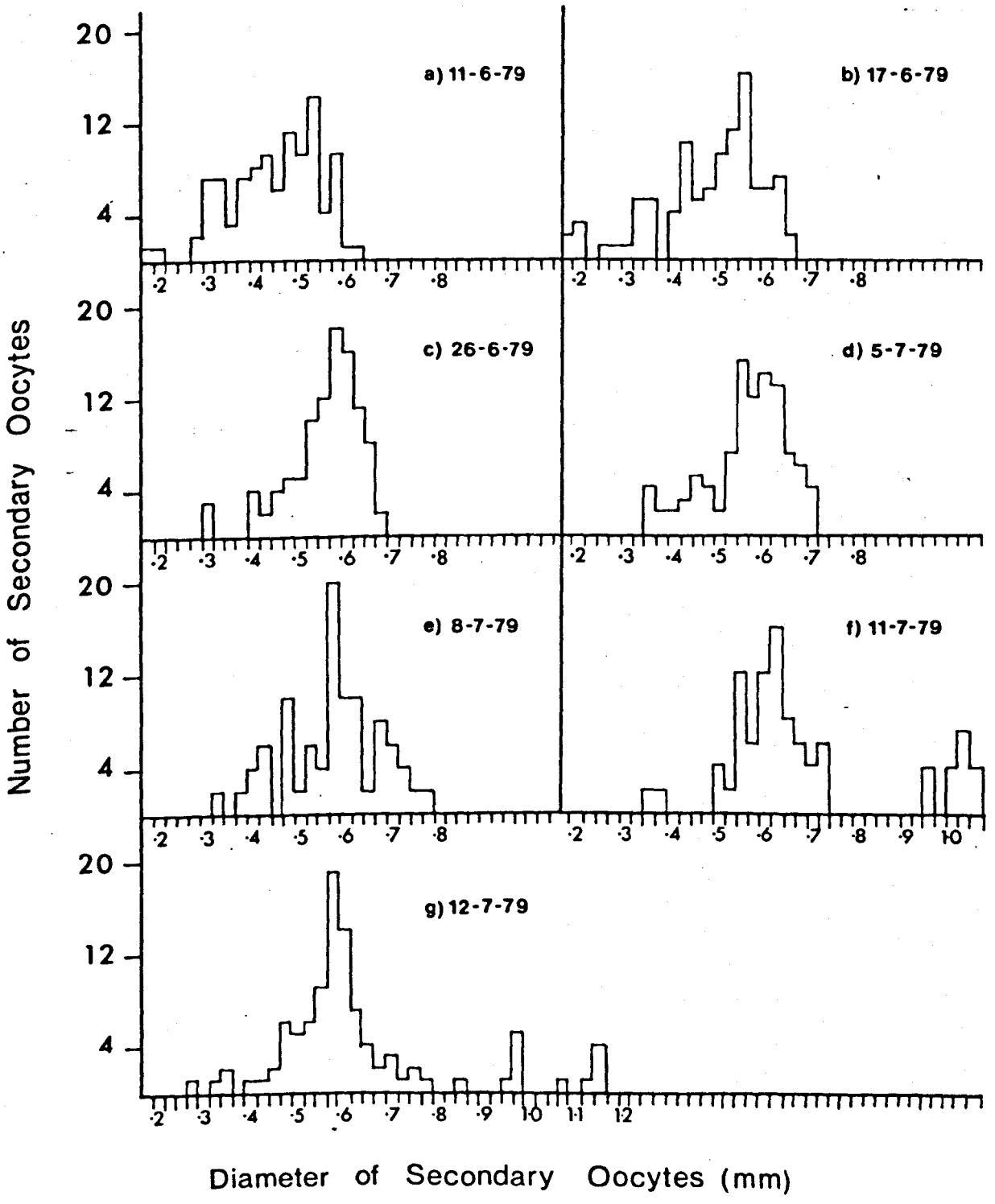
MATERIALS & METHODS

A catheter was made by attaching 40cm of flexible silicone-rubber tubing (3mm internal diameter) to a 20ml disposable syringe barrel. The tube was rounded at its distal end by attaching a tube-restrictor (taken from a water-dispenser) and rubbing both the tube and restrictor with glass-paper until the end of the tube was smooth and rounded (see Fig. 14).

Sampling began approximately three weeks before the expected spawning date of the broodstock, initially occurring once every three days. However, the frequency was increased to once every two days approximately one week before the first expected ovulation. The catheter tube was gently pushed into the oviduct of the female and as far into the lumen of the ovary as possible. Samples were initially taken from

Fig. 15. Changes in the size-frequency distribution of secondary oocytes during the month prior to a female turbot's first ovulation of the season. The sample size in (a) to (g) is 100 oocytes.

Fig. 15.


a) 11-6-79
b) 17-6-79
c) 26-6-79
d) 5-7-79
e) 8-7-79
f) 11-7-79
g) 12-7-79
Number of Secondary Oocytes
Diameter of Secondary Oocytes (mm)

SOME OF THE PRE-OVULATORY STAGES OF TURBOT EGGS.

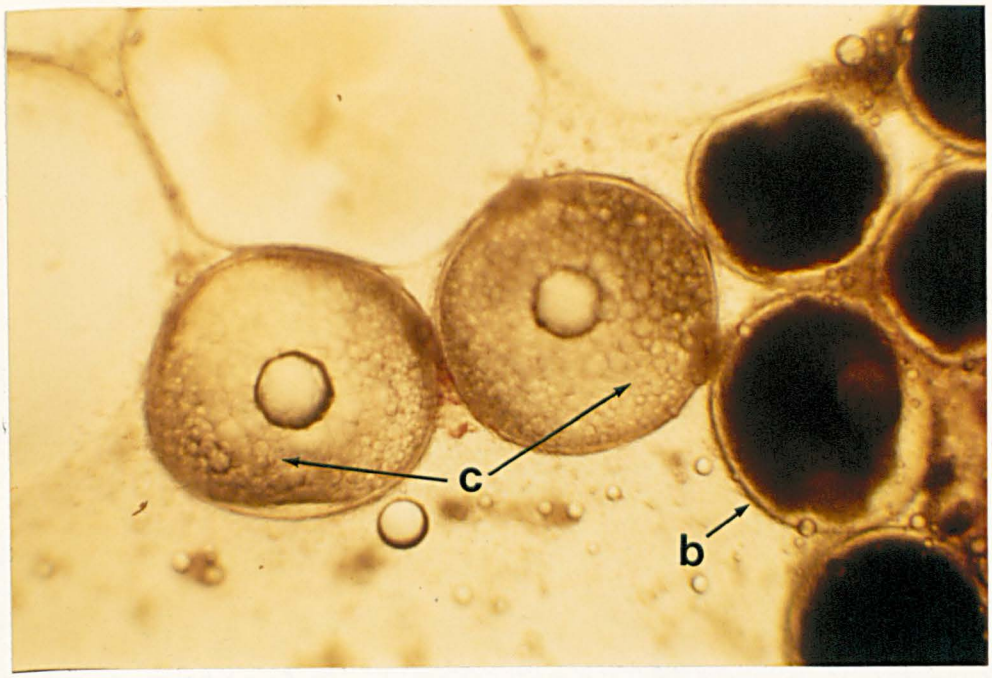


Plate XIII.

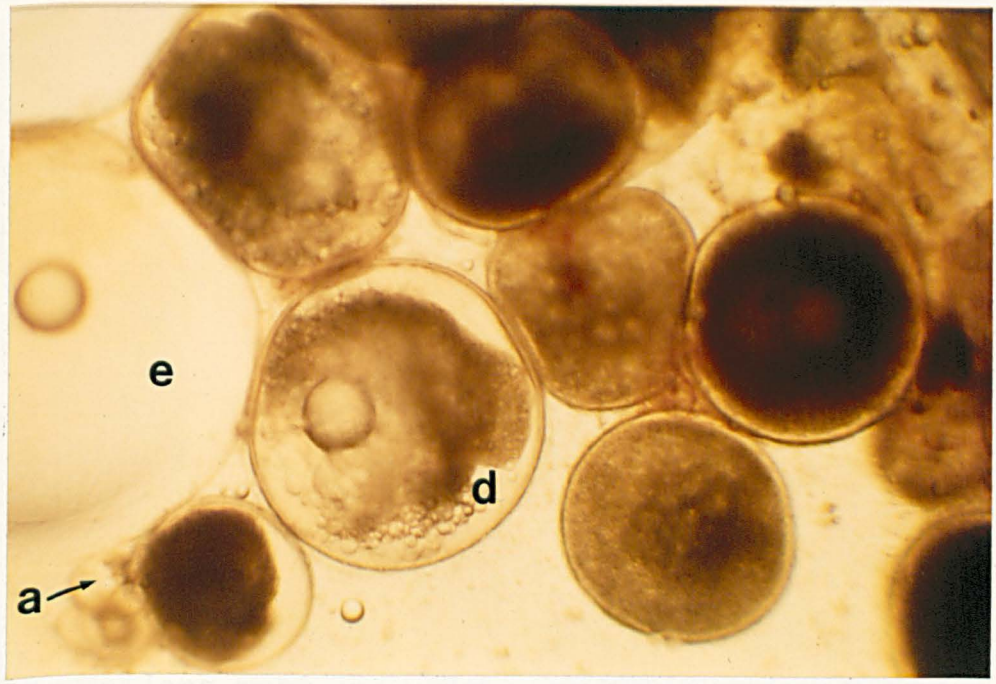


Plate XIV.

a - primary oocyte.

b - secondary oocyte.

c - egg beginning to turn hyaline; oil-droplet formed.

d - egg becoming more hyaline

e - ovulated egg.

the anterior and posterior parts of the ovaries and compared but later, since no difference could be found between anterior and posterior oocyte size-distribution, samples were taken by applying suction whilst slowly drawing the catheter tube back along the length of the ovary. A sample of approximately 2cm^3 volume was taken each time. MS222 was used to anaesthetise the fish for the first sampling procedure but was found to be unnecessary, and so was abandoned in later samplings.

Small sub-samples were taken from the extracted ovary tissue and the oocytes were teased free with seekers. 100 secondary oocytes were measured using an Olympus BHB microscope with a graticuled eyepiece (40 grats. ≡ 1mm). Two diameters were measured for each oocyte and an average taken. A histogram showing the frequency-distribution of secondary oocyte diameter was then drawn for each sampling date. Photographs of oocytes were also taken, using a Fujica camera attached to the microscope, in order to aid later oocyte-comparisons.

RESULTS

Fig. 15 shows the changes in the size-frequency distribution of secondary oocytes during the month prior to one female's first ovulation of the season. (In actual fact, 12 catheterisations were made and histograms plotted - that is, on every third day - but only 7 are shown here because the changes were very gradual). Ovulated eggs were present in the ovary lumen on 11-7-79. By 12-7-79 some had already begun to over-ripen, becoming large and misshapen. Plates XIII & XIV show the appearance of oocytes at different stages of maturity.

DISCUSSION

It proved very difficult to use these histograms and photographs to predict the time of a fish's next ovulation, particularly as it was feared that sampling more than once a day might seriously damage the female's ovary. These experiments were therefore abandoned in favour of the second method of ovulation-prediction (see following sub-section) which proved to be far more successful.

2. OVULATION-PREDICTION USING PLOTS OF PERCENTAGE FERTILISATION AND HATCH AGAINST TIME:

MATERIALS & METHODS

A female which was very close to her first ovulation of the season was carefully checked every day to see whether hyaline eggs could be hand-stripped by gentle pressure over her ovary. (The female had not been subjected to catheterisation). When it was possible to obtain eggs by stripping, freshly-stripped milt was added immediately. After at least 3.5 hours, a small sample of eggs were taken from the floating, surface-layer of eggs. Two lots of 400 eggs each were counted out from each sample, examined under an Olympus BHB microscope and the number of dividing eggs was noted. This figure was used to compute the percentage fertilisation of the egg-batch.

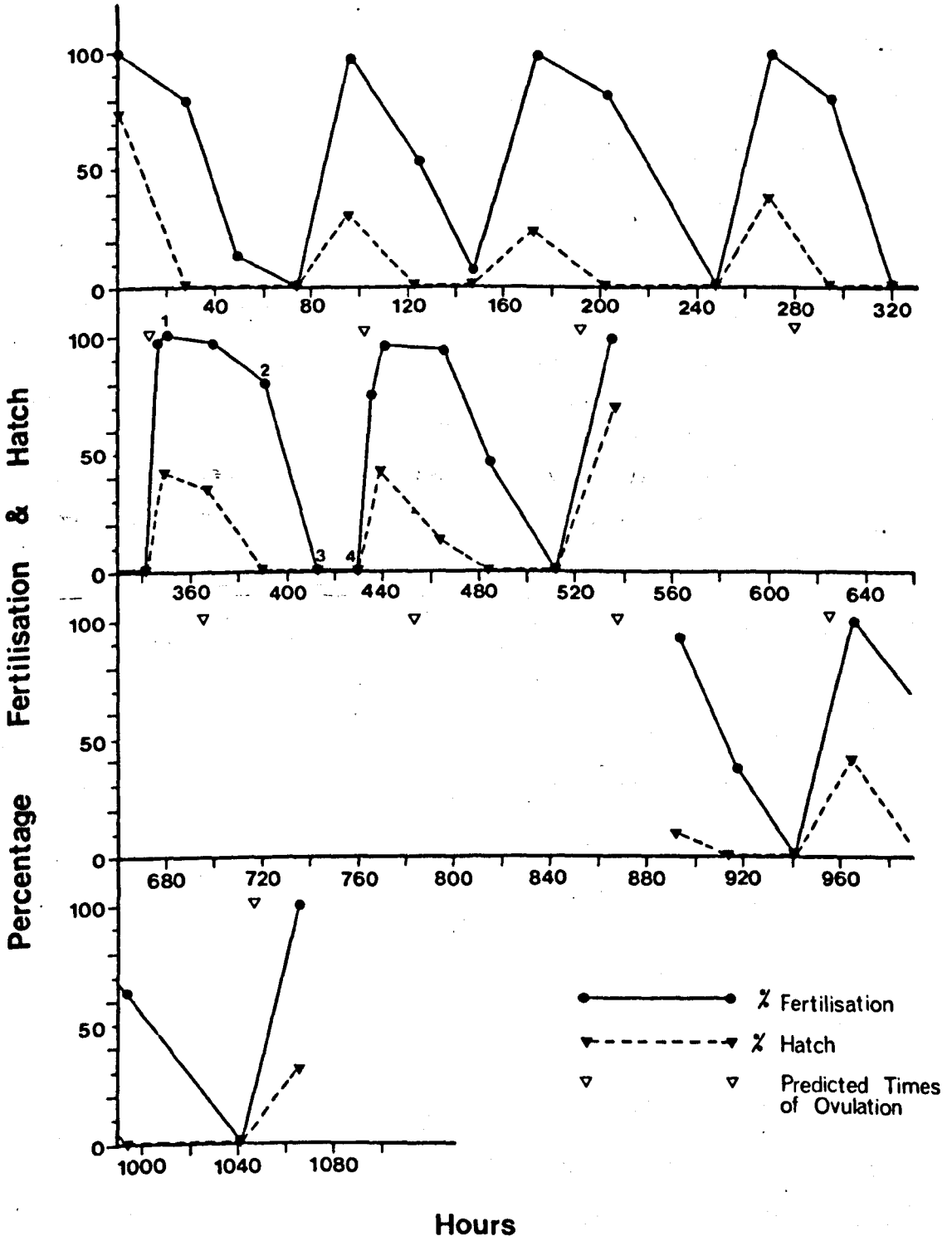
The two sets of 400 eggs were then incubated at 12.5°C in 33cm x 23cm x 21cm plastic tanks containing static seawater with streptomycin sulphate and sodium penicillin at 50ppm. until they hatched on Day 6. When hatching was completed (Day 7) the number of hatched larvae in the two tanks were counted, a mean taken, and this figure used to compute the percentage hatch of the egg-batch.

The female was stripped every day, and the percentage fertilisation and percentage hatch of each egg-batch were plotted against time - zero hour being the time at which the first lot of eggs were stripped from the female. The same male was used to inseminate each egg-batch.

This procedure was continued for two weeks (the fish being stripped at a different time each day to prevent en-

Fig. 16. An ovulation-prediction chart for turbot female, "Black Fleck". The percentage fertilisations and hatches were computed from duplicate samples of 400 eggs. Each point on the graph therefore represents a mean of duplicates. A sudden rise in percentage fertilisation and hatch indicates the presence of freshly-ovulated eggs. The numbers 1 to 4 inserted over the 5th. ovulatory peak correspond to Plates XV to XVIII. Zero on the x axis simply indicates the start of the experiment. See text for full details of the ovulation-prediction method.

Fig.16. Ovulation-Prediction Chart.



trainment) by which time an ovulatory "pattern" emerged from the results (see Fig. 16). An increase in percentage fertilisation to greater than 90% was taken to indicate a freshly-ovulated batch of eggs, and an average time-interval between the first four ovulations depicted in Fig. 16 was estimated. This estimated time-interval was used to predict the approximate time the next ovulation would occur and the fish was stripped every hour over a 3 hour period which encompassed the expected time of ovulation.

This was repeated for one further ovulation and then the female was left for 2.5 weeks, only being stripped twice a week at the same time as the other spawning females. After this time, daily stripping was again resumed to see if the female had retained the same time-interval between ovulations.

This daily stripping procedure was repeated for 15 other females from 3 different broodstocks. Percentage fertilisation and percentage hatch were measured as before but the sample sizes were reduced to two lots of 200 eggs and these were incubated in smaller plastic containers (400ml capacity) due to space-limitations in the egg-incubation room.

RESULTS

Fig. 16 shows the "pattern" obtained by plotting the percentage fertilisation and the percentage hatch of egg-batches stripped from one particular female and fertilised by the same male. The plot demonstrates a clear-cut ovulatory rhythm. The "ovulatory period" (i.e., the time interval between two ovulations) was computed to be 86.5 hours by tak-

TIME OF STRIPPING (hours)	% FERTILISATION	BATCH-WEIGHT (g)
342 (1:00p.m.)	0.00	10
346 (5:00p.m.)	87.80	7
347 (6:00p.m.)	76.00	20
349 (8:00p.m.)	99.75	100

Table 11a. Results of sampling over the 5th ovulation monitored in Fig. 16.

TIME OF STRIPPING (hours)	% FERTILISATION	BATCH-WEIGHT (g)
431.5 (6:30a.m.)	0.00	5
435 (10:00a.m.)	76.70	40
440 (3:00p.m.)	97.25	92

Table 11b. Results of sampling over the 6th ovulation monitored in Fig. 16 (see text for details).

THE APPEARANCE OF EGGS AT DIFFERENT STAGES OF OVER-RIPENING WITHIN THE LUMEN OF THE OVARY: Plates XV - XVIII correspond to the numbers 1-4 inserted over the 5th. ovulatory peak in Fig. 16.

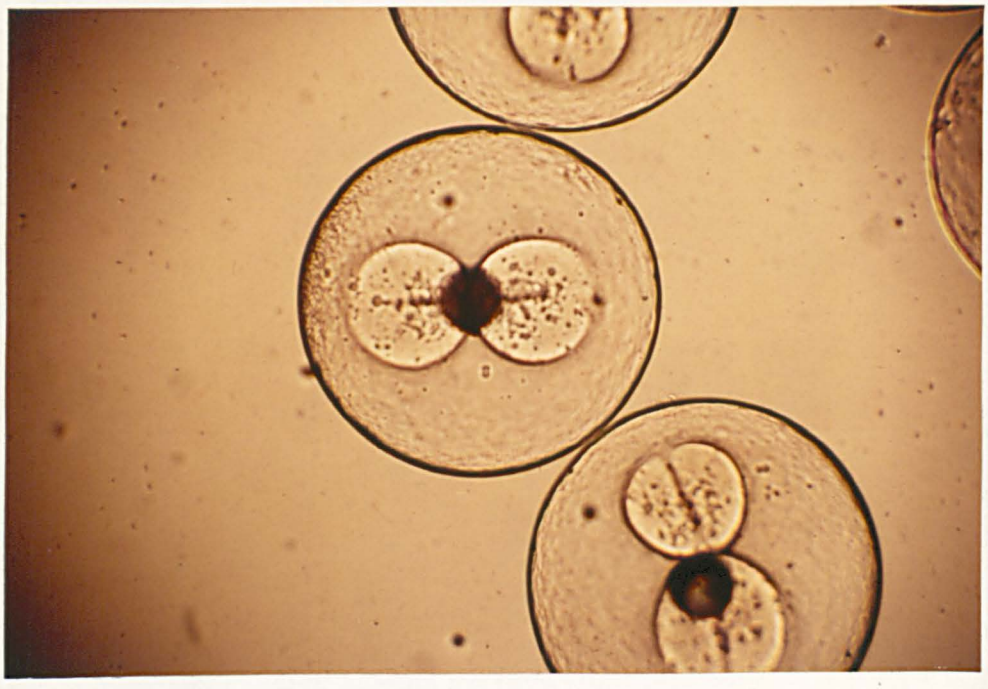


Plate XV. Good quality eggs stripped approximately 3 h from the start of ovulation.

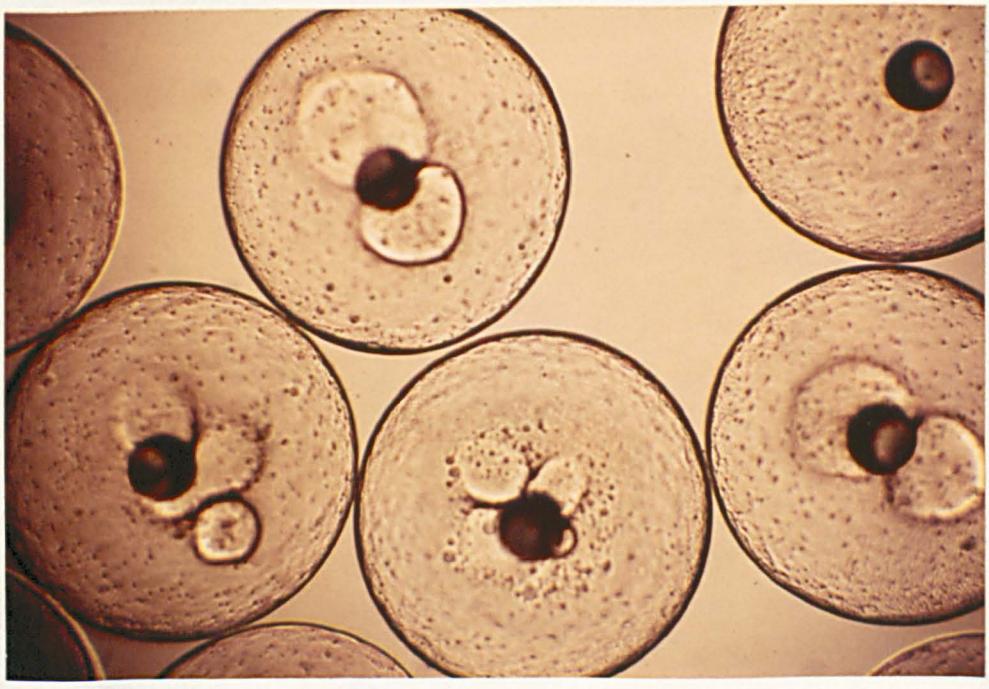


Plate XVI. Poor quality eggs stripped approximately 45 h from the start of ovulation.

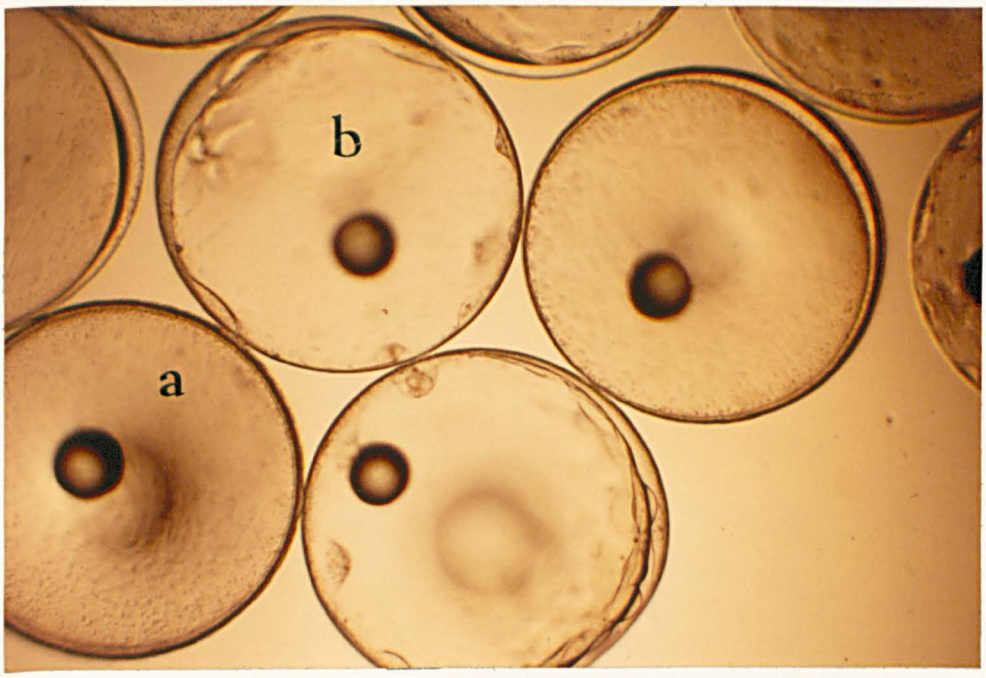


Plate XVII. Infertile eggs stripped approximately 67 h from the start of ovulation.

a - "spotted, infertile" egg(s.i.). **b** - "dimpled" egg.

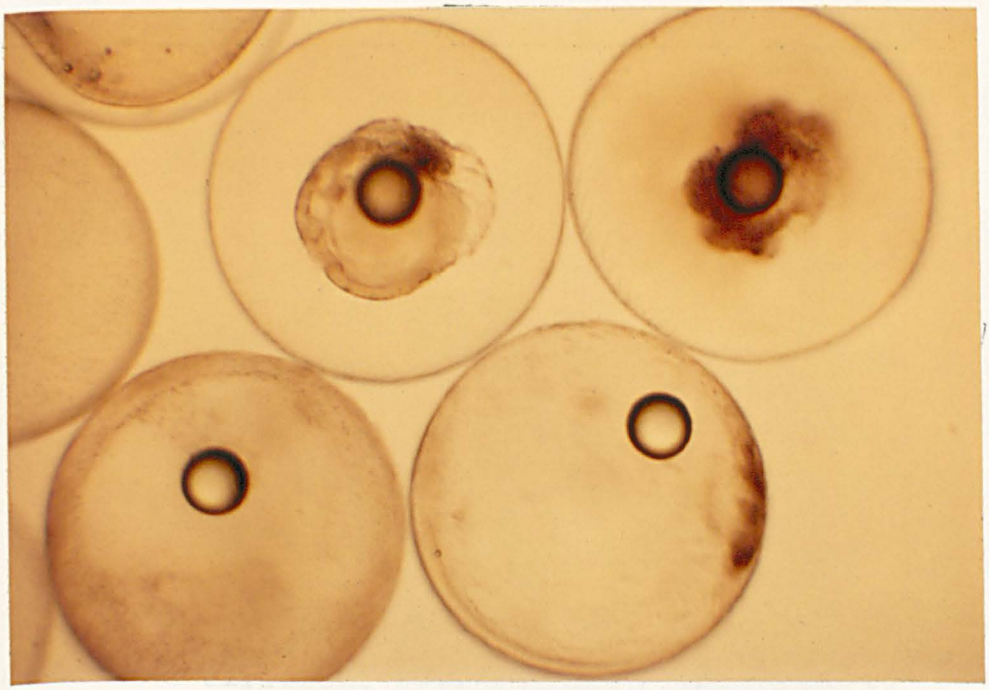


Plate XVIII. Sunken, infertile eggs stripped approximately 85 h from the start of ovulation.

ing a mean of the intervals between the first four ovulations shown in Fig. 16. The time of the fifth ovulation was thus predicted to be 346 hours from the start of the experiment (i.e., 5p.m. of the 14th. day).The female was therefore stripped at 342h (1p.m.), 346h (5p.m.), 347h (6p.m.), and 349h (8p.m.). The percentage fertilisations and weights of the different egg-lots are shown in Table 11a.

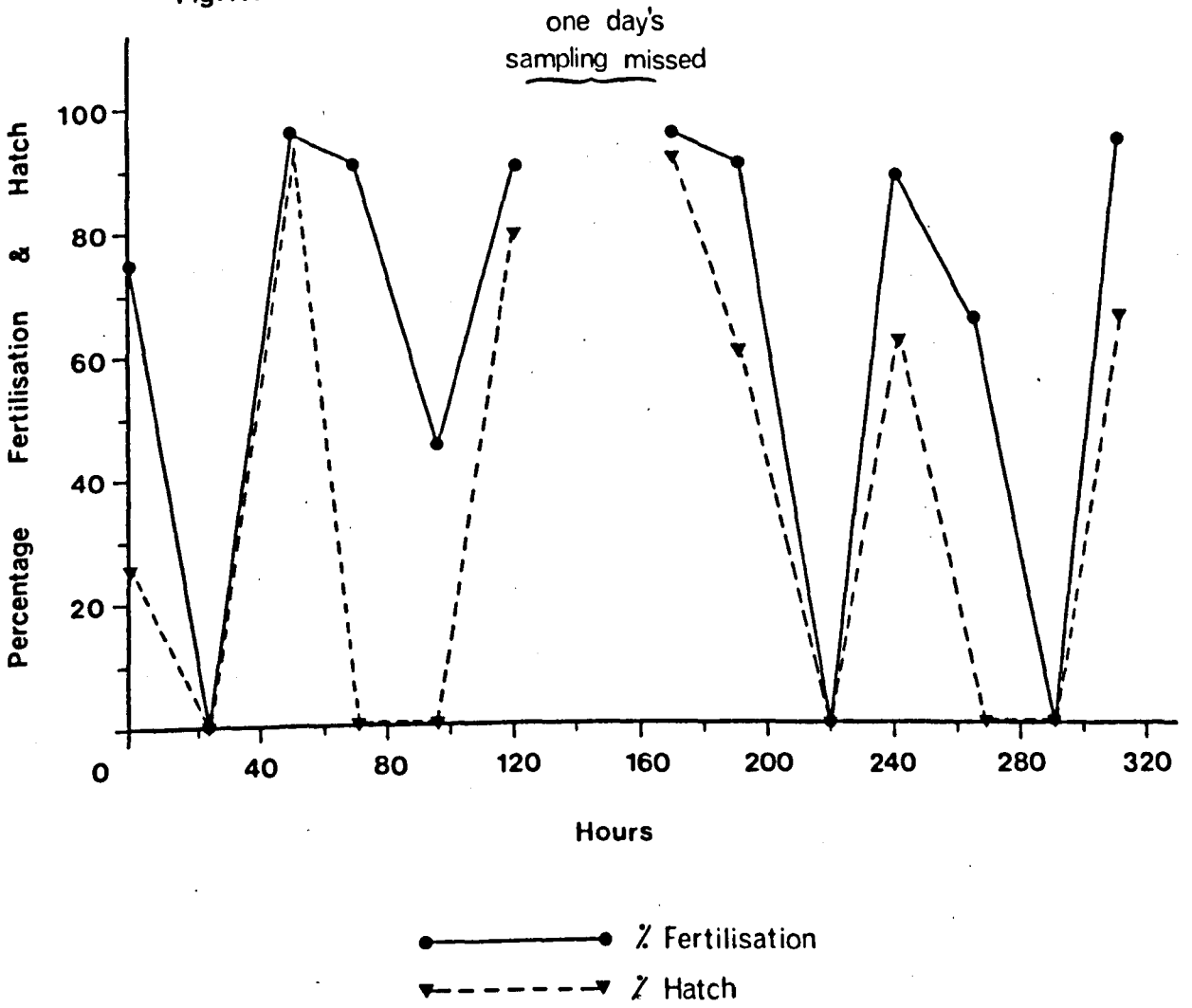
Similarly, the sixth ovulation was predicted to occur at 432.5 hours from the start of the experiment (i.e., 7a.m. of the 18th. day). The female was again stripped over her expected ovulation time and the percentage fertilisations and weights of the egg-lots are shown in Table 11b. From these results, it would appear that it takes approximately 4-6 hours for a complete batch of turbot eggs to be ovulated.

When daily stripping was resumed after subjecting the female to the more usual twice-weekly stripping programme for a fortnight (the eggs simply being stripped and discarded to prevent severe overcrowding of the ovary), she was still found to have the same ovulatory period as at the start of the experiment 5.5 weeks before.

In addition to the female having a constant ovulatory period, the eggs themselves showed a relatively constant rate of ageing. This meant that eggs stripped at any time during the ovulatory cycle could be aged to the nearest 24 hours, thus giving an estimate of when the female last ovulated. Since the ovulatory period of the fish was known, the next ovulation-time could then be predicted. Plates XV to XVIII show these different over-ripening stages of turbot eggs. The photographs correspond to the numbers 1-4 inserted over the fifth ovulation peak in Fig. 16. This rate of

Fig. 17. An ovulation-prediction chart for Female "VCV". The percentage fertilisations and hatches were computed from duplicate samples of 200 eggs. Each point on the graph therefore represents a mean of duplicates. A sudden rise in percentage fertilisation and hatch indicates the presence of freshly-ovulated eggs. Zero on the x axis simply indicates the start of the experiment.

Fig.17.


one day's
sampling missed
Percentage Fertilisation & Hatch
100
80
60
40
20
0
40
80
120
160
200
240
280
320
Hours
% Fertilisation
% Hatch

Figs. 18 & 19. Ovulation-prediction charts for Females LPM (1980) and XXX respectively. The percentage fertilisations and hatches were computed from duplicate samples of 200 eggs. Each point on the graph therefore represents a mean of duplicates. A sudden rise in percentage fertilisation and hatch indicates the presence of freshly-ovulated eggs. Zero on the x axis simply indicates the start of the experiment.

Fig.18.

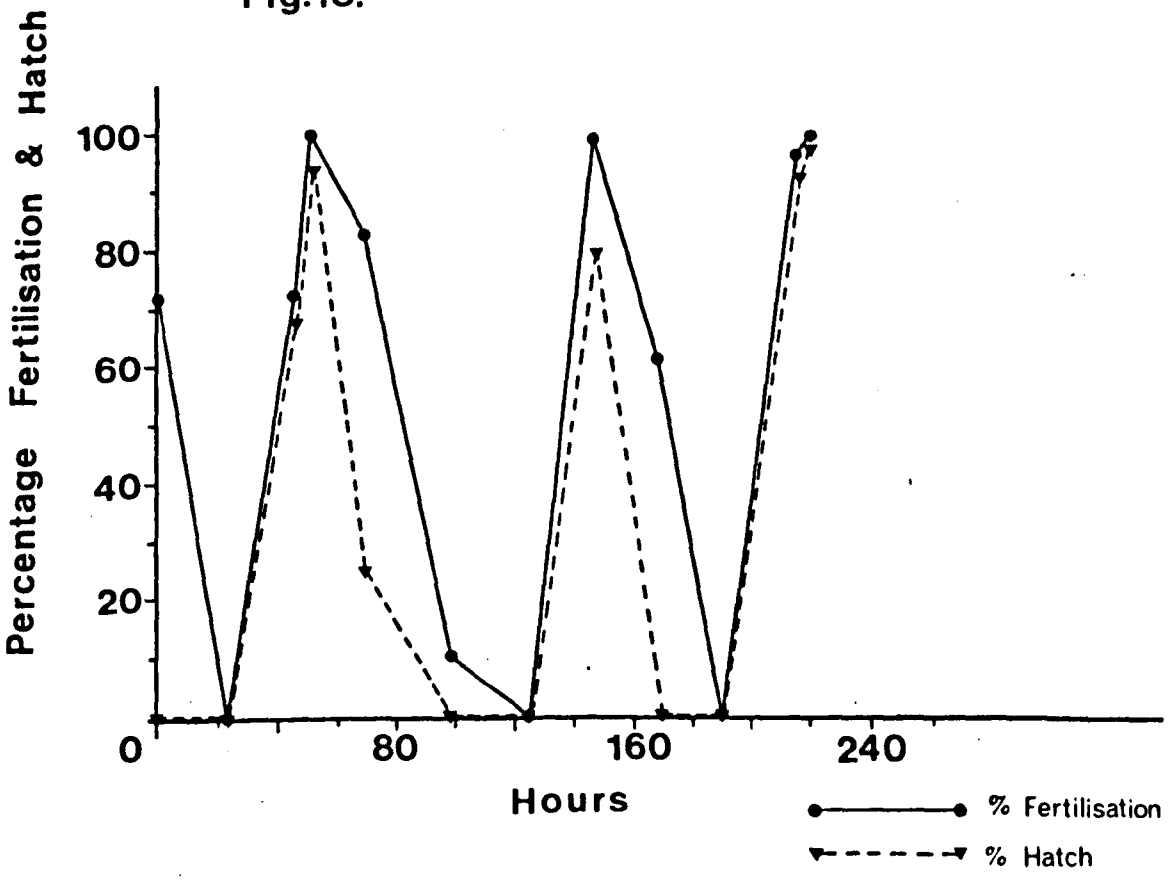

Percentage Fertilisation & Hatch
100
80
60
40
20
0
80
160
240
Hours
% Fertilisation
% Hatch

Fig.19.

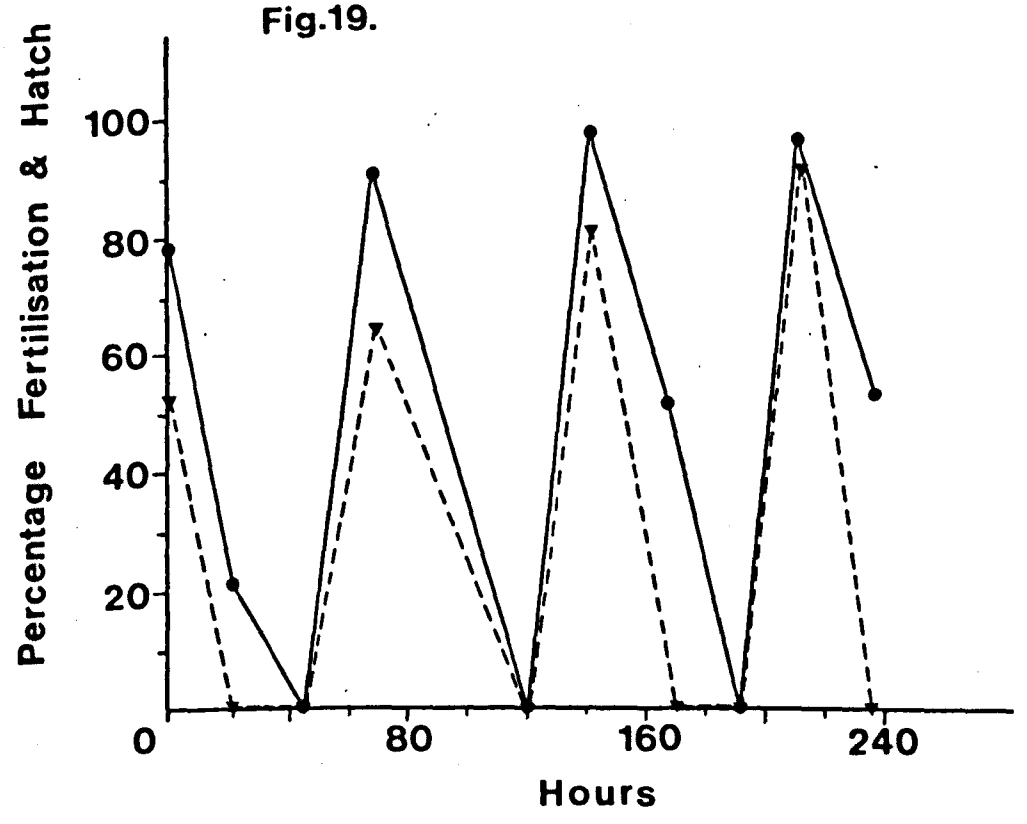

Percentage Fertilisation & Hatch
100
80
60
40
20
0
80
160
240
Hours

Fig. 20. An ovulation-prediction chart for Female "VVV", demonstrating the alternation of long and short ovulatory cycles in this turbot female. The percentage fertilisations and hatches were computed from duplicate samples of 200 eggs. Each point on the graph therefore represents a mean of duplicates. A sudden rise in percentage fertilisation and hatch indicates the presence of freshly-ovulated eggs. Zero on the x axis simply indicates the start of the experiment.

Fig. 20.

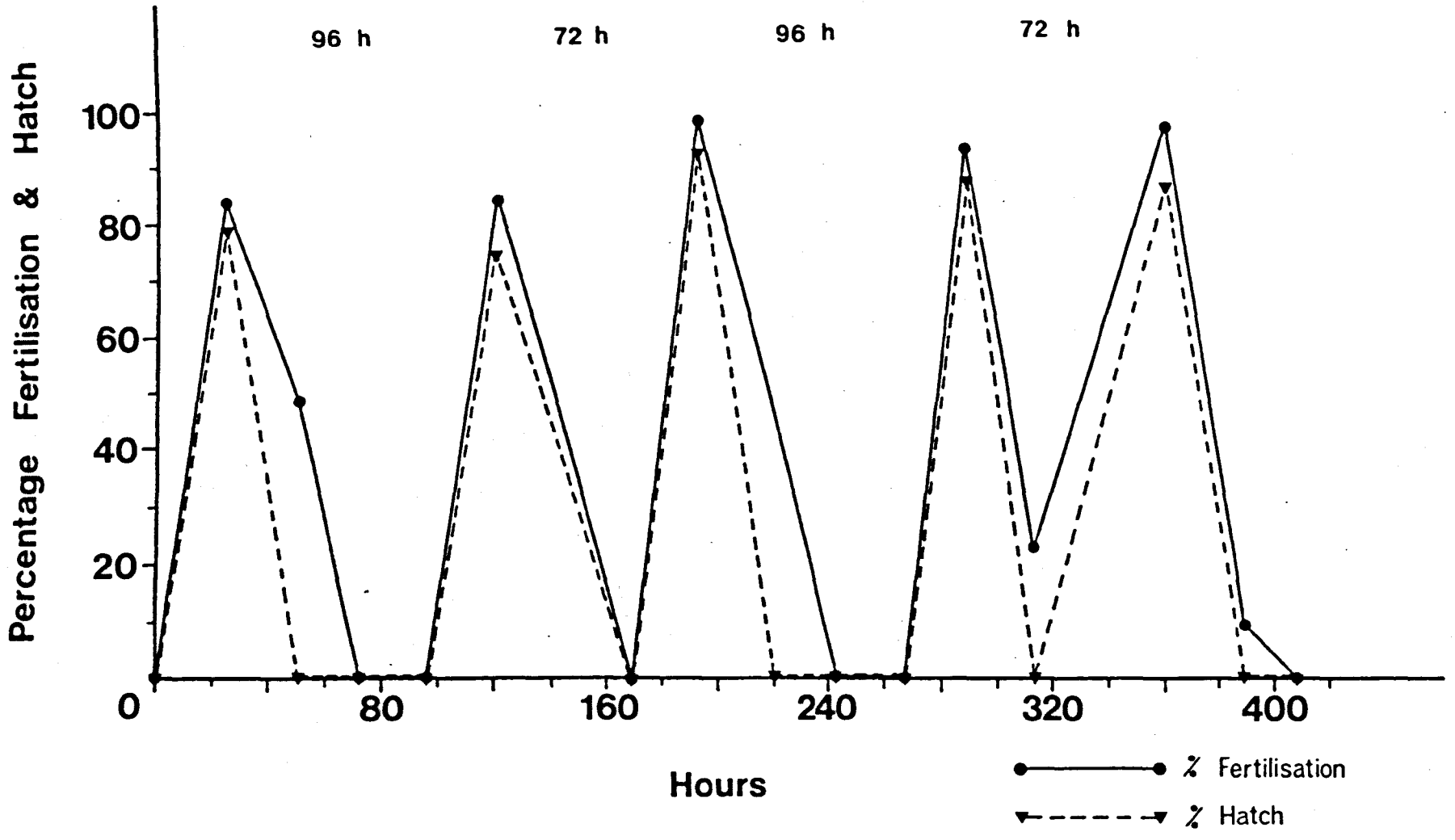
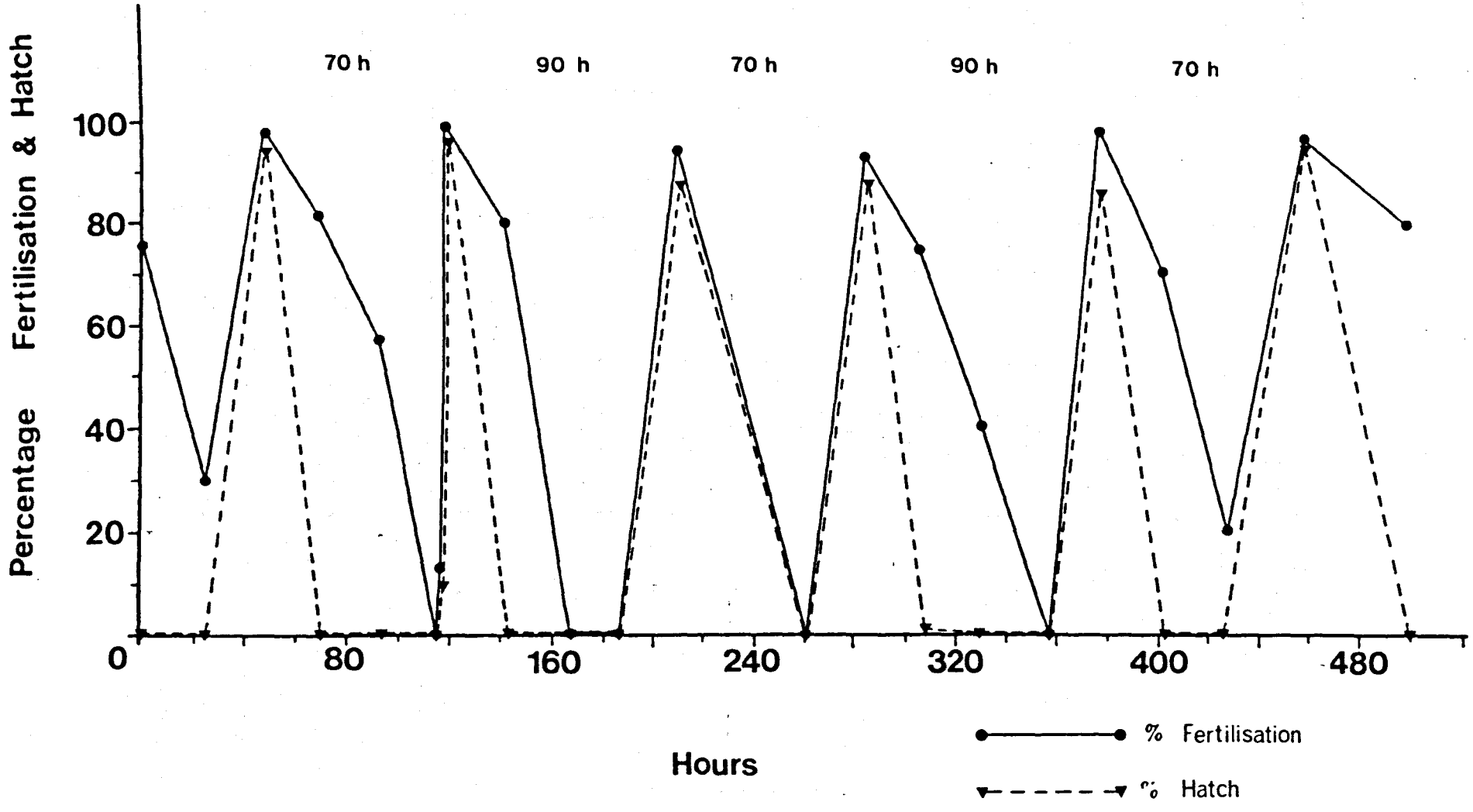

96 h
72 h
96 h
72 h
Percentage Fertilisation & Hatch
100
80
60
40
20
0
80
160
240
320
400
Hours
% Fertilisation
% Hatch

Fig 21. An ovulation-prediction chart for Female "VIV", demonstrating the alternation of long and short ovulatory cycles in this turbot female. The percentage fertilisations and hatches were computed from duplicate samples of 200 eggs. Each point on the graph therefore represents a mean of duplicates. A sudden rise in the percentage fertilisation and hatch indicates the presence of freshly-ovulated eggs. Zero on the x axis simply indicates the start of the experiment.

Fig. 21.


70 h
90 h
70 h
90 h
70 h
Percentage Fertilisation & Hatch
100
80
60
40
20
0
80
160
240
320
400
480
Hours
% Fertilisation
% Hatch

FEMALE	YEAR	WEIGHT (kg)	LENGTH (cm)	OVULATORY PERIOD (h)
XO	1980	2.00	40.0	93 (±2)
OΛ*	1980	2.90	45.6	72/100
XC	1980	3.27	47.5	62 (±2)
XCX	1980	3.50	54.0	90 (±5)
XXX	1980	4.20	50.5	70
II	1980	4.98	55.2	83 (±2)
VVV*	1980	5.30	59.0	96/72
VCV	1980	5.30	61.0	63 (±2)
Black Spot	1980	5.60	58.2	≃70 (2 Ovulations)
VIV*	1980	5.80	58.5	90/70
VCV	1981	6.10	62.5	67 (±2)
CO	1980	6.20	59.3	85 (±3)
VVI	1980	6.50	61.5	60 (±4)
LPM	1980	6.70	62.5	85
BWM	1980	6.70	64.0	≃92 (2 Ovulations)
Black Bar	1981	7.00	59.5	85 (±6)
Black Fleck	1980	7.10	62.0	86.5
Black Spot	1981	7.30	60.5	65-70
BWM	1981	7.50	65.5	≃95 (2 Ovulations)
Black Fleck	1981	8.00	62.7	113 (±2)

Table 12. The ovulatory periods and sizes of 16 different turbot females (four females were monitored for two consecutive years). The limits of uncertainty (figures in parentheses) do not necessarily indicate fluctuations in a female's ovulatory period, they merely reflect the frequency of sampling. Asterisks indicate those females with alternating long and short ovulatory periods. Females monitored for two ovulations only are noted.

over-ripening was found to be very similar for eggs from other turbot females.

It is also clear from Fig. 16 that although the percentage fertilisation of eggs can remain quite high (greater than 80%) for 30 to 35 hours after ovulation, the over-ripening process affects the "quality" of the first divisions so that after 18 to 20 hours, very few eggs survive to hatching.

Figs. 17 to 21 show the percentage fertilisation and hatch plots for 5 other turbot females. It can be seen from these figures that although each female has a regular ovulatory rhythm, the ovulatory period varies between individuals. This is further demonstrated in Table 12 which gives the ovulatory periods of 16 females computed from percentage fertilisation and hatch plots in the same manner as described above. (4 females were monitored for 2 consecutive years). No relationship was found between the size of a female and the length of her ovulatory cycle. In addition, the cycles of individual females appeared to vary from year to year, but no firm conclusions could be drawn on this account due to insufficient data.

Figs. 20 & 21 clearly show that a few turbot females have alternately long and short ovulatory periods rather than one constant period. The results of further investigations into this phenomenon are given in sub-section 5.

DISCUSSION

Each of the females monitored closely in this study appeared to have a regular ovulatory rhythm but the time-period between ovulations varied between individuals - the shortest being 60($\pm$ 4) hours and the longest 113($\pm$ 2) hours. However, the ovulatory period of most females seemed to fall between 80

Fig. 22. A demonstration of how Shearwater's twice weekly stripping programme may have given a false impression of some female turbots' breeding value. The fact that most turbot appear to ovulate every 3.5 days during the spawning season resulted in some females being continually stripped when the eggs in the lumens of their ovaries were over-ripe (and were thus labelled as "Bad Breeders"). Conversely, other females were usually stripped just after their ovulations (and were therefore labelled as "Good Breeders").

For further explanation, see text.

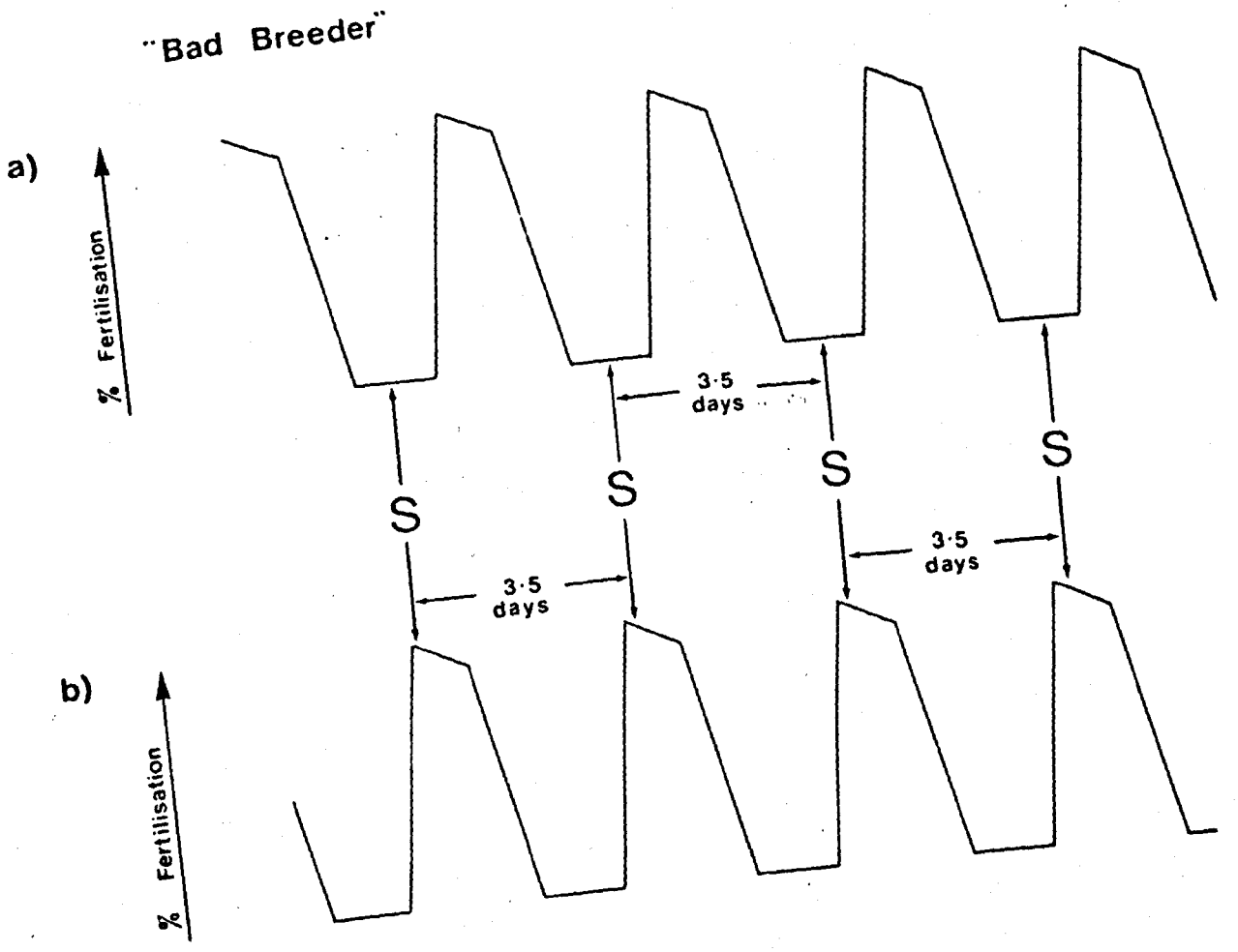

"Bad Breeder"
a)
% Fertilisation
3·5 days
S
S
S
S
3·5 days
3·5 days
b)
% Fertilisation
"Good Breeder"

Fig. 22.

and 90 hours; that is, most females ovulated every 3.5 days.

Since all females used to provide eggs for the hatchery were originally stripped routinely twice a week, this 3.5 day ovulatory period could explain why some females usually gave good eggs, some females always gave infertile eggs, whilst the egg-quality of other females varied considerably. If a female was initially stripped just after her ovulation, it was likely that the next time she was stripped 3-4 days later, her eggs would again be freshly-ovulated and therefore of good quality. If, however, a female's initial stripping was 1.5-2.5 days after her ovulation, not only would all the eggs of that particular batch be infertile but probably the eggs of most of her later batches since the female would continue to be stripped at this time in her cycle (see Fig. 22). Those females whose egg-quality varied considerably probably had ovulatory periods which were greater or shorter than 3.5 days. The twice-weekly stripping programme thereby gave a false impression of some females' breeding-value. This is further confirmed by the fact that fish labelled as "good-breeders" one year did not necessarily perform well the next (and vice versa), and by "bad-breeders" sometimes giving good quality eggs if they were stripped outside the usual twice-weekly routine.

Therefore, in order to genetically select female turbot broodstock, it is necessary to compare the different percentage hatches obtained from the females' freshly-ovulated egg-batches (e.g., female "Black Fleck" did not usually give a hatch of greater than 50%, whereas female "VIV" always gave very good hatches - see Figs. 16 & 21 respectively). It is useless to select females by comparing eggs at different stages of over-ripening.

FEMALE	TOTAL EGG WEIGHT (g)	NUMBER AT HATCH $\times 10^{-3}$	% FROM 1st. DIVISION	% FROM TOTAL	
VVI	1513	302	31.0	20.0	ROUTINE TWICE-WEEKLY STRIPPING REGIME (1979)
Black Spot	All Dead	---	---	---	
Black Bar	2960	270	16.0	9.0	
LPM	2244	108	13.5	5.0	
101	1444	305	34.9	21.0	
VVI	904	630	86.0	69.5	STRIPPING USING OVULATION PREDICTION CHARTS (1980)
Black Spot	521	145	33.5	28.0	
Black Bar	1346	185	25.0	13.5	
LPM	618	125	32.5	20.0	
101	783	142	25.5	18.0	

Table 13. Comparison of the 1979 and 1980 egg and yolk-sac larval production figures of 5 female turbot, all from Brood-stock 1. (Figures computed by Mr. N. Fullerton, Shear-water Fish Farming Ltd..)

Ovulation-prediction by plotting quality of eggs' first division against time (see sub-section 4 for further details) has already proved useful in general hatchery procedure. Although females need to be stripped frequently at first in order to determine their ovulatory period, this method ultimately saves time because the eggs and larvae obtained are usually of better viability. In addition, because of the higher percentage hatches obtained from freshly-ovulated eggs, sufficient larvae are obtained by stripping only 3 or 4 females at any one time, rather than routinely stripping all the females twice a week. Usually 3 or 4 females are monitored closely for a few weeks, then a different group of females is chosen for the next few weeks, and so on until the end of the spawning season. This helps to alleviate the stressing of females by continual stripping.

The advantages of the ovulation-prediction stripping-method is reflected in Table 13 which compares the 1979 and 1980 egg and yolk-sac larval production figures of 5 female turbot. (Figures computed by Mr. N. Fullerton, Shearwater Fish Farming Ltd..) In 1979 a routine twice-weekly stripping programme was used, so large quantities of eggs were collected. However, many egg-batches gave low percentage hatches. In 1980, ovulations were predicted in the manner described above so these 5 females were subjected to stripping for a shorter period of time (note the lower total egg-weights) but, in general, both the percentage hatch and actual number of yolk-sac larvae were higher than those in 1979.

Fewer female broodstock are required when ovulation-prediction methods are employed, but it is helpful to increase the number of male fish in the tank to ensure that suff-

icient milt is available whenever one of the monitored females ovulates fresh eggs.

3. ATTEMPTS TO SYNCHRONISE THE OVULATORY-RHYTHMS OF SEVERAL FEMALES BY HORMONE INJECTIONS:

Once it became apparent that turbot females showed precise ovulatory rhythms, it was decided to investigate the feasibility of synchronising the ovulations of several fish by the administration of hormone injections. Having several females ovulating in synchrony would reduce both the number of man-hours spent hand-stripping fish and the amount of disturbance and stress to which the broodstocks would be subjected, since the fish could all be stripped at the same time on the same day rather than on several different days. It would also facilitate the stocking of Shearwater's large 10,000 l larval-rearing tanks with larvae of the same age. Obviously, synchronisation would only be of commercial advantage if the hormones used were effective, cheap and easily administered. Bearing this in mind, a primer dose of salmon pituitary extract followed by injections of either a crude preparation of 17α-hydroxy 20β dihydroprogesterone(17αOH 20βP) or 17α-hydroxyprogesterone(17α OH P) were decided upon for the following reasons.

17αOH 20βP has been shown to be highly effective in inducing oocyte maturation and ovulation in trout, both *in vitro* (Fostier *et. al.*, 1973) and *in vivo* (Jalabert *et. al.*, 1976), and carp (Jalabert *et. al.*, 1977), and in causing *in vitro* oocyte maturation in northern pike and goldfish (Jalabert, 1976). It also has some maturation-inducing activity in winter flounder (*Pseudopleuronectes americanus*) oocytes incubated in plasma (Campbell, 1975). Similarly, the *in vitro* studies of Goetz and Theofan (1979) led them to suggest that 17α OH 20βP may stimulate natural germinal vesicle migration and

breakdown, and ovulation of yellow perch oocytes. Schmidt and Idler (1962) found both 17α OH 20β P and 17α OH P to be present in high concentrations in mature and postspawned Oncorhynchus nerka. However, Campbell et. al.(1976) have demonstrated that plasma concentrations of 17α OH P rise from 3.5ug/100ml in pre-mature winter flounder females to 53(.26)µg/100ml in maturing fish, whereas the concentration of 17α OH 20β P does not increase. They therefore suggest that, in this species, 17α OH P may be involved in oocyte maturation rather than 17α OH 20β P. Taking all of this into consideration, it was decided to test the effects of both steroids on oocyte maturation and ovulation in turbot.

Although 17α OH P is available cheaply, pure 17α OH 20β P is extremely expensive and its use could not be justified in this experiment. It was therefore decided to follow Jalabert et. al.'s (1977) example and make a crude preparation (approximately 70%) of 17α OH 20β P from 17α OH P using methods described by Norymberski and Woods (1955) and Smith et. al.(1962).

A priming dose of gonadotropin was used because previous workers have shown that this procedure greatly increased the efficiency of the subsequent steroid injection (e.g., Jalabert et. al., 1977). Although the gonadotropin used (i.e. salmon pituitary extract) was costly, only very small amounts were used for "priming" and so the expense was not great.

It must be noted that when this experiment was attempted, ovulation-prediction charts had been drawn for only 5 fish. The ovulation periods of all these females had fallen between 80 and 90 hours. It was therefore postulated at this point that ovulatory periods were very similar for all indiv-

iduals. That is, if the initial ovulations were synchronised, the ovulatory rhythms of the females would remain reasonably "in phase" for several weeks. After studying a greater number of fish's rhythms, it was realised that this was untrue. Unfortunately, it had not been possible to postpone the hormone-experiment until the "constant ovulatory period" hypothesis had been tested because this was the only time of year at which there were sufficient numbers of mature female turbot to make the trials statistically valid.

MATERIALS & METHODS

Experimental Fish:

46 fish (34 females; 12 males) that were known to have spawned during the summer of 1979 were transported by air from the Shearwater on-growing site at Wylfa, Northern Anglesey to the Isle of Man hatchery in January, 1980. The fish were transported in large polythene bags one-third full of seawater. These were sealed with metal tags after oxygen had been "injected" into the water and into the air-space above.

On arrival at the hatchery, the fish were put into a 7.6m x 3.8m rectangular metal tank that already contained an established summer-spawning broodstock consisting of 20 fish. The two broodstocks were kept separate by a mesh partition constructed across the width of the tank. All fish were subjected to a light/dark regime of increasing photoperiod which was scheduled to induce spawning in June of that year.

The transported fish were allowed to settle down undisturbed except for feeding and cleaning until April when they were freeze-branded for identification purposes, sexed,

weighed and measured.

It was surprising that most of these fish were small - the majority being of 2-3kg weight and 40-45cm length - since all of them were at least 3 years old. However, this was fortuitous as far as the hormone experiment was concerned since they required smaller doses of hormones and were less commercially valuable than the other large broodstock females at the Port Erin hatchery which had well-established spawning records.

Injected Material:

Injections were made intraperitoneally and the doses were adjusted so that each fish received 1ml of injected material per kg of fish body weight.

The salmon pituitary extract was made from acetone-dried pituitary powder (International Enzymes Ltd., Syndel Laboratories) in the following manner. The required amount of pituitary powder was homogenised in 15-30 volumes (w/v) of physiological saline (8‰ NaCl) using a glass-teflon homogeniser. This extract was centrifuged at 14,000rpm for 15 minutes, and the resultant pellet discarded. Additional saline solution was added to the supernatant to give an extract concentration equivalent to 4mg of pituitary powder per ml of physiological saline. Fresh extract was made for each day of use.

The crude 17αOH 20βP was prepared by the same method used by Jalabert et. al.(1977); that is, by selective reduction of 17αOH P's 20-ketone groups with sodium borohydride in methanol-dimethylformamide (Norymberski & Woods, 1955; Smith et. al., 1962). This procedure was most kindly performed by Dr. Andrew Grimm of Bangor, U.C.N.W.. The crude

17αOH 20βP contained approximately 30% of 17αOH P and over-reduced derivatives. The 17αOH P was purchased from Sigma.

Both steroids were dissolved in pure ethanol and then, just before injection, they were precipitated as a suspension in 8‰ saline so that there was 20% ethanol in the injected product (Jalabert et. al., 1977).

Determination of Stage of Oocyte-Maturation:

Oocyte-maturation stages of the female fish were determined both by external examination and catheter sampling. The scale of external development used was as follows:

0 - no development.

1 - ovary beginning to swell.

2 - ovary swollen; vent red.

3 - ovary very swollen; spawning imminent.

4 - "running" with hyaline eggs.

Ovary samples were taken using a catheter as in sub-section 1, and the oocytes were "cleared" with a 1:1 (v/v) mixture of methylated spirits and acetic acid. This treatment made the nuclei visible under the microscope so permitting the oocyte development stage to be assessed using a similar scheme to Jalabert et. al.(1977):

A - nucleus at the centre of oocyte.

B - migrating nucleus.

C - nucleus at periphery of oocyte.

D - mature oocyte; after germinal vesicle breakdown, before ovulation.

Plates XIX to XXII show some of these maturity stages.

The samples had to be dealt with immediately after adding the clearing solution since its action continued, eventually making the nuclei difficult to see. Therefore, sev-

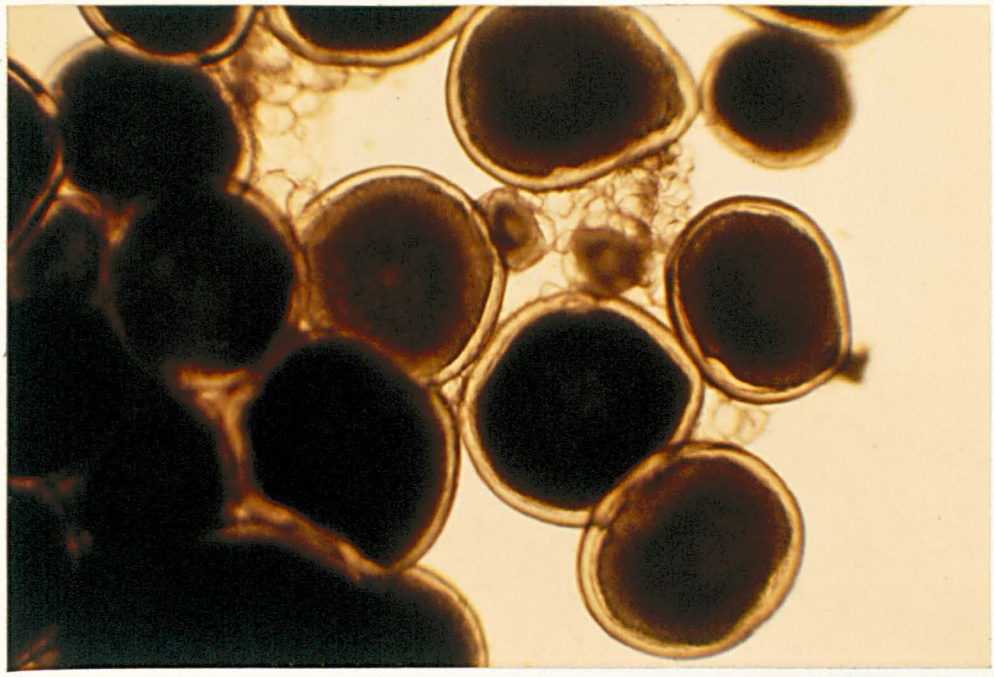


Plate XIX. Stage A oocytes, i.e. centred nuclei.

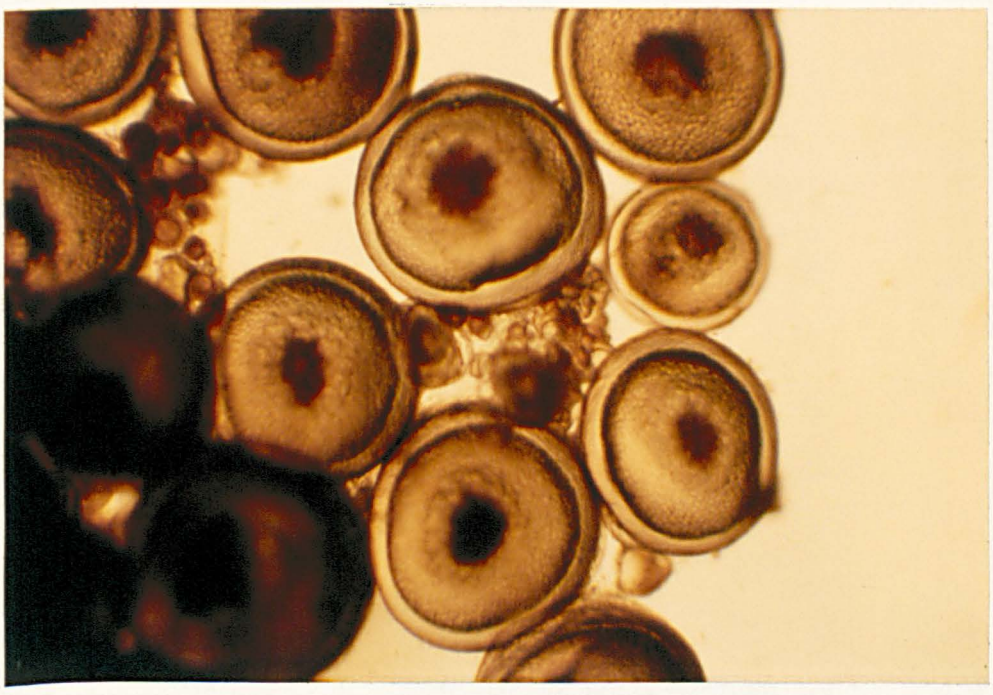


Plate XX. The same oocytes as above "cleared" with methylated spirits/acetic acid.

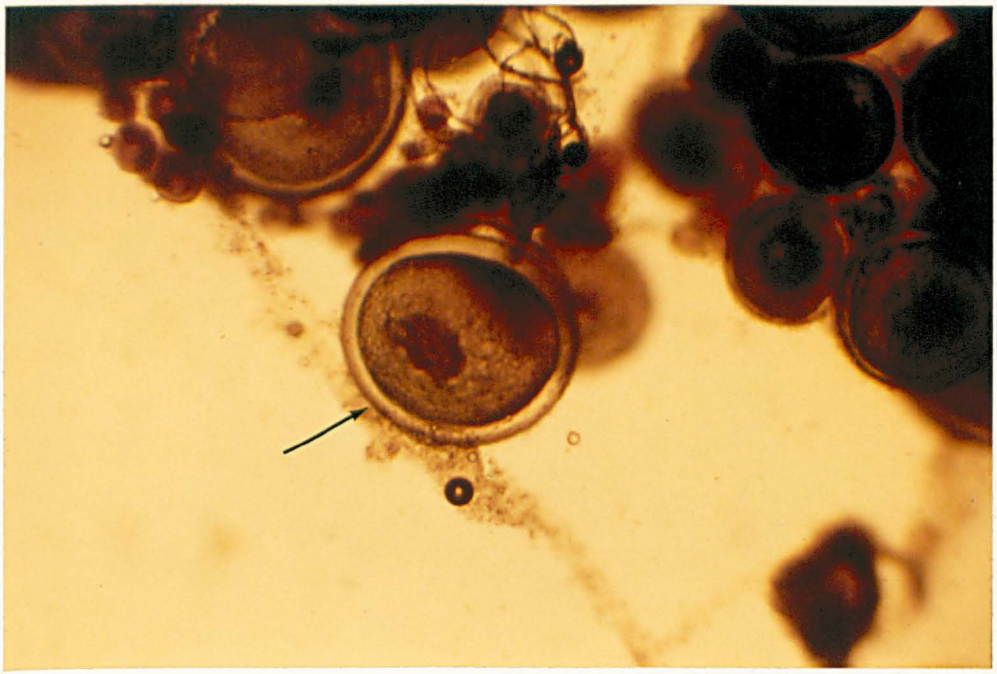


Plate XXI. Stage B oocyte, i.e. with a migrating nucleus.

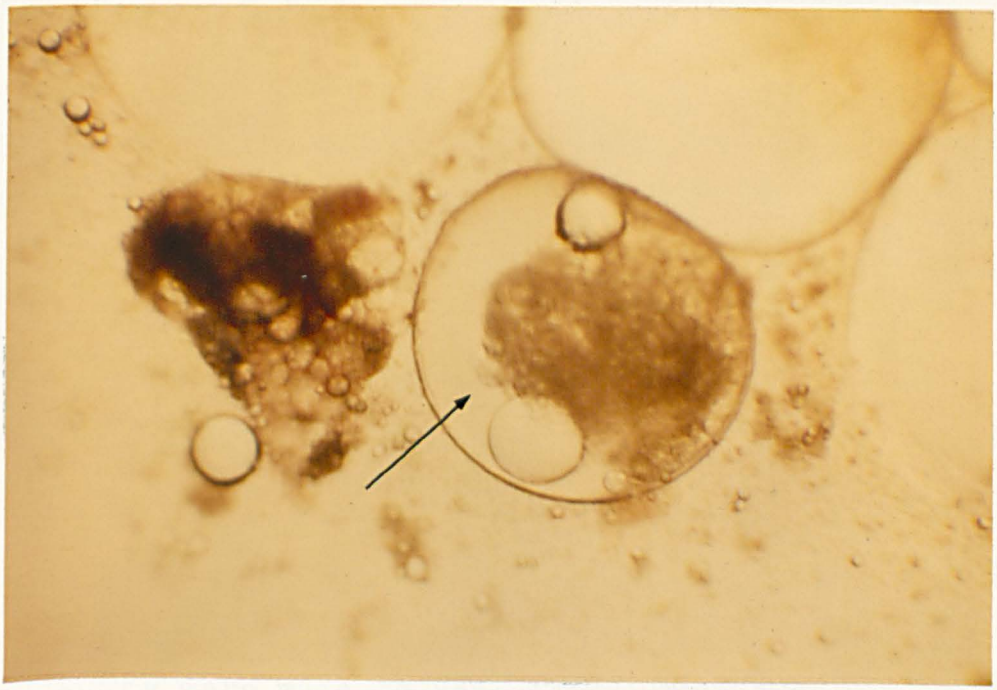


Plate XXII. Stage D oocyte, i.e. mature, pre-ovulatory oocyte.

eral subsamples of the extracted material were quickly scanned using an Olympus BHB microscope, and a note made of the most abundant oocyte stage and the most advanced stage. Photomicrographs were also taken for later reference.

Preliminary Trial:

On 9th. May, 3 females were selected which were all at maturity stage 1,A (i.e., approximately 4 weeks away from their expected first ovulation). Each weighed 2kg. They were injected with the equivalent of 4mg/kg of acetone-dried salmon pituitary powder (A.D.S.P.) on Day 1, followed by doses of 1mg/kg of 17αOHP on Days 2 and 3.

Attempts were made to hand-strip ripe eggs from the females on Days 3 to 8, inclusive.

Experiment 1:

On 24th. June, 8 fish were chosen whose maturity stages and weights were as follows:

Control Fish			Experimental Fish		
	Maturity Stage	Weight		Maturity Stage	Weight
1)	2,A	6.2kg	5)	2,A	3.1kg
2)	1-2,A	3.2kg	6)	1-2,A	2.8kg
3)	2,A	3.3kg	7)	2-3,B	1.9kg
4)	2-3,B	4.2kg	8)	2-3,B	3.1kg

On Day 1, the experimental fish were injected with 4mg/kg of A.D.S.P., followed by a dose of 2mg/kg of 17αOHP on Day 2. The control fish were given saline injections (8‰ NaCl) of 1ml/kg on Day 1 and 1ml/kg of saline containing 20% ethanol on Day 2.

Attempts were made to hand-strip eggs from both control and experimental fish on the next consecutive 6 days.

Experiment 2:

On 29th. June, 9 fish were selected with maturity stages and weights as follows:

Control Fish			Experimental Fish					
			Group A			Group B		
	Mat/St	Wt.		Mat/St	Wt.		Mat/St	Wt.
1)	2,A	2.8kg	4)	2,A	3.2kg	7)	2,A	2.1kg
2)	2,A	4.9kg	5)	2,A	3.3kg	8)	2,A	2.9kg
3)	1-2,A	1.8kg	6)	1-2,A	3.6kg	9)	1-2,A	2.9kg

On Day 1, both groups of experimental fish were injected with 4mg/kg of A.D.S.P.. On Day 2, Group A experimental fish were given injections of 4.4mg/kg of 17αOHP and Group B fish were given crude 17αOH 20βP injections as follows:

Fish 7) 4.9mg/kg

Fish 8) 3.0mg/kg

Fish 9) 4.9mg/kg

The 3 control fish were given saline injections on Day 1 and 20% ethanol in saline injections on Day 2.

Attempts were made to handstrip eggs from both control and experimental fish for the next six consecutive days.

An ovulation-synchronisation experiment using 8 control fish and two groups of 8 experimental fish (one for 17αOHP injections, and one for 17αOH 20βP injections) was planned. However, this was abandoned due to the failure of these initial trials, and the realisation that some females had different ovulatory periods- that is, even if several females' ovulations were initially synchronised they would slip out of phase after a few ovulations.

	FEMALE	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6
CONTROL FISH	A	Saline	Saline	*	---	*	---
	B	Saline	Saline	---	---	---	---
	C	Saline	Saline	---	---	---	---
	D	Saline	*	---	---	*	---
EXPERIMENTAL FISH	E	Primer	17α OH P	---	---	---	---
	F'	Primer	17α OH P	---	---	---	---
	G	Primer	*	---	---	---	*
	H	Primer	17α OH P	---	---	---	*

Table 14. The sequence of injections given to control and experimental fish in Experiment 1, and the results of monitoring these fish for a further four days. Asterisks indicate the presence of freshly ovulated eggs in a female's ovary.

	FEMALE	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6
CONTROL FISH	I	Saline	Saline	*	---	---	*
	J	Saline	Saline	---	---	---	---
	K	Saline	Saline	---	---	---	---
EXPERIMENTAL GROUP A.	L	Primer	17α OH P	---	---	---	---
	M	Primer	17α OH P	---	---	---	---
	N	Primer	17α OH P	---	---	---	---
EXPERIMENTAL GROUP B.	O	Primer	17α 20β P	---	---	*	---
	P	Primer	17α 20β P	---	---	---	---
	Q	Primer	17α 20β P	---	---	---	---

Table 15. The sequence of injections given to control and experimental fish in Experiment 2, and the results of monitoring these fish for a further four days. Asterisks indicate the presence of freshly ovulated eggs in a female's ovary.

RESULTS

Preliminary Trial:

Ovulations were not induced in any of the females used in this trial. In fact, all three fish's ovaries regressed somewhat after the injections and the females only came into spawning condition about 8-9 weeks later, right at the end of the season.

Experiment 1:

As can be seen from Table 14, the experiment was completely unsuccessful. The ovulations which occurred appeared quite random in nature and must have been spontaneous rather than induced by the administered hormones.

Experiment 2:

Table 15 shows the unsuccessful nature of this experiment. Neither 17αOH P nor 17αOH 20βP injections induced ovulations in any of the females.

DISCUSSION

It is obvious from the results presented here that 17α OH P and 17αOH 20βP given at the stated concentrations do not induce ovulation in turbot. In fact, in the preliminary trials, 17αOH P actually seemed to have an inhibitory effect on oocyte maturation. It is possible that this inhibition was in part due to the females' oocytes only being at the first stages of maturity when the hormone was administered since Riley and Jones (pers. comm.) found that turbot at early stages of maturity also regressed their ovaries when given HCG injections which were intended to induce ovulation.

4. OVULATORY RHYTHMS OF FEMALES KEPT IN A CONSTANT-LIGHT REGIME:

Several species of batch-spawning fish have been shown to have rhythmic periodicity of reproductive behaviour with spawning confined to a limited period of day or night (Ahlstrom, 1943; Daugs, 1965; Egami, 1954; Forselius, 1957; Gamulin & Hure, 1956; Legault, 1958; Marshall, 1967; Robinson & Rugh, 1943). Light has been shown to be a principal factor in the precise timing of spawning in some of these species; for example, Oryzias latipes (Egami, 1954; Robinson & Rugh, 1943); the zebra fish, Brachydanio rerio (Legault, 1958), and Trichopsis vittatus and T. pumilus (Marshall, 1967).

In all of these examples, ovulation occurred no more than a few hours prior to egg-laying (provided tank conditions were optimal -Egami, 1954; Robinson & Rugh, 1943). Accordingly ovulation times also show a periodicity. In addition, Stacey et. al.(1979a & b) have shown that both the preovulatory gonadotropin surge and the ovulation of the goldfish are synchronised by photoperiod, ovulation occurring at approximately the same time of day at temperatures from 12-16°C.

"Round-the-clock" plankton samples taken by Simpson (1972) indicated that plaice in the southern North Sea spawn between 1900 and 0600h in early January, extending to 1100h by mid-February. Although dab in the same area spawned throughout the day and night, more eggs were released between midnight and noon. Simpson also found that the spawning of sprat in the Irish Sea in January and early March was limited to the period 2200h to noon with most eggs being released between midnight and 0400h. Further plankton samples taken

by Simpson indicated that the spawning of pilchard in the English Channel in June was almost completely limited to the period 2000 to 0200h. Finally, Jones (1972) states that the few natural spawnings which occurred in his experimental turbot broodstock all took place at night.

In view of all this evidence in favour of diel ovulatory and spawning behaviour in fish, it was rather surprising to find that female turbot ovulated at various times during the day and night (see Tables 11a & b), apparently only dependent on some form of endogenous rhythm and completely ignoring the "on-off" stimuli of their light/dark regime (18h light : 6h dark). It was therefore decided to investigate this phenomenon further by monitoring the ovulatory rhythms of two females placed in a constant light regime (24h light: 0h dark).

MATERIALS & METHODS

The ovulatory rhythms of two female turbot subjected to the usual 18h light : 6h dark photoperiod were monitored for four days by stripping eggs from them daily. Actual percentage fertilisation figures were not computed for this experiment. Instead, the degree of over-ripening of the eggs was assessed by observing their first and second cleavages under the microscope and comparing these with the "ageing-scale" described on page 63 and in Plates XV to XVIII. This was a very accurate and rapid method of determining the day on which ovulation occurred, since only freshly ovulated eggs showed really good quality first divisions.

After four days, the two fish were subjected to a continuous light regime (24h light : 0h dark). In order to do

Fig. 23. Tank-system for "Constant Light" Experiment.
(Not drawn to scale; see text for tank dimensions.)

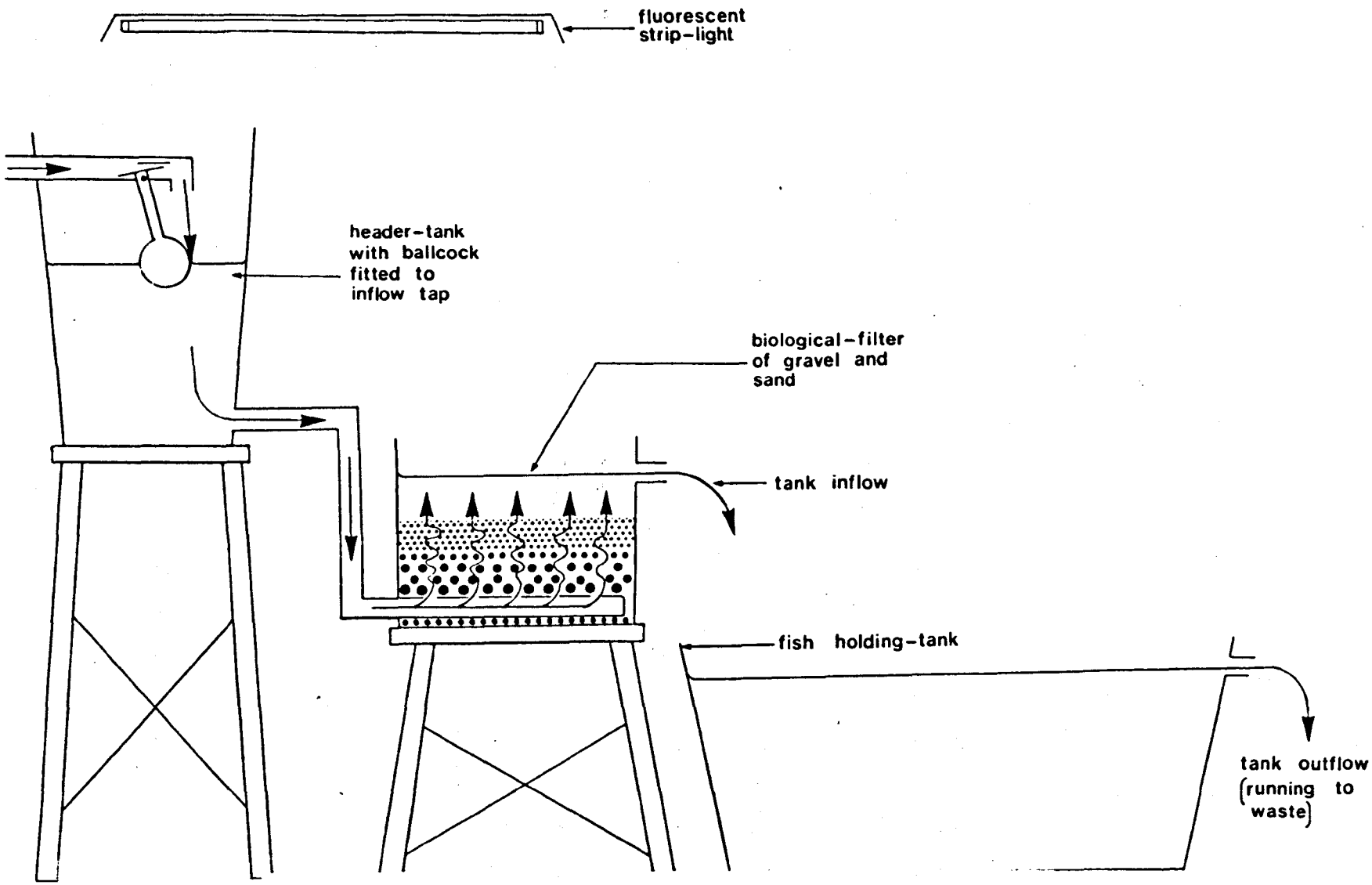

fluorescent
strip-light
header-tank
with ballcock
fitted to
inflow tap
biological-filter
of gravel and
sand
tank inflow
fish holding-tank
tank outflow
(running to
waste)

Fig. 23

this the fish had to be isolated from the rest of the broodstock and placed in a 1.22m x 0.6m x 0.6m fibreglass tank which had seawater flowing through it at a rate of 3 l/minute. The seawater passed through a biological gravel filter beforehand (see Fig. 23) and aeration was supplied to air-stones placed at either end of the fish-tank. The water in the fish-tank was kept at 14.0(±0.5)°C for the duration of the experiment (i.e., the same temperature as the water in the main broodstock tank) and continuous lighting was provided by a fluorescent strip-light placed in the corner of the room so that the fish received a diffuse light which was similar to that over the main broodstock-holding tanks.

Although it would have been preferable to monitor the females' ovulatory cycles for at least two weeks before subjecting them to a constant light regime, this was not possible because the fish were coming to the end of their spawning season, and so time was limited.

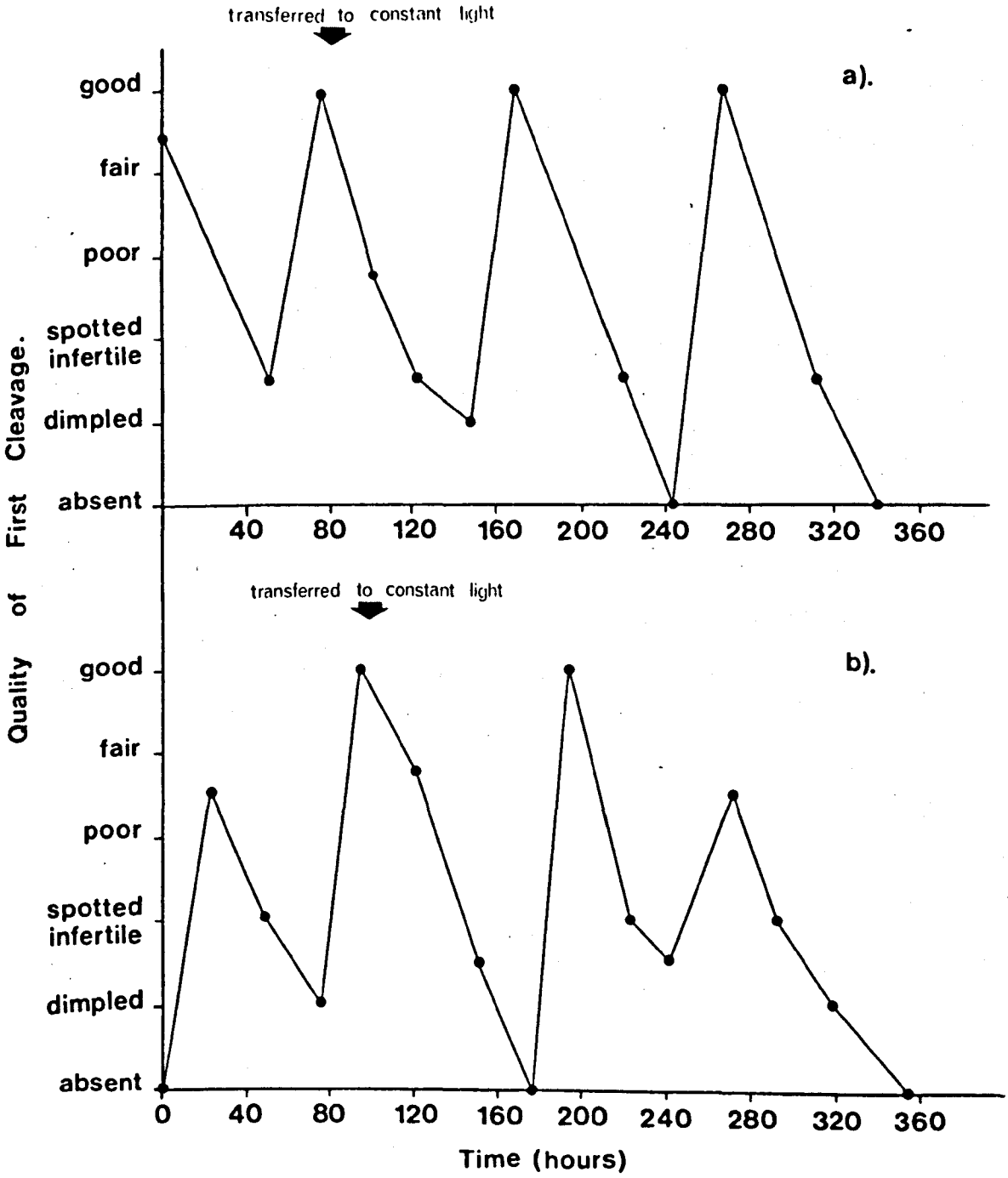
Monitoring of the fish's ovulatory rhythms continued in exactly the same manner for a further two weeks - that is, the fish being stripped daily and the quality of their eggs being assessed under the microscope.

RESULTS

Transferring two females from their usual 18h light : 6h dark regime into one of constant light did not have any discernible effect on either female's ovulatory rhythm (see Figs. 24a & b).

Figs. 24a & b. Ovulation-prediction charts of 2 turbot females transferred from an 18h Light : 6h Dark regime to one of constant light (see arrows on graphs). Turbot egg-quality declines rapidly after ovulation, so "good quality" eggs on the graphs denotes the presence of freshly-ovulated eggs in the females' ovarian lumens. Plates XV to XVIII show the appearance of the different quality stages of turbot eggs used in the y axes of these figures.

Fig.24.


transferred to constant light
a).
good
fair
poor
spotted infertile
dimpled
absent
40
80
120
160
200
240
280
320
360
transferred to constant light
b).
good
fair
poor
spotted infertile
dimpled
absent
0
40
80
120
160
200
240
280
320
360
Time (hours)
Quality of First Cleavage.

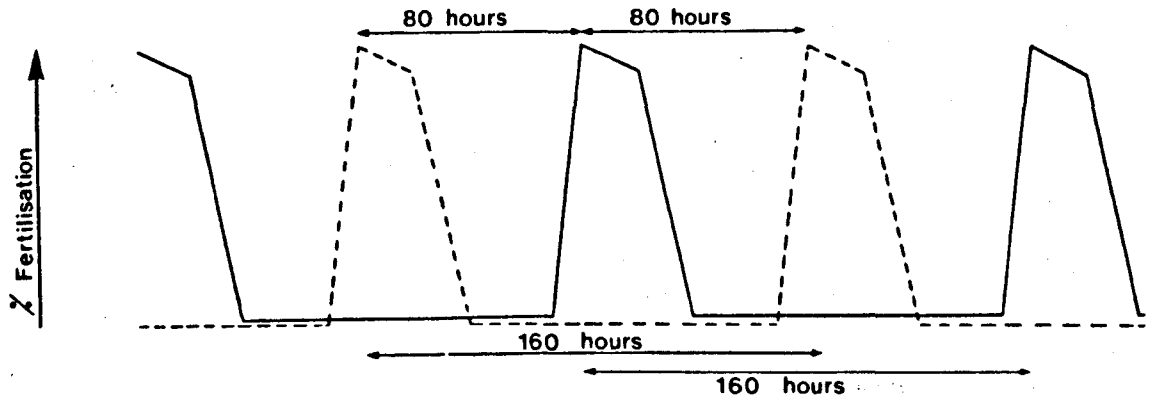
DISCUSSION

The results of this experiment suggest that turbot females do not depend on any "lights on/off" stimuli to act as pacemakers for their ovulatory rhythms. However, it was only possible to monitor the two experimental females for 3-4 ovulations in constant light (they both stopped ovulating at this point) and so the experiment did not completely disprove rhythm entrainment. It is possible that the ovulatory rhythms were simply "free-running" whilst the females were in constant light.

Nonetheless, since all females seem to ovulate successive egg-batches at completely different times of the day and night, it seems safe to assume that ovulation is not triggered by any "lights on/off" stimuli. However, even though ovulation does not seem to be confined to a limited period of the day or night, there is no reason to assume that natural spawning behaviour shows the same rhythmicity.

Fig. 25. A hypothetical explanation of the fact that a few turbot females have alternating long and short ovulatory cycles instead of one constant ovulatory period as in the majority of cases. The hypothesis assumes that the paired ovaries of turbot ovulate alternately. That is, if the ovulatory period of a female was 80 hours, then each of her ovaries would ovulate every 160 hours; the ovaries being 80 hours out of phase with each other if the female showed "regular ovulations" (as depicted in (a)). If, however, the phasing was not quite "correct" the female would appear to have alternating long and short ovulatory cycles (as depicted in (b)).

a) Female with "Regular Ovulations"



b) Female with "Alternate Ovulations"

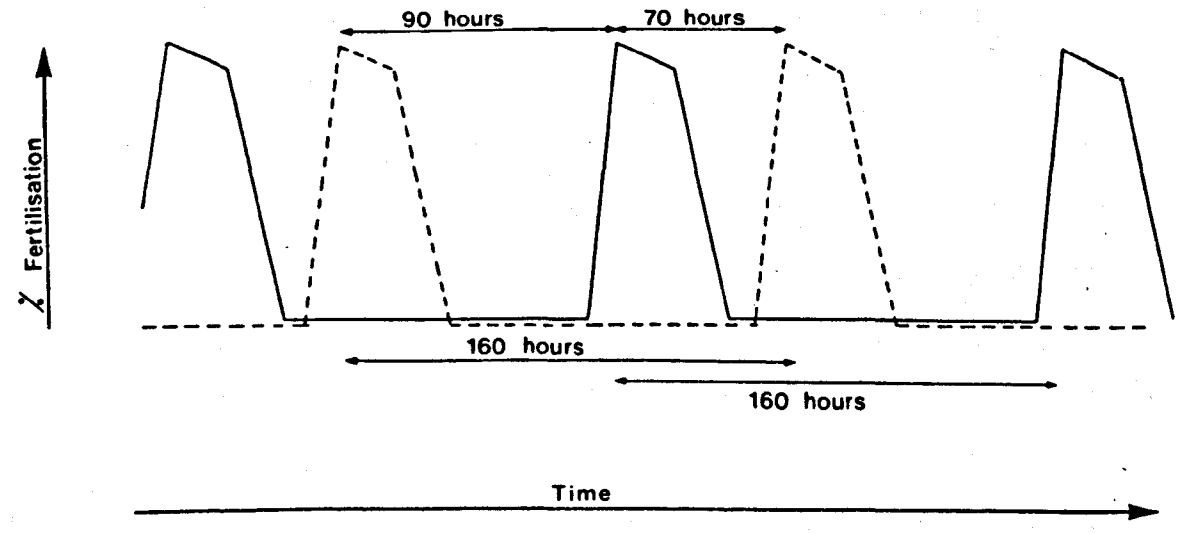


Fig. 25.

5. WHETHER AN INDIVIDUAL'S OVARIES OVULATE SIMULTANEOUSLY OR ALTERNATELY:

Although in the majority of cases the ovulatory period of an individual female remained remarkably constant throughout the spawning season, the occasional female showed an alternation of long and short intervals between her ovulations (see Figs. 20, 21 & 24b). This was observed in only 3 fish, but in each case the alternating pattern was very precise. For example, the fish "VIV" ovulated after 70 hours then after 90 hours, after 70 hours then after 90 hours, and so on (see Fig. 21).

This strange phenomenon seemed to suggest that the paired ovaries of the female turbot might ovulate alternately. That is, if the ovulatory period of a female was 80 hours then each of her ovaries would ovulate every 160 hours, the ovaries being 80 hours out of phase with each other (see Fig. 25a). If, however, the phasing was not quite "correct" the female would appear to have alternating long and short time-intervals between ovulations (see Fig. 25b).

This seemed unlikely because turbot ovaries are in much greater contact with each other and "fused" to a greater degree than the paired ovaries of higher mammals, which do show alternate ovulations. In addition, no mention of a similar phenomenon occurring in other fish species could be found in the literature. However, the alternating pattern was so clear-cut that it was decided that a closer investigation was warranted.

The phenomenon was studied in three ways:

a) A dye-mark experiment.

b) Catheterisation experiments.

c) The examination of the ovaries of a female sacrificed just prior to her ovulation.

MATERIALS & METHODS

a) Dye-Mark Experiment:

A solution of "fast-green" was made by dissolving 1g of "fast-green" in 50ml of physiological saline (8% NaCl in distilled water). Then, a female turbot which was 1 day away from her next ovulation was anaesthetised with MS222 and a catheter tube (see Fig. 14) was introduced into her oviduct. The tube was gently pushed as far back into the lumen of the upper (i.e., left) ovary as possible. It was known that the catheter had entered the upper ovary, as opposed to the lower one, because it could be seen moving underneath the fish's skin. A few ml of "fast-green" solution were taken up in a 20ml disposable syringe barrel and injected into the ovary lumen by attaching the barrel to the catheter tube protruding from the oviduct. The catheter was then removed.

The fish was hand-stripped daily and the resultant eggs were examined for traces of dye.

b) Catheterisation Experiments:

Female "VIV" was taken a few hours after ovulation and all her ovulated eggs were stripped away by hand. A catheter tube was then introduced into her upper ovary and a sample of oocytes removed by suction. A clean catheter tube was then introduced into the lower ovary (this time the tube could not be seen moving underneath the skin of the upper side) and another oocyte sample removed.

100 secondary oocytes from each of these two samples

were teased free with seekers and their diameters measured under the microscope using a graticuled eyepiece (2 diameters at right angles to each other were measured per oocyte and a mean taken). A frequency-distribution diagram of oocyte diameters was drawn for each ovary and compared.

This procedure was repeated two days later when the female was approximately 12 hours away from her next ovulation.

c) Comparing Ovaries of a Sacrificed Female:

In the previous method, it was assumed that if the catheter could be seen moving beneath the skin of the fish's upper side it must have entered the upper ovary, and if it could not be seen in this way it must have entered the lumen of the lower ovary. However, this introduced an element of uncertainty into the results. When the frequency-distribution diagrams for both ovaries were almost exactly the same, it was decided to sacrifice one of the smaller females and compare her two ovaries to make certain that the oocyte samples of the previous experiment had not been taken from the same ovary.

A small female which was about 12 hours away from her next ovulation was culled and her ovaries removed by dissection. Frequency-distribution diagrams of the diameters of 200 secondary oocytes were drawn for each ovary and compared (100 oocytes being taken from the anterior and posterior lobes of each ovary).

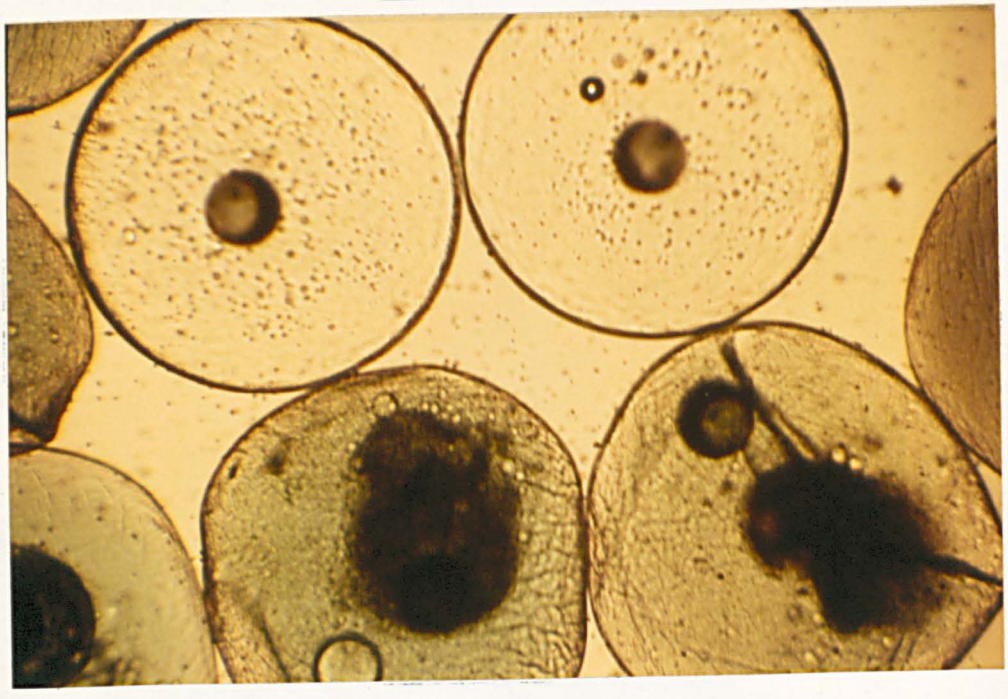


Plate XXIII. Showing clear, freshly-ovulated eggs (above) and dyed, over-ripe eggs (below). See text for details of this experiment.

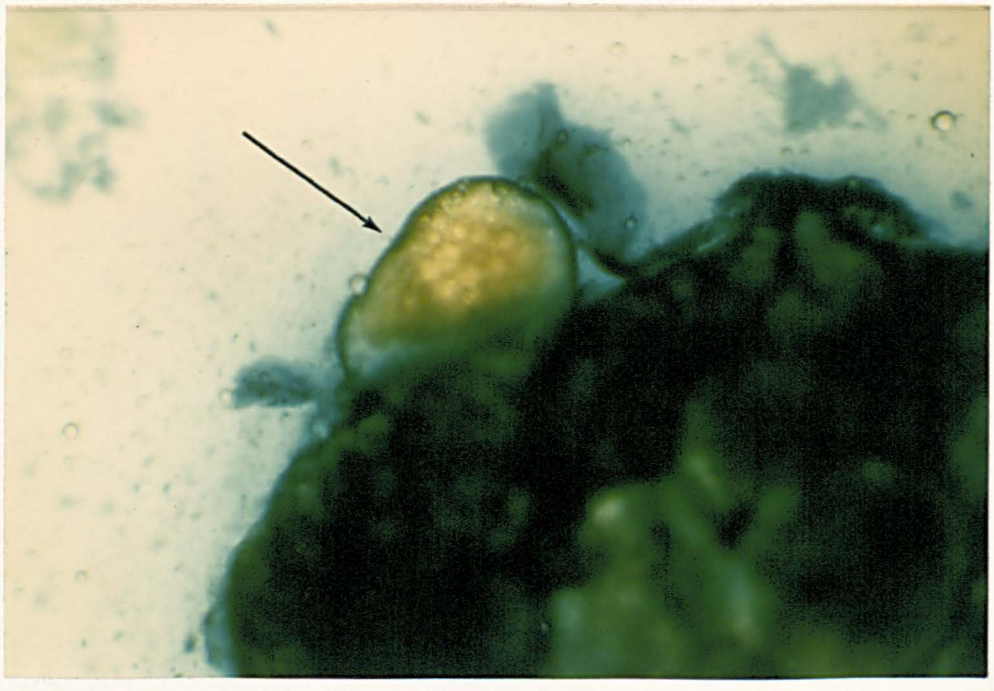


Plate XXIV. Showing that unovulated oocytes teased from the ovarian tissue were not dyed by the "fast-green". See page 83 for details.

a) Dye-Mark Experiment:

The eggs that were hand-stripped from the experimental female the day after dye had been introduced into her upper ovary consisted of a mixture of fresh eggs (ovulated during the night) and some very over-ripe eggs (left over from the previous ovulation 3-4 days earlier). All the over-ripe eggs had taken up the "fast-green" dye, whereas the freshly-ovulated eggs were completely clear (see Plate XXIII).

Initially, it was thought that this result was indicative of the female's ovaries ovulating alternately. It was very disappointing when the female stopped ovulating at this point because it was hoped that the next batch of eggs that were stripped would confirm this result by the freshly-ovulated eggs being dyed turquoise (coming from the upper, left ovary), and the over-ripe eggs remaining from the previous ovulation being clear (coming from the lower, right ovary). However, when further checks were made into the validity of this method, it became apparent that the dye only marked eggs that had already been ovulated into the ovary-lumen when the dye was introduced. It did not dye unovulated eggs within the body of the ovary. (This was found by taking a small ovarian sample from another female by catheterisation and placing it in a solution of "fast-green" dye in physiological saline for a few hours. Only the outer tissues of the ovary and ovulated eggs were stained. As shown in Plate XXIV, unovulated oocytes teased from the ovarian tissue were not dyed). The results of the dye-mark experiment therefore failed to indicate whether or not turbot ovaries released their eggs alternately.

Fig. 26. Size-frequency distributions of secondary oocytes from the left and right ovaries of 1 turbot female 12 hours before her next ovulation. A sample-size of 100 oocytes was used from each ovary.

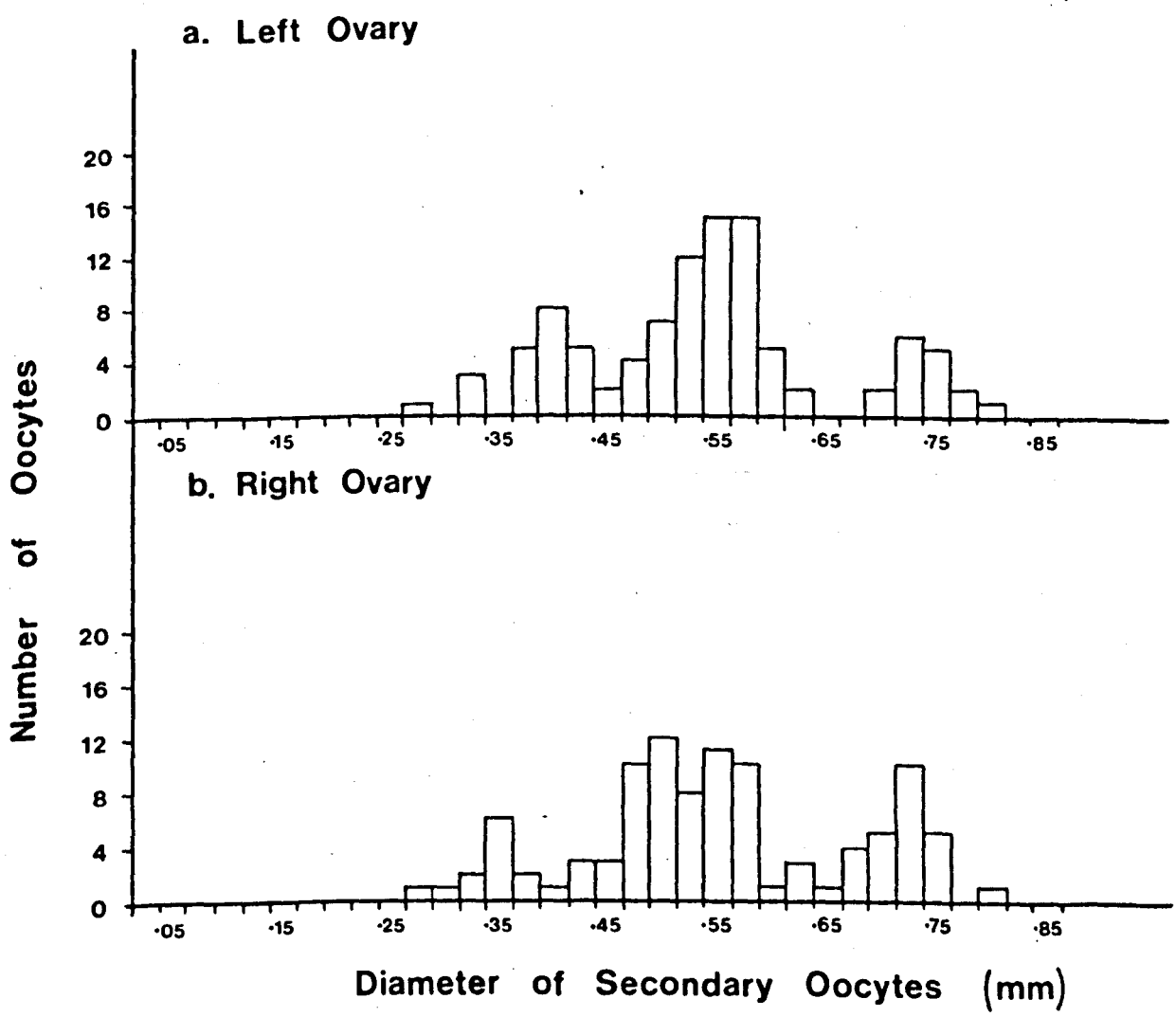

a. Left Ovary
b. Right Ovary
Number of Oocytes
20
16
12
8
4
0
·05
·15
·25
·35
·45
·55
·65
·75
·85
Diameter of Secondary Oocytes (mm)

Fig.26.

Fig. 27. Size-frequency distributions of secondary oocytes from the left and right ovaries of 1 turbot female a few hours after her ovulation (and after hand-stripping). A sample-size of 100 oocytes was used from each ovary.

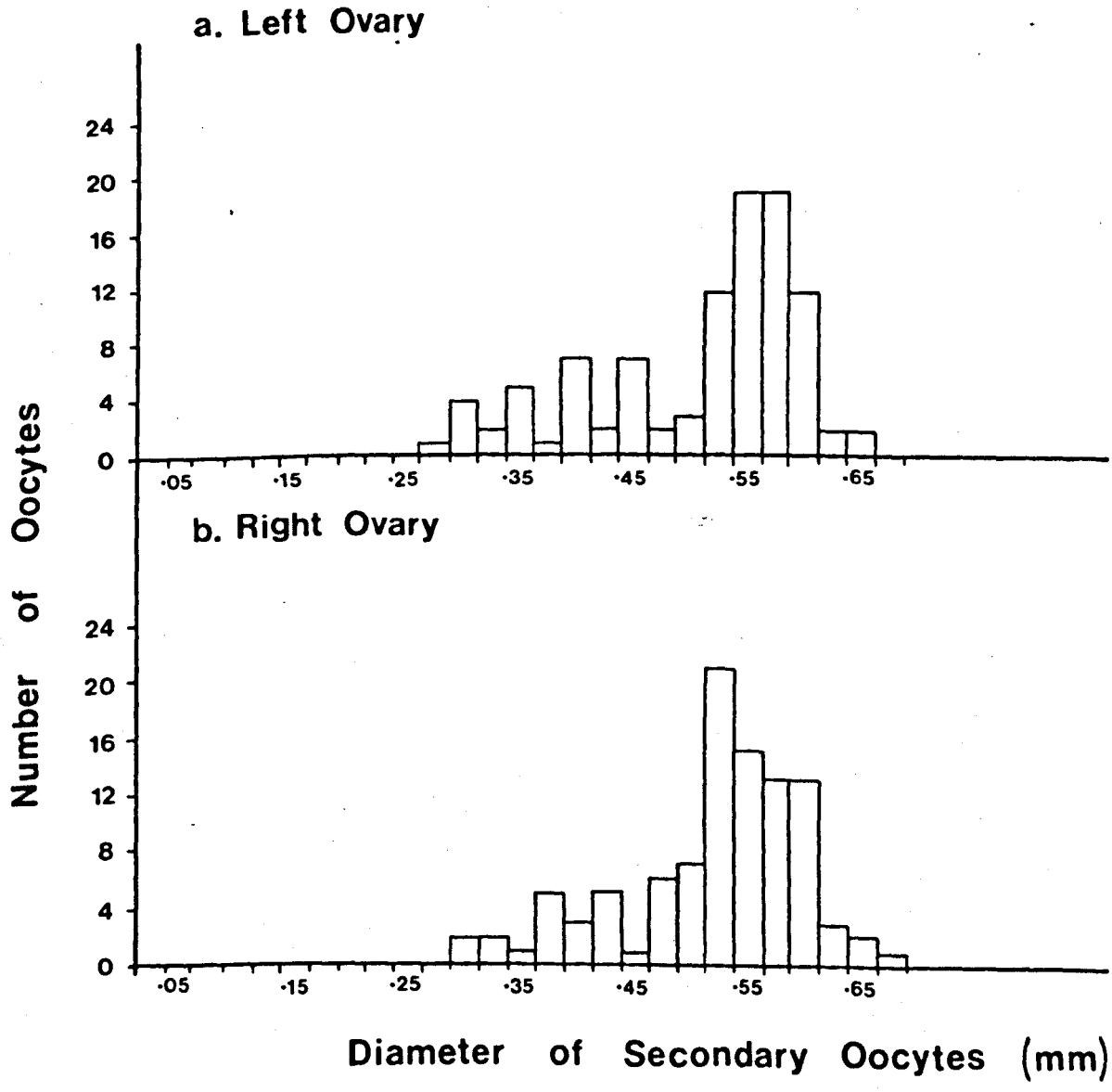

a. Left Ovary
b. Right Ovary
Number of Oocytes
0
4
8
12
16
20
24
·05
·15
·25
·35
·45
·55
·65
Diameter of Secondary Oocytes (mm)

Fig.27.

Fig. 28. Size-frequency distributions of secondary oocytes in the left and right ovaries of a female turbot sacrificed approximately 12 hours before her next ovulation. 2 samples of 100 oocytes were measured from each ovary - 1 from the anterior lobe and 1 from the posterior lobe.

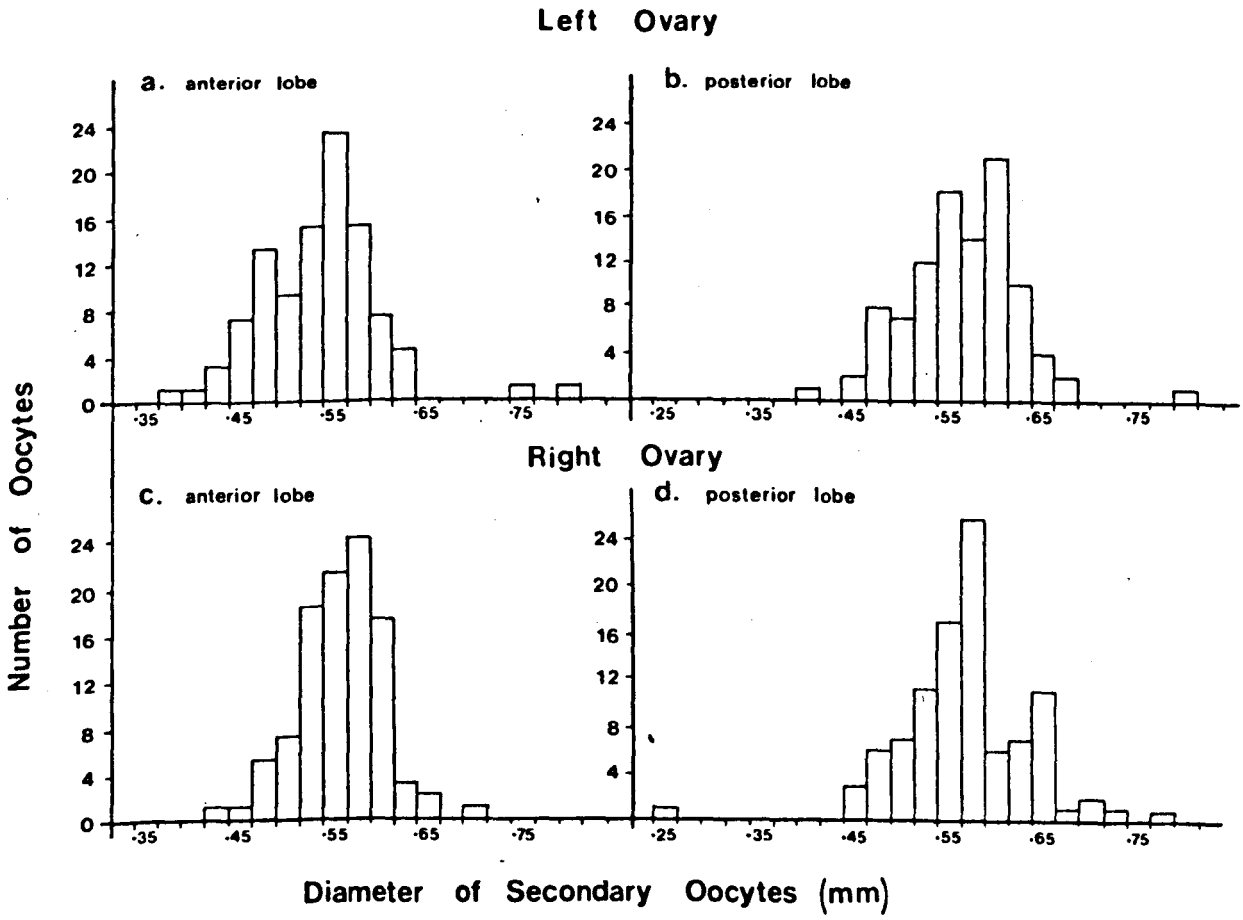

Left Ovary
a. anterior lobe
b. posterior lobe
Right Ovary
c. anterior lobe
d. posterior lobe
Number of Oocytes
Diameter of Secondary Oocytes (mm)
0
4
8
12
16
20
24
·25
·35
·45
·55
·65
·75

Fig. 28.

b) Catheterisation Experiments:

No significant difference could be seen in the size-frequency distribution of unovulated oocytes taken from the upper (left) and the lower (right) ovaries of female VIV, either before or after ovulation (see Figs.26 & 27).

The similarity between the upper and lower ovary samples indicates that both ovaries ovulate in synchrony.

c) Comparing Ovaries of a Sacrificed Female:

There was no significant difference in the size-frequency distribution of unovulated oocytes in either ovary of the culled female,i.e. oocytes were at the same stages of development in both ovaries (see Figs. 28a,b,c & d). Therefore, the paired ovaries of female turbot appear to ovulate in synchrony.

DISCUSSION

Since the validity of the "dye-mark" experiment was later discredited, it can be assumed from the results of the catheterisations and culling that turbot females' paired ovaries ovulate in synchrony and "alternation of ovulation" is not the answer to the peculiar long and short ovulatory periods observed in some females. These alternating ovulatory periods must be a manifestation of alternating long and short hormone-release cycles, but exactly how they are caused and why they should be so regular cannot at present be explained.

6. WHETHER NATURALLY-SPAWNING TURBOT SHOW RHYTHMIC SPAWNING ACTIVITY:

The experiments described in the previous sub-sections demonstrated that captive female turbot have very regular ovulatory rhythms which vary between individuals and, apparently, are not cued by "lights on/off" stimuli. However, the hand-stripped broodstocks at the Port Erin hatchery are in very unnatural holding conditions, so much so that their natural spawning behaviour is suppressed. The author was therefore curious to find out if a female's endogenous ovulatory rhythm would (i) be reflected in rhythmic spawning activity if she were allowed to spawn, and her eggs be fertilised, naturally; or (ii) whether courtship behaviour could override the basic ovulatory rhythm and induce ovulation in some way. (Presumeably, a female's oocytes would have to be at a well-advanced stage of maturity before induction of this nature could be possible).

Bearing in mind that eggs become inviable about 18 hours after their ovulation due to ageing processes, some form of synchronisation must occur between the male and female before successful fertilisation can take place. If endogenous ovulatory rhythms do occur in wild-spawning, it might be necessary for close pair-bonding between a male and female over an extended period of time. In this way the female could indicate to the male by behavioural and/or pheromonal signals when she was about to ovulate, and thus initiate courtship. If, however, courtship itself is capable of inducing ovulation in females, such close pair-bonding would not be necessary.

It is interesting that Manx fishermen say that on the rare occasions they net turbot, there are usually two fish

Fig. 29. Dimensions of the outside water storage pond.

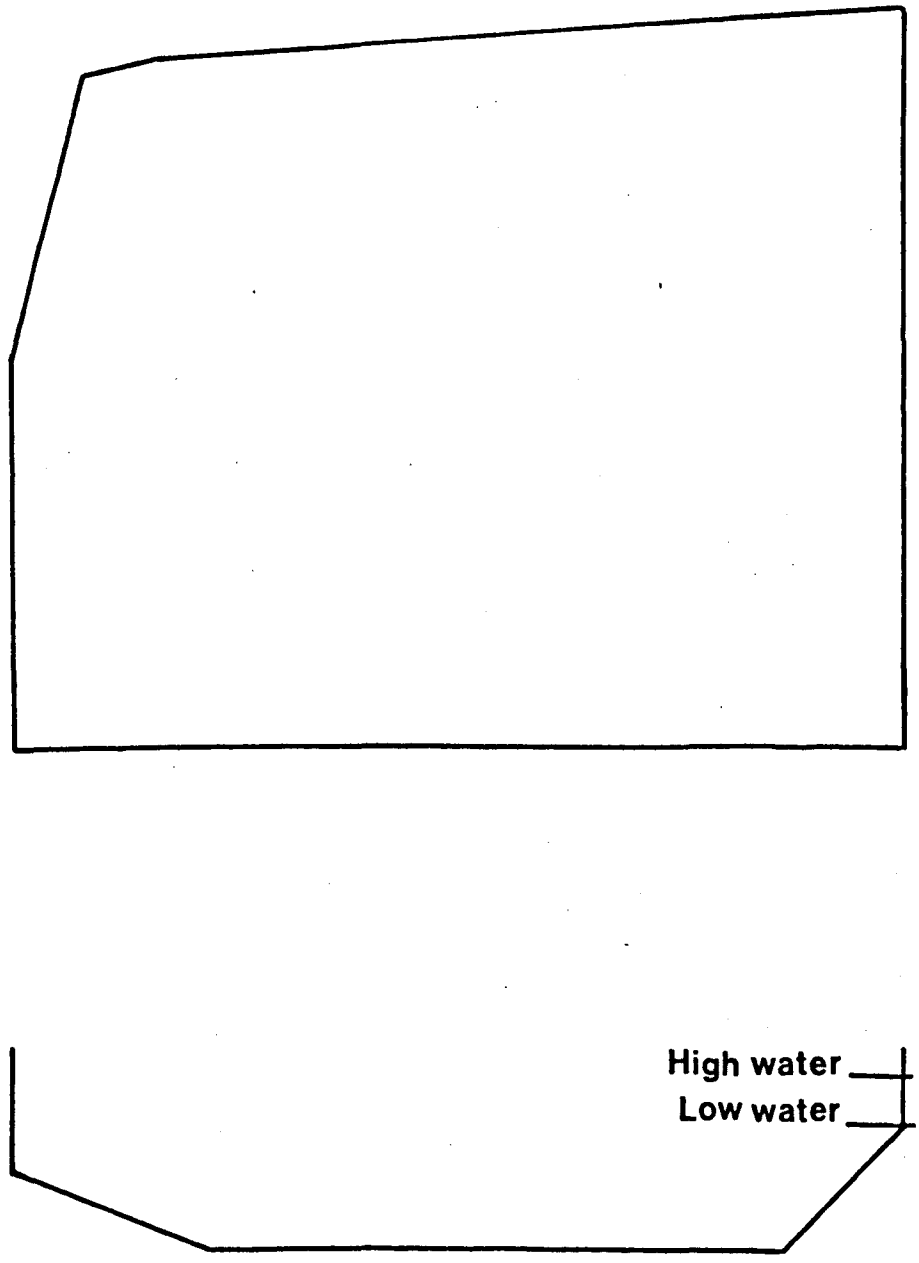
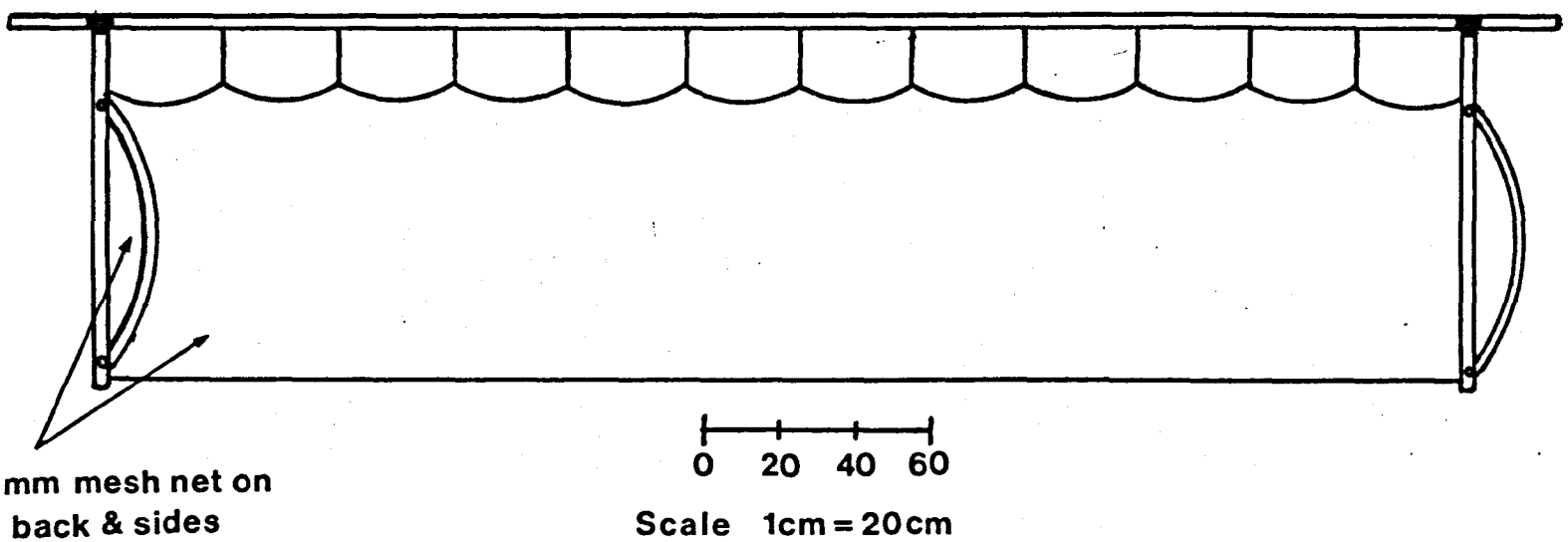


Fig. 29.

Fig. 30. The egg-collecting net used in the experiment reported in sub-section 6.

Fig.30.



taken in close proximity to each other, and these are generally a male and female (Bowers, pers. comm.). Possibly, then, the natural spawning behaviour of turbot does involve a lengthy pair-bond.

The following experiment attempted to monitor whether a naturally-spawning male and female turbot showed regular, rhythmic spawning activity.

MATERIALS & METHODS

A female that was approximately two weeks away from her first spawning of that season, and whose ovulatory rhythm had been monitored very closely the previous year, was selected for the experiment. On 27-5-81, she was put into an outside water-storage pond along with two ripe males. The dimensions of the pond are shown in Fig. 29. Fresh seawater was pumped into its base at each high tide until the water-depth was 2.25m. Since the pond acted as a reservoir for the University of Liverpool's Marine Biological Station, the water-level gradually dropped after each pumping as water was used. The lowest water-level monitored during the experiment was 1.60m.

The three fish were left undisturbed in the pond for a week, and then the water surface was skimmed daily to a depth of approximately 0.5m using the net shown in Fig. 30 (mesh size = 1mm). A handnet (24cm x 9 cm) was used to skim eggs close to the pond walls.

Any eggs that were collected in this fashion were put into clean seawater and inspected under the microscope.

DATE	TIME	EGG-QUALITY
11-6-81	12 a.m.	Poor quality - some spotted, infertile.
15-6-81	12 a.m.	Freshly-ovulated - "water-activated".
28-6-81	1 p.m.	Freshly-ovulated - "water-activated".
13-7-81	4 p.m.	Fair-poor quality - "water-activated".
17-7-81	6 p.m.	Fair-poor quality - "water-activated.

Table 16. The occurence of naturally spawned eggs in the outside pond. (See Section IV for details of the "water-activation reaction).

RESULTS

Table 16 indicates the days on which eggs were skimmed from the surface of the pond. Although most of these eggs appeared to be quite freshly-ovulated, none had been fertilised, they were simply "water-activated" (see Section IV for further details of this phenomenon). No clear spawning rhythm was apparent.

DISCUSSION

The males were "running" with ripe milt when they were put into the pond, and Girin (1979) obtained natural spawning and fertilisation behaviour in turbot held in a smaller pond than the one used in this experiment (Girin's pond had a volume of $40m^3$). Therefore the fact that the males did not fertilise any eggs is probably due to all the experimental fish, male and female, being hatchery reared and not used to being allowed to spawn naturally. It is also possible that the presence of two males in the experimental pond interfered with normal spawning or pre-spawning activity.

The fact that no regular spawning rhythm was apparent by inspecting eggs floating at the water surface does not necessarily mean that females lose their endogenous ovulatory rhythm. The female could have retained ovulated eggs in the lumen of her ovary without spawning them, as happens in captive, hand-stripped females. If this was the case, the eggs would have been over-ripe when they were eventually released and so would have sunk to the bottom of the pond, thus escaping the sampling net.

In summary, no conclusions can be drawn from this experiment due either to the apparent absence of natural spawn-

ing activity in the experimental male turbot, or to the experimental conditions.

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SECTION IV

INTRODUCTION

The previous section dealt with the ageing of ovulated oocytes inside the lumen of the ovary prior to hand-stripping. This final section reports an investigation into how long male and female turbot gametes are able to retain their viability after they have been stripped from the fish, and whether contact with seawater affects their rate of ageing.

The experiments were prompted chiefly by some sperm cryopreservation trials that were being carried out at Shearwater's Port Erin hatchery. Since the deep-frozen milt samples were rather valuable, it seemed foolish to thaw them for use until it was certain that sufficient quantities of freshly ovulated eggs were available. Therefore, the females were usually stripped in advance. However, the milt samples took a few minutes to thaw, so it was necessary to find out how long unactivated and seawater- activated eggs could retain their fertility.

Another reason for these investigations is the fact that if several female turbot are being "hand-stripped" at the same time, it is much quicker and easier to make a "pool" of milt (from about three males) right at the beginning of the operation and use this to fertilise all subsequent eggs that are stripped. Since this method of "hand-stripping" was used routinely at Port Erin, it was important to determine how long "stripped" milt remains viable.

MATERIALS & METHODS

1. PILOT STUDY:

An initial test was carried out to indicate how long "stripped", unfertilised eggs could retain their fertility if they were not activated by seawater.

Eggs from a female turbot which had ovulated within the previous 10 hours were stripped by hand into a 33cm x 23cm x 21cm dry, plastic tank. The time of ovulation was determined as described earlier (see Section III, p 63). Three samples of approximately 3g each of these eggs were transferred to dry, plastic beakers of 400ml capacity.

Milt, freshly stripped from two males, was added to approximately 10ml of clean seawater and stirred. (The same two males were used to supply milt throughout the experiment). 1 ml of this milt suspension was added to one sample of eggs and 300ml of seawater at 14°C were poured in immediately afterward.

After 50 minutes, the two males were again stripped and 1 ml of freshly-made milt suspension was added to the second sample of eggs, immediately followed by 300ml of seawater. This process was repeated for the third egg-sample after a further 77 minutes. At the end of the experiment, approximately 3g of fresh eggs were stripped from the female to act as a control. These were fertilised immediately by adding 1 ml of fresh milt-suspension and 300ml of seawater.

After second cleavage (approximately 3 hours after fertilisation), 200 eggs were counted out from each sample-beaker. Any over-ripe eggs (i.e., eggs from an earlier ovulation) were not included in the sample. The percentage fertilisation

for each treatment was computed by counting the number of dividing eggs in each 200 egg-sample.

Each sub-sample was then incubated in separate 400ml plastic beakers of seawater containing sodium penicillin and streptomycin sulphate at 50ppm.. The beakers were put in an egg-incubation room at 12.5°C until the larvae hatched. When hatching was completed (Day 7) the numbers of hatched larvae were counted and the figures used to compute the percentage hatch for each treatment.

2. MILT-VIABILITY EXPERIMENTS:

Experiments were carried out to investigate how long milt remains viable after it has been stripped from the male adult. The ageing of both unactivated ("dry") and seawater-activated ("wet") milt was studied.

Eggs were stripped from a female which had recently ovulated and a few grams of them placed in 11 dry, plastic beakers of 400ml capacity. Seawater was not added until after milt-addition. The eggs were therefore in an unactivated state prior to fertilisation.

Milt was stripped from two males, mixed and then divided into two equal amounts. Seawater was added to one of the milt samples in a ratio of 1:1 (v/v). The other milt sample was not activated by seawater contact. At set intervals (see Tables 18a & b), the two types of milt ("wet" and "dry" milt) were added to two different samples of unactivated eggs, immediately followed by 300ml of seawater at 14°C. 2ml of "wet" milt, as opposed to 1 ml of "dry" milt, were used in the fertilisations to compensate for the dilution factor.

At the end of the experiment, fresh milt was stripped

from the same two males and used to fertilise a final beaker of stored, unactivated eggs. This served to test that the unactivated eggs had not lost their fertility during the course of the experiment.

Three hours after milt-addition, the percentage fertilisation for each treatment was assessed, the sub-samples incubated, and the percentage hatch computed in exactly the same manner as in the pilot study.

This experiment was repeated twice more, on different dates, using different males and females. However, in these later experiments only percentage fertilisation was monitored.

3. EGG-ACTIVATION EXPERIMENTS:

A series of trials to investigate how long unfertilised, seawater-activated turbot eggs retained their fertility were carried out as follows. Eggs were stripped from a female that had recently ovulated and were left unactivated in a 33cm x 23cm x 21cm dry, plastic container until milt had been taken from two males and mixed with approximately 15ml seawater. (The same two males were used throughout each experiment.) The plastic container was then three-quarters filled with seawater and at set intervals, approximately 2-3g samples of the activated eggs were lifted from it, put into 400ml plastic beakers and fertilised with 2ml milt solution. The seawater-levels were adjusted to 300ml in all beakers.

In the first trial, the same milt solution was used throughout the experiment and its viability tested at the end by using it to fertilise some freshly-stripped eggs. However, in later trials a fresh milt solution was made

every 10minutes to safeguard against milt ageing and losing its viability over the course of the experiment.

In trial (a), eggs were stripped from the same female 24 hours after the initial stripping and subjected to the same procedure. This was to investigate whether over-ripening of ovulated eggs inside the lumen of the ovary prior to hand-stripping had any effect on their fertility-period once they were seawater-activated. (The ovulation cycles of the adult female had been carefully studied prior to the experiment so that her ovulation times could be predicted to within a few hours.)

Each treatment in trial (d) was run at two different temperatures (11°C and 15.0°C) to check whether temperature of the activating seawater had any effect on the fertility-period of stripped, activated eggs.

Three hours after milt-addition, the percentage fertilisations were assessed by counting the number of dividing eggs in a 200 egg sample taken from each treatment. Again, any over-ripe eggs were ignored.

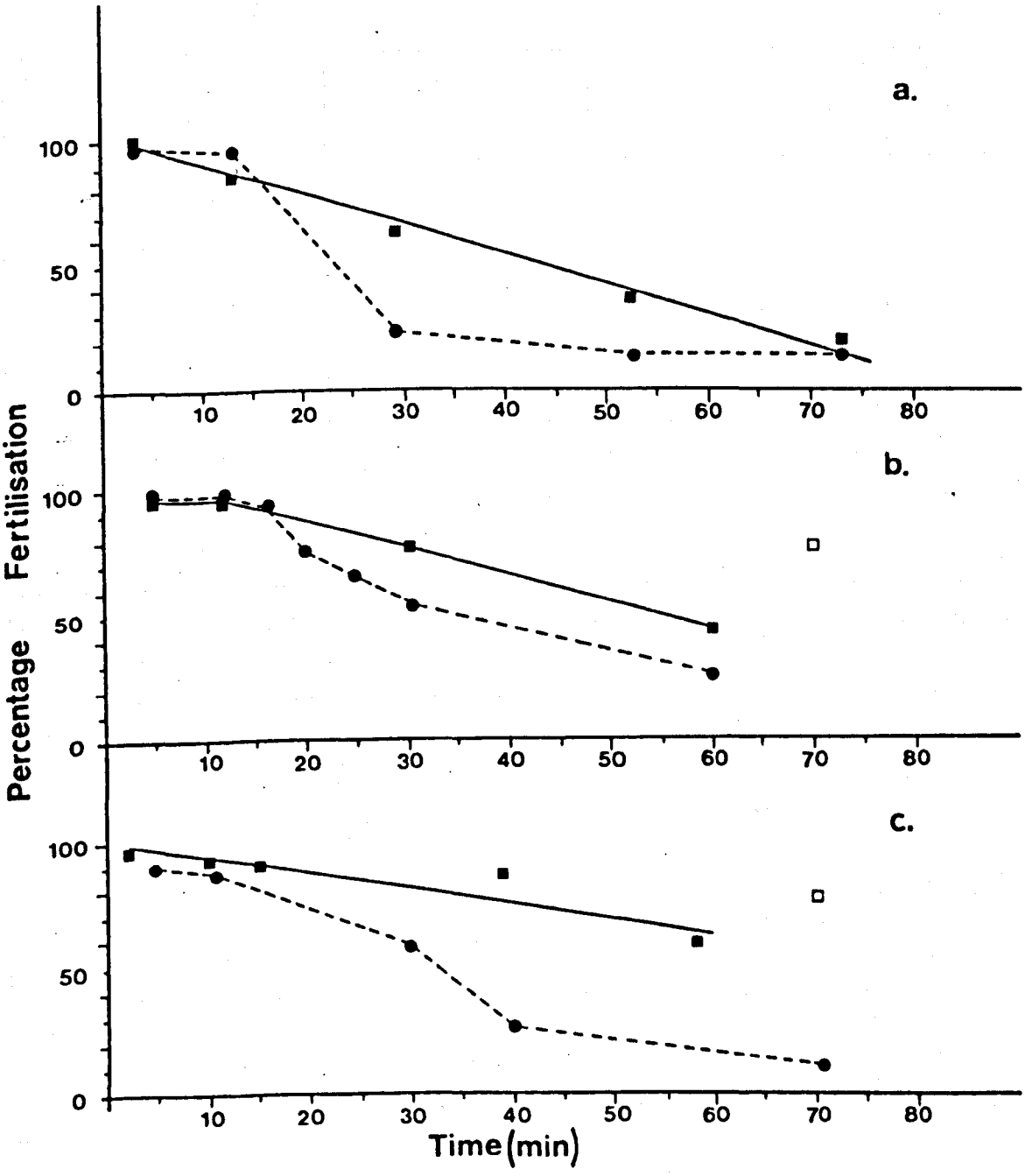
TIME-PERIOD LEFT STANDING IN OVARIAN FLUID AFTER STRIPPING	% FERTILISATION	% HATCH
0 minutes	94.6	73.5
50 minutes	87.4	64.1
127 minutes	88.5	60.9
FRESH EGGS & FRESH MILT STRIPPED AT 127 MINUTES AFTER START OF EXPERIMENT	80.7	56.9

Table 17. The percentage fertilisations and hatches obtained from eggs which had been left standing in their ovarian fluid (without seawater contact) for varying periods of time after "stripping".

Fig. 31a, b & c. The percentage fertilisations achieved in 3 separate experiments using "wet" and "dry" milt that had been left standing for different periods after stripping. Percentage fertilisations were computed by counting the number of dividing eggs in a 200 egg sub-sample.

- ● "wet" (or seawater-activated) milt
- ■ "dry" (or unactivated) milt
- □ % fertilisation obtained by the dry-stored eggs (used throughout the experiments) being inseminated with freshly-stripped milt at the end of each experiment. This procedure was carried out to check that fertility of the eggs had not declined markedly over the course of the experiments.

Fig. 31.


a.
b.
c.
Percentage Fertilisation
100
50
0
10
20
30
40
50
60
70
80
Time (min)

a) Seawater activated ("wet") milt.

TIME FROM STRIPPING AND ACTIVATION	% FERTILISATION	% HATCH	% HATCH / % FERTILISATION
3 minutes	97.4	90.7	0.93
13 minutes	94.5	93.0	0.98
28 minutes	21.8	19.7	0.90
52 minutes	13.0	13.0	1.00
73 minutes	12.2	12.2	1.00

b) Unactivated ("dry") milt.

TIME FROM STRIPPING	% FERTILISATION	% HATCH	% HATCH / % FERTILISATION
3 minutes	98.3	98.3	1.00
13 minutes	85.1	79.0	0.93
28 minutes	62.3	59.1	0.95
52 minutes	35.4	34.8	0.98
73 minutes	18.2	18.2	1.00

Table 18. Showing the ratios of percentage fertilisation : percentage hatch in the first milt-viability experiment. (Corresponding to Fig. 31a.)

RESULTS

1. PILOT STUDY:

Table 17 presents the percentage fertilisations and percentage hatches obtained from the different treatments in the pilot study. Clearly, freshly-ovulated turbot eggs can be stored in ovarian fluid outside the female's body for at least two hours and still retain their fertility.

2. MILT-VIABILITY EXPERIMENTS:

Fig.31a,b,c shows the percentage fertilisations achieved in three separate experiments using "wet" and "dry" milt that had been left standing for different periods after stripping. "Wet" milt produces good percentage fertilisation for approximately 15 minutes after stripping and activation, and then its viability declines rather quickly, levelling out at about 30-40 minutes from stripping. In contrast, the "dry" milt has a slower, constant rate of ageing. However, variations in the ageing rate occur between the milt from the three different males, especially in the "wet" milt.

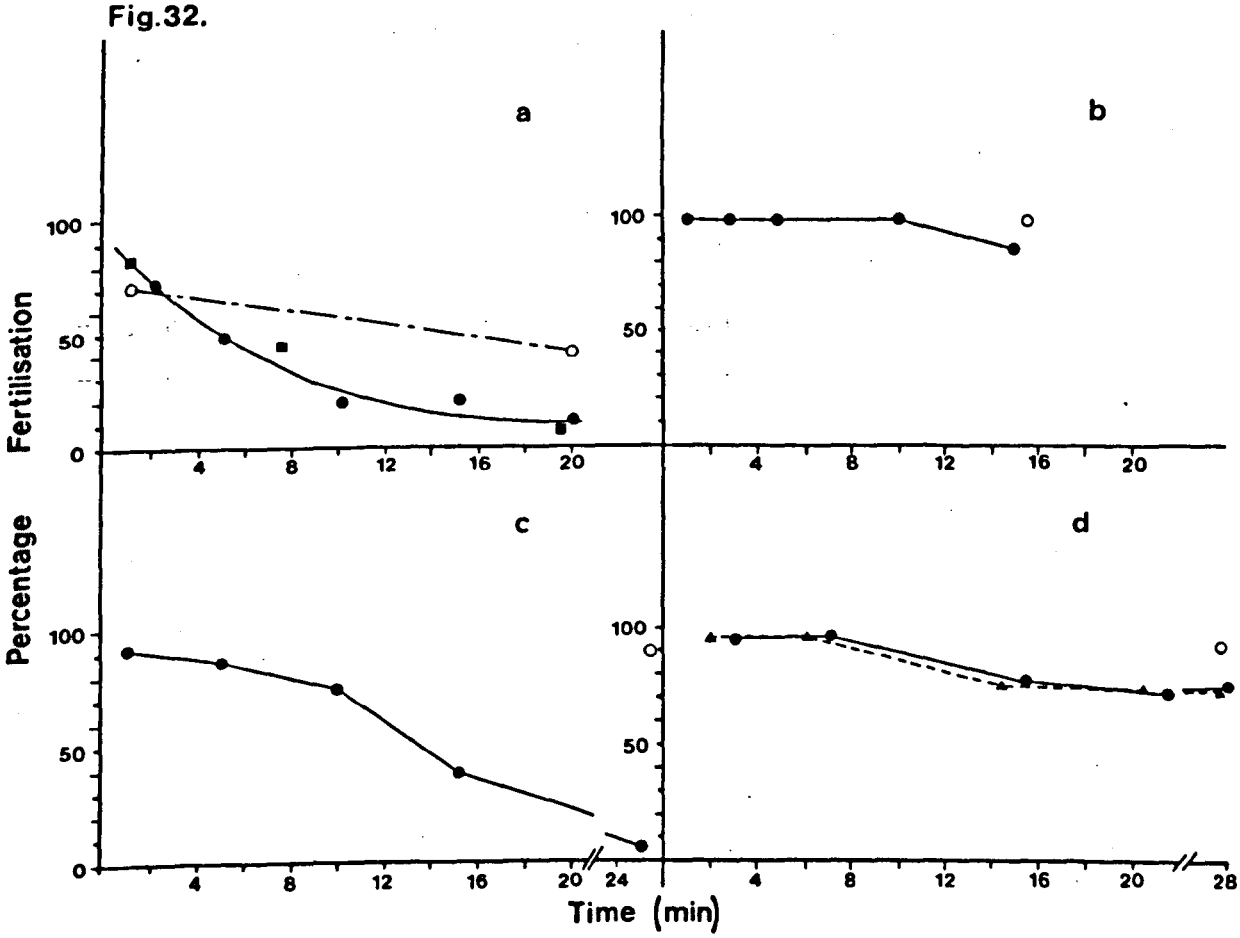
The percentage hatch: percentage fertilisation ratios were very similar for all of the milt treatments in the first experiment (see Table 18).

3. EGG-ACTIVATION EXPERIMENTS:

Fig.32 shows the rates of decline in fertility of seawater-activated turbot eggs hand-stripped from four different females. The different egg-batches appear to

Fig. 32a, b, c & d. The rates of decline in fertility of sea-water activated turbot eggs hand-stripped from 4 different females. Trial (a) compares the fertility decline rates of freshly-ovulated eggs (•) and the same eggs which had been left in the female's body for 18 to 20 hours longer (■). Trial (d) compares the fertility decline rate of eggs stripped and fertilised at 11°C (▲) with that of eggs stripped and fertilised at 15°C (•). The open circles (O) in Trial (a) indicate the percentage fertilisations obtained by inseminating freshly stripped eggs with fresh milt at the beginning and end of the experiment. (The same milt suspension was used throughout this trial). The open circles (O) in Trials (b), (c) & (d) denote the percentage fertilisations obtained from dry-stored eggs (from the same batches used in each of the experiments) being inseminated with freshly stripped milt at the end of the trials. (In these later 3 trials fresh milt suspension was made every 10 minutes to safeguard against milt ageing and losing its viability over the course of the trials).

Fig.32.


a
b
c
d
Fertilisation
Percentage
100
50
0
4
8
12
16
20
24
28
Time (min)

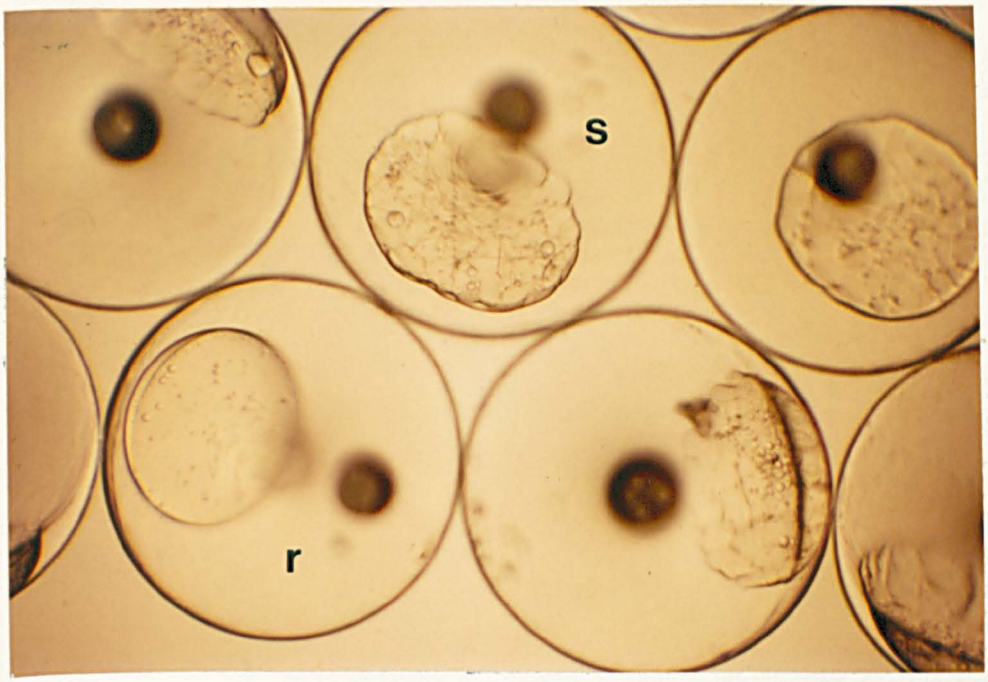


Plate XXV. Comparing water-activated and fertilised eggs.

r - water-activated egg.

S - fertilised egg (of the same age as "r") which is at the 32-64 cell stage.

have different rates of fertility-loss. The fish eggs monitored in the first experiment were especially different (see Fig 32a).

There did not appear to be any significant difference in the rate of fertility decline of freshly-ovulated eggs or of the same eggs that had been left in the female's body for 18 to 20 hours longer (see Fig. 32a). There was also no apparent difference between the fertility decline rates of eggs stripped and fertilised at two different temperatures (see Fig. 32d).

It was noted that all the "infertile" eggs in these trials had, in fact, been "water-activated". This phenomenon has been described in detail for other teleost species by Rothschild (1957), Yamamoto (1961) and many others. Basically it involves the formation of a perivitelline space, due to emptying of cortical granules (induced by water contact), but no division takes place. The cortical reaction usually renders the egg impenetrable to sperm due to closure of the micropyle (but not by a sudden internal block to sperm as in sea-urchin eggs (Yamamoto, 1961).). Plate XXV compares a freshly-ovulated "seawater-activated" egg with an egg of the same age but which has been fertilised and is at the 32 to 64 cell stage.

DISCUSSION

Yamamoto (1961) states that although anisotonicity of the spawning medium is not the sole cause of water-activation, isotonicity is important in keeping ova in a fertilisable state for longer periods. However, he points out that although goldfish eggs can retain their fertility after being stored in ovarian fluid outside the female's body for several hours, they are activated by immersion in almost isotonic Ringer's solution, though at a slower rate than in fresh water (Yamamoto, 1948 & 1954 - both cited by Yamamoto, 1961). Since similar results have been found in the dace and ayu (Kanoh & Ito, 1953; Ito, 1953 both cited by Yamamoto, 1961), Yamamoto suggests that "the eggs are in an inhibitory state because of the presence of an inhibitor either within them or in the ovarian fluid or both." It is unfortunate that in the experiments described in this section, no trials were conducted to determine whether turbot eggs are activated by isotonic Ringer's solution, due to time-limitations and the death of several spawning females.

It is quite probable that changes in pH may also play an important part in the activation reaction. For example, Billard et, al.(1974) found that higher percentage fertilisations were obtained in rainbow trout eggs when the diluant used in artificial insemination was between pH 8.5 and 9.5.

In the light of this earlier work, the results of the pilot study seem to be quite straightforward. The eggs were stored in ovarian fluid which would have had the same osmol-

arity and pH as the eggs, and which may or may not have contained an "egg-inhibitor". Therefore, no activation shock occurred and the eggs retained their capacity to be fertilised. "Dry-stripping" of eggs at the Shearwater hatchery has since proved useful when conducting insemination trials with cryopreserved milt, and helps to conserve supplies of freshly-stripped milt (see Introduction to this section).

The "water-activation" reactions described by Rothschild (1958), Yamamoto (1961) and others also helps to explain the progressive loss in percentage fertility with time in turbot eggs which were stripped directly into seawater (see Figs. 32a to d). Presumeably, as the interval between water-contact and sperm-addition increased, progressively more eggs became "water-activated" so that they were impenetrable to sperm. However, it is rather surprising that the fertility-decline rates appeared to vary between eggs from different females. The eggs used in the first egg-activation experiment had an especially different rate of fertility decline (see Fig. 32a). Since these eggs were stripped from an Easter/summer spawning female whilst the eggs monitored in Figs. 2b and c were stripped from winter-spawning females, it was feared that the lower ambient temperatures might be causing the winter eggs to have lower activation rates. Trial (d) was therefore run at two different temperatures. However, there was no significant difference between the two activation rates (see Fig. 32d), so it would appear that temperature was not the cause of the different rates of fertility-loss observed in different female's eggs. This is in contrast to Yamamoto's (1944) work with Oryzias latipes where a higher temperature caused the disintegration of cortical gra-

nules to occur at a faster rate.

It was originally thought that the rate of fertility-loss might be affected by the length of time the oocytes had been retained in the ovary after ovulation. It had been observed that freshly-ovulated eggs showed first cleavage in just under 2.5 hours from fertilisation whilst older eggs that had been retained in the ovary for about 24 hours and which only showed "fair" quality divisions (applying Lincoln's (1976) egg-quality scale), did not start to divide until about 4 hours after fertilisation. It was therefore postulated that the degree of "oocyte-overripeness" might affect the rate of water-activation in a similar manner. However, this is not supported by the results depicted in Fig. 32a. The reason for the apparent discrepancy between eggs from different females is therefore unclear.

Yamamoto (1954b) found that although the cortical reaction induced by water-activation was complete after 5 minutes in goldfish eggs, activated eggs inseminated after 5 minutes still showed 7% fertilisation (as determined by their normal development). He therefore concluded that in the goldfish the micropyle of some eggs remains open for a short period after activation, permitting sperm entry even though the cortical reaction had occurred. The results depicted in Fig. 32a to d seem to suggest that the micropyle of some turbot eggs also remains open for a period after water-activation, so permitting fertilisation to occur. If the percentage of eggs with late-closing micropyles altered between females, this might offer an explanation to the apparent difference in rates of fertility-loss in different females. However, many more trials need to be run to investigate whether this

is a real phenomenon or an artefact.

The results of the milt-viability experiments showed that the percentage of eggs fertilised with seawater-activated milt declined markedly after approximately 15 minutes, although it had remained very high until then. However, the viability of "dry", unactivated milt declined more steadily. Possibly this is a reflection of different metabolic rates in the two types of milt. Water-activation, brought about by changes in osmotic pressure (e.g., Yamamoto, 1961), changes in pH (Petit et. al.,1974 - cited by Billard et. al., 1974), or the removal of some form of inhibitor or "androgamone" (Hartmann, 1944 - cited by Rothschild, 1958), induces sperm-motility and therefore must increase the metabolic rate of the sperm. However, "dry" turbot milt is, presumeably, inactive(sperm was not observed under the microscope in its undiluted form) and therefore should have a lower metabolic rate. It therefore seems logical that "dry", unactivated milt should lose its viability at a slower, more constant rate.

Nikolsky (1963 - cited by Blaxter, 1969) suggests that sperm motility is short lived where spawning takes place in fast-flowing water (c.f., 10-15 seconds in Oncorhyncus; 230-290 seconds in sturgeon, which spawn in slower flows; and several hours or days in herring, spawning at sea). It is debateable whether turbot milt fits Nikolsky's hypothesis, although it certainly has a much longer life than milt of the freshwater species. This might, however, be due to the higher osmolarity of seawater (Yamamoto, 1961).

Since the percentage hatch : percentage fertilisation ratios achieved with turbot milt left standing for long per-

iods after stripping were just as high as those ratios obtained from milt used within a few minutes of stripping it can be concluded that "old" milt (whether "wet" or "dry") does not have a detrimental effect on subsequent egg-development provided it is capable of fertilisation.

In view of the results of the milt- and egg-activation experiments it is adviseable to add milt and eggs to seawater simultaneously if the "wet" method of fertilisation is employed. Probably the easiest way to do this is to add half the milt to the seawater-filled stripping tank; immediately hand-strip the female's eggs into the water, and then add the remainder of the milt.

Due to time-limitations, the experimental work in this section had to be cut short. There are several other experiments which need to be performed. Although stripping eggs into isotonic Ringer's solution in an attempt to prolong fertilisability may have very limited applications in general hatchery procedure (it is far easier to let the eggs stand in their ovarian fluid), it would be very interesting to investigate different methods of short-term gamete storage - for example, by applying Blaxter's (1955) method for herring gametes in which he stored them dry at 4°C for several days. Storing sperm in buffered diluants is another possibility which needs investigating. If short-term storage of turbot milt proved successful, it would not only quicken the artificial insemination procedure but would also alleviate stress to the males caused by continual stripping. Similarly, short-term storage of unfertilised eggs could produce more efficient stocking of hatched larvae. If a large batch of good eggs is stripped from a female, a significant

proportion of the hatched larvae will usually be discarded due to inadequate stocking facilities or larval food supplies. If large batches of eggs could be divided, half fertilised immediately and the rest stored for a few days prior to fertilisation, the hatching and stocking times of the larvae would thus be "staggered" and so more of the larvae could be used in the hatchery.

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CONCLUDING REMARKS

The causes and effects of variations in fish egg quality present a research field of considerable dimensions and it must be clear to the reader that the work described in this thesis has barely scratched the surface of this complex topic. This is particularly obvious in Sections II and IV where the reported experiments are no more than preliminary investigations which will hopefully provide a basis for more detailed work in the future.

Additional topics for future investigations could include a full comparison of hatchery and "wild-spawned" turbot eggs; determining the effect of different broodstock-holding conditions and diets on the viability and chemical composition of the females' eggs; techniques allowing short-term storage of gametes; the possibility of inducing "hybrid-vigour" by crossing turbot from different geographical areas (Shearwater hope that they will be able to obtain hybrid strains of European and Black Sea turbot sometime in the future); and finally, determining how the turbot's ovulation-cycles integrate into their natural spawning regime. It is difficult to anticipate how this ovulatory periodicity is adaptive to the natural environment of the turbot since the females' ovulations do not appear to be synchronised in any way. However, the very fact that this mechanism has evolved strongly suggests that it must convey some advantage to the parents and/or offspring. Therefore, before the ovulatory-cycles can be fully understood, more information is required concerning the natural spawning behaviour of turbot.

On a more academic level, elucidation of the hormonal

sequence controlling the ovulation-cycles presents a complex task in itself. It would also be very interesting to determine whether similar ovulatory-cycles occur in other batch-spawning flatfish.

APPENDIX Ia

Data concerning the relationship between the mean egg-diameter of a particular egg-batch and the mean length, yolk-sac index and the yolk sac : larval length ratio of the emergent larvae. (See Section I(2) and Figs. 5 & 6).

MEAN EGG-DIAMETER: (mm)	MEAN LARVAL LENGTH: (mm)	MEAN YOLK-SAC INDEX: (mm^2)	MEAN YSI/LL RATIO: (mm)
0.985(0.012)	2.450(0.065)	0.747(0.051)	0.305
1.023(0.007)	2.559(0.099)	0.771(0.068)	0.301
1.014(0.011)	2.519(0.049)	0.840(0.028)	0.334
1.028(0.011)	2.710(0.083)	0.783(0.071)	0.289
1.053(0.010)	2.733(0.068)	0.834(0.059)	0.305
1.004(0.009)	2.401(0.069)	0.848(0.043)	0.353
0.994(0.009)	2.379(0.058)	0.855(0.039)	0.359
1.039(0.013)	2.634(0.049)	0.836(0.030)	0.318
1.001(0.010)	2.656(0.045)	0.747(0.032)	0.281
0.997(0.009)	2.664(0.091)	0.702(0.192)	0.264
0.997(0.008)	2.506(0.078)	0.768(0.034)	0.307
1.020(0.014)	2.534(0.072)	0.864(0.050)	0.334
1.040(0.014)	2.623(0.064)	0.870(0.041)	0.332
0.979(0.010)	2.465(0.056)	0.821(0.040)	0.333
0.983(0.009)	2.478(0.050)	0.811(0.044)	0.327
0.962(0.009)	2.276(0.088)	0.765(0.036)	0.336
1.010(0.013)	2.414(0.063)	0.909(0.040)	0.377
1.035(0.008)	2.658(0.060)	0.743(0.045)	0.280
0.975(0.008)	2.563(0.083)	0.716(0.071)	0.279
0.951(0.013)	2.503(0.054)	0.704(0.056)	0.281
0.979(0.011)	2.538(0.096)	0.666(0.039)	0.263
0.978(0.009)	2.370(0.049)	0.789(0.032)	0.333

APPENDIX Ia (cont'd)

MEAN EGG-DIAMETER: (mm)	MEAN LARVAL LENGTH: (mm)	MEAN YOLK-SAC INDEX: (mm^2)	MEAN YSI/LL RATIO: (mm)
0.999(0.010)	2.459(0.059)	0.731(0.151)	0.297
1.011(0.006)	2.721(0.067)	0.790(0.039)	0.290
1.014(0.012)	2.558(0.038)	------	---
1.069(0.010)	2.676(0.044)	0.844(0.048)	0.316
0.970(0.010)	2.518(0.069)	0.656(0.040)	0.261
0.996(0.010)	2.443(0.086)	0.638(0.078)	0.261
0.947(0.010)	2.421(0.113)	0.568(0.092)	0.235
1.009(0.011)	2.589(0.136)	0.609(0.098)	0.235
0.997(0.011)	2.418(0.086)	------	---
0.946(0.013)	2.484(0.106)	0.589(0.095)	0.237
0.979(0.017)	2.578(0.108)	0.661(0.074)	0.256
0.896(0.007)	2.503(0.096)	------	---
1.040(0.009)	2.756(0.084)	0.753(0.045)	0.273
1.046(0.007)	2.681(0.067)	0.809(0.041)	0.302
1.062(0.012)	2.683(0.065)	0.913(0.063)	0.340
1.050(0.012)	2.820(0.098)	0.771(0.079)	0.274
0.991(0.007)	2.666(0.104)	0.735(0.069)	0.276
0.997(0.012)	2.686(0.078)	0.724(0.079)	0.270
0.998(0.007)	2.455(0.080)	0.832(0.053)	0.339
0.981(0.010)	2.498(0.090)	0.751(0.078)	0.301

APPENDIX Ib

Data from experiments comparing growth of early and late season larvae. (See Section I(4) and Figs. 7a, b & c).

FEMALE I (data corresponding to Fig. 7a)

Early Season Larvae: eggs stripped on 11-11-80; mean egg-diameter = 1.062(0.012)

TIME FROM FIRST HATCH (hours)	MEAN LARVAL LENGTH (mm)	MEAN YOLK-SAC INDEX (mm^2)
3.0	2.683(0.065)	0.913(0.063)
27.5	3.118(0.052)	0.627(0.082)
58.0	3.348(0.085)	0.404(0.031)
★ 82.0	3.539(0.072)	0.129(0.043)
	3.534(0.109)	0.110(0.018)
	3.523(0.101)	0.119(0.119)
127.5	3.968(0.117)	Depleted
	3.898(0.112)	"
	3.976(0.080)	"
151.5	4.124(0.151)	"
	4.243(0.208)	"
	4.233(0.142)	"
173.5	4.454(0.256)	"
	4.449(0.134)	"
	4.518(0.208)	"
202.0	4.921(0.274)	"
	4.933(0.234)	"
	4.603(0.268)	"
226.0	4.959(0.274)	"
	5.161(0.273)	"
	4.666(0.292) - none remaining	"

Late Season Larvae: eggs stripped on 13-12-80; mean egg-diameter = 0.998(0.007)

TIME FROM FIRST HATCH (hours)	MEAN LARVAL LENGTH (mm)	MEAN YOLK-SAC INDEX (mm^2)
4.5	2.455(0.080)	0.801(0.052)
29.75	2.823(0.134)	0.409(0.044)
49.0	2.964(0.113)	0.338(0.111)

FEMALE I, Late Season Larvae: (cont'd)

TIME FROM FIRST HATCH (hours)	MEAN LARVAL LENGTH (mm)	MEAN YOLK-SAC INDEX (mm^2)
★74.5	3.224(0.034) 3.216(0.084) 3.218(0.050)	0.088(0.017) 0.088(0.009) 0.086(0.007)
124.0	3.691(0.110) 3.626(0.249) 3.660(0.138)	Depleted " "
144.75	3.949(0.194) 3.843(0.347) 3.895(0.213)	" " "
198.75	4.433(0.390) 4.919(0.234) One tank collapsed.	" "
217.0	4.510(0.338) 4.755(0.348)	" "

FEMALE II (data corresponding to Fig. 7b)

Early Season Larvae: eggs stripped on 18-11-80; mean egg-diameter = 1.050(0.012)

TIME FROM FIRST HATCH (hours)	MEAN LARVAL LENGTH (mm)	MEAN YOLK-SAC INDEX (mm^2)
3.0	2.820(0.098)	0.771(0.079)
32.0	3.201(0.073)	0.557(0.092)
51.0	3.379(0.050)	0.365(0.050)
★73.5	3.510(0.069) 3.500(0.063) 3.473(0.038)	0.088(0.015) 0.087(0.010) 0.081(0.012)
104.0	3.825(0.063) 3.783(0.084) 3.700(0.092)	Depleted " "
148.5	4.155(0.182) 4.201(0.130) 3.990(0.187)	" " "
205.0	4.456(0.210) 4.586(0.395) 4.500(0.305)	" " "
246.75	4.632(0.237) 4.611(0.251) One tank collapsed.	" "

FEMALE II

Late Season Larvae: eggs stripped on 11-12-80; mean egg-diameter = 0.997(0.001)

TIME FROM FIRST HATCH (hours)	MEAN LARVAL LENGTH (mm)	MEAN YOLK-SAC INDEX (mm^2)
6.0	2.686(0.078)	0.724(0.079)
33.0	2.924(0.066)	0.449(0.062)
58.5	3.156(0.087)	0.241(0.096)
★79.5	3.404(0.083) 3.451(0.085) 3.428(0.034)	0.092(0.015) 0.087(0.011) 0.087(0.010)
103.5	3.586(0.081) 3.570(0.052) 3.577(0.061)	Depleted " "
151.5	4.099(0.198) 4.185(0.100) One tank collapsed.	" "
173.5	4.190(0.281) 4.451(0.112)	" "
227.5	4.583(0.284) 5.135(0.305)	" "
242.5	5.303(0.265) 5.413(0.288)	" "

FEMALE III (data corresponding to Fig. 7c)

Early Season Larvae: eggs stripped on 7-11-80; mean egg-diameter = 1.046(0.001)

TIME FROM FIRST HATCH (hours)	MEAN LARVAL LENGTH (mm)	MEAN YOLK-SAC INDEX (mm^2)
3.0	2.681(0.067)	0.809(0.041)
24.5	3.055(0.065)	0.554(0.025)
51.5	3.301(0.096)	0.356(0.030)
★76.0	3.256(0.112) 3.485(0.196) 3.549(0.053)	0.092(0.012) 0.100(0.022) 0.087(0.008)
103.0	3.703(0.139) 3.685(0.097) 3.674(0.073)	Depleted " "

FEMALE III, Early Season Larvae: (cont'd)

TIME FROM FIRST HATCH (hours)	MEAN LARVAL LENGTH (mm)	MEAN YOLK-SAC INDEX (mm^2)
146.5	4.096(0.284)	Depleted
	4.135(0.184)	"
	4.024(0.167)	"
174.0	4.428(0.332)	"
	4.389(0.109)	"
	4.366(0.078)	"
200.0	4.544(0.044)	"
	4.801(0.217)	"
	4.686(0.377)	"
219.0	4.961(0.290)	"
	4.940(0.326)	"
	One tank collapsed.	
245.0	5.334(0.508)	"
	5.301(0.465)	"

Late Season Larvae: eggs stripped on 10-12-80; mean egg-diameter = 0.991(0.001)

TIME FROM FIRST HATCH (hours)	MEAN LARVAL LENGTH (mm)	MEAN YOLK-SAC INDEX (mm^2)
3.5	2.666(0.104)	0.735(0.069)
57.0	3.328(0.110)	0.275(0.033)
★82.0	3.398(0.115)	0.063(0.010)
	3.531(0.048)	0.063(0.008)
	3.462(0.094)	0.063(0.001)
101.0	3.515(0.110)	Depleted
	3.645(0.092)	"
	One tank collapsed.	
125.5	3.688(0.137)	"
	3.830(0.080)	"
175.0	4.169(0.158)	"
	4.305(0.230)	"
196.5	4.531(0.167)	"
	4.534(0.410)	"

★ = larvae transferred into triplicate experimental tanks.

Standard deviations in parentheses.

APPENDIX Ic

Data concerning the relationship between the size of a female turbot and her maximum mean egg-diameter in that particular spawning season. (See Section I(5) and Figs. 8 & 9.)

MAX. MEAN EGG-DIAMETER (mm)	WEIGHT OF MOTHER (kg)	LENGTH OF MOTHER (cm)	CONDITION INDEX (K)
0.990(0.013)	2.16	43.3	2.66
1.065(0.017)	6.70	62.5	2.74
1.028(0.008)	6.20	61.2	2.70
1.053(0.010)	2.27	45.5	2.41
1.041(0.009)	4.80	58.5	2.40
1.027(0.020)	4.30	56.7	2.36
1.024(0.010)	4.77	53.0	3.20
1.067(0.008)	5.30	60.5	2.39
1.035(0.013)	4.10	57.1	2.20
0.999(0.010)	3.52	50.3	2.77
1.050(0.015)	5.60	58.2	2.84
1.030(0.013)	4.50	55.5	2.63
1.018(0.020)	3.98	53.2	2.64
1.010(0.011)	2.50	40.3	3.82
0.974(0.025)	4.55	54.8	2.76
1.028(0.011)	5.60	62.5	2.29
1.033(0.012)	6.70	64.0	2.56
1.053(0.010)	4.40	55.0	2.64
1.050(0.009)	5.70	57.5	3.08
1.039(0.013)	5.50	60.0	2.55
1.065(0.015)	6.10	61.0	2.69
1.078(0.011)	7.10	62.0	2.98
0.986(0.009)	2.80	40.0	4.38
1.015(0.010)	6.20	59.3	2.97

APPENDIX Ic (cont'd)

MAX. MEAN EGG-DIAMETER (mm)	WEIGHT OF MOTHER (kg)	LENGTH OF MOTHER (cm)	CONDITION INDEX (K)
0.980(0.013)	4.20	50.5	3.26
0.992(0.015)	1.90	40.0	2.97
1.031(0.012)	3.10	46.2	3.14
0.959(0.010)	2.30	43.6	2.78
0.999(0.020)	3.10	50.0	2.48
1.002(0.011)	5.50	58.5	2.75
1.004(0.006)	3.70	52.8	2.51
1.037(0.012)	4.50	57.0	2.43
0.947(0.009)	3.60	53.7	2.32
1.040(0.009)	5.60	59.3	2.69
1.010(0.015)	3.00	54.0	1.91
1.034(0.017)	4.70	57.1	2.52
0.962(0.009)	5.30	56.0	3.02
1.036(0.016)	5.00	56.5	2.77
1.021(0.011)	3.00	53.5	2.35
1.019(0.010)	3.60	54.0	2.29
1.023(0.007)	4.50	59.0	2.19
1.040(0.009)	6.50	61.5	2.79
1.060(0.008)	7.30	60.5	3.30
1.056(0.010)	7.00	59.5	3.32
1.050(0.012)	7.00	64.0	2.67
1.062(0.012)	8.50	63.0	3.40

APPENDIX Id

Estimations of captive turbot fecundity which take the seasonal decline in egg-diameter into consideration.

FEMALE'S BRAND	BODY WEIGHT (kg)	FECUNDITY
VVO	5.60	1.049×10^6 eggs
V	2.16	0.879×10^6 eggs
VVV	4.50	2.286×10^6 eggs
VIV	5.00	2.827×10^6 eggs
XC	2.27	2.615×10^6 eggs
LPM	6.00	4.735×10^6 eggs
BWM	5.40	3.402×10^6 eggs
Black Spot	4.30	3.080×10^6 eggs
101	3.90	0.994×10^6 eggs
White Fleck	4.00	1.884×10^6 eggs
Black Bar	4.20	1.605×10^6 eggs
VCV	4.50	4.095×10^6 eggs
Black Fleck	5.90	2.567×10^6 eggs
X	3.52	3.449×10^6 eggs
CCI	3.60	3.852×10^6 eggs
XXO	5.50	2.475×10^6 eggs
VΛ	4.77	1.813×10^6 eggs
VVI	4.10	2.173×10^6 eggs

(See Section I(7) and Fig. 10).

APPENDIX IIa

Data from the Marsh and Weistein standard plot of lipid weight versus optical density.

Blanks =	0.00	20µg T =	0.275
	0.00		0.265
	0.00		0.270
5µg T =	0.06	28µg T =	0.41
	0.06		0.37
	0.06		0.39
10µg T =	0.130	40µg T =	0.55
	0.150		0.52
	0.135		0.60

(where T = tripalmitin standard).

A linear regression line was fitted to the data. The relationship between lipid weight and optical density is expressed by the equation:

$$y = 0.0141\,x - 0.00529$$

(i.e., when $x = 40$; $y = 0.5577$)

(and when $x = 20$; $y = 0.2760$)

The correlation coefficient (r) = 0.9997

APPENDIX IIb

A print-out from the Supergrator III computing integrator.

(The names of the fatty acids were added by hand later).

(Female III, phospholipid fraction.)

RUN PARAM

INIT PK WDTH	0.1
MIN PK SIZE	200.
SENSITIVITY	70.

TIMED EVENTS

TYPE	TIME	VALUE
2	0.01	
3	2.00	

CALC PARAM

TYPE CALC	1
TYPE RESULTS	1
SYS FACTOR	1.
MIN REPORT	0.2

IDENT PARAM

CAL TABLE NO	0

RAW DATA

NO	TIME	AREA / HEIGHT		
1	2.75	5365	TT	
		657		
2	3.09	1607	TT	14:0
		175		
3	4.08	873	TT	
		82		
4	5.25	27566		16:0
		1626		
5	5.85	3320	TT	16:1ω9+7
		202		
6	7.04	2971	BV	
		131		
7	9.22	8300	BV	18:0
		275		
8	10.13	18306	VV	18:1ω9+7
		553		
9	12.38	3118	VB	18:2ω6
		58		
10	16.55	1291	BV	18:3ω3
		27		
11	17.91	1477	VB	20:1ω9+7
		28		
12	20.69	239	BV	
		9		
13	21.27	1594	VV	18:4ω3
		11		
14	25.90	5184	VB	20:4ω6
		61		
15	32.36	10187	BV	20:5ω3
		97		+ 22:1ω11,9+7
16	34.93	281	VV	
		9		
17	35.70	1904	VB	22:5ω3
		25		
18	54.11	225	VV	
		5		
19	63.79	31117		22:6ω3
		182		
20	70.35	409		
		3		
TOTAL		125334		

NORMALIZATION

NO	TIME	PCT CONC		
1	2.75	4.281	TT	
2	3.09	1.283	TT	14:0
3	4.08	0.697	TT	
4	5.25	22.000		16:0
5	5.85	2.650	TT	16:1ω9+7
6	7.04	2.371	BV	
7	9.22	6.623	BV	18:0
8	10.13	14.610	VV	18:1ω9+7
9	12.38	2.488	VB	18:2ω6
10	16.55	1.031	BV	18:3ω3
11	17.91	1.179	VB	20:1ω9+7
13	21.27	1.272	VV	18:4ω3
14	25.90	4.136	VB	20:4ω6
15	32.36	8.128	BV	20:5ω3
16	34.93	0.225	VV	+ 22:1ω11,9+7
17	35.70	1.520	VB	22:5ω3
19	63.79	24.830		22:6ω3
20	70.35	0.327		
TOTAL		99.630		

minus spurious peaks = 90.467

APPENDIX III

Data from the ovulation-prediction charts described in Section III. (See also Figs. 16-21 and Table 12).

Female "XO"

Time from Start of Experiment (hours)	% Fertilisation
0.0	89.2
22.0	75.3
44.5	0.0
69.0	0.0
98.5	80.8
120.0	48.0
143.0	21.4
168.0	0.0
191.0	55.0
212.0	83.0
236.5	6.5
261.5	0.0
285.0	0.0

Female "O "

Time from Start of Experiment (hours)	Description of Eggs
0.0	all sunken
21.5	fair/poor
48.5	s.i.
74.0	dimpled
93.5	good
120.0	fair/poor
149.0	s.i./dimpled
173.0	all sunken
194.5	good
221.0	s.i.
241.0	s.i./dimpled
270.0	fair/poor
290.0	s.i.
315.0	dimpled
355.0	no eggs - fish spent

Female "XC"

Time from Start of Experiment (hours)	% Fertilisation	% Hatch
0.0	98.0	88.0
26.0	59.4	0.0
50.8	15.7	0.0
52.5	0.0	0.0
55.0	90.6	89.0
75.0	42.8	42.6
99.5	0.0	0.0
127.0	67.7	60.0
147.5	40.0	0.0
170.0	0.0	0.0

Female "XC" (cont'd)

Time from Start of Experiment (hours)	% Fertilisation	% Hatch
192.0	73.10	62.7
219.0	83.70	0.0
243.5	97.40	96.0
267.0	74.11	73.1

Female "XXX"

Time from Start of Experiment (hours)	% Fertilisation	% Hatch
0.0	78.55	70.0
21.5	21.85	0.0
44.0	0.00	0.0
68.5	90.50	65.5
119.5	0.00	0.0
142.5	98.00	95.5
167.5	50.00	0.0
190.5	0.00	0.0
211.5	96.90	96.5
236.0	52.50	0.0

Female "II"

Time from Start of Experiment (hours)	% Fertilisation	% Hatch
0.0	85.60	0.0
45.8	0.00	0.0
71.3	95.25	64.6
96.0	36.95	0.0
120.3	0.00	0.0
144.3	81.30	74.9
192.8	0.00	0.0
215.3	0.00	0.0
237.5	68.40	22.3
263.8	63.60	0.0
308.3	42.30	40.0
311.8	95.50	91.0

Female "VVV"

Time from Start of Experiment (hours)	% Fertilisation	% Hatch
0.0	0.00	0.0
24.5	84.25	79.1
50.5	38.90	0.0
71.8	0.00	0.0
96.5	0.00	0.0
121.0	85.10	75.3
169.0	0.00	0.0

Female "VVV" (cont'd)

Time from Start of Experiment (hours)	% Fertilisation	% Hatch
192.5	98.5	93.3
219.0	48.6	0.0
243.0	0.0	0.0
267.0	0.0	0.0
289.0	94.0	90.0
313.0	23.5	0.0
360.5	98.3	86.9
389.0	10.0	0.0
408.5	0.0	0.0

Female "VCV" (1980)

Time from Start of Experiment (hours)	% Fertilisation	% Hatch
0.0	74.2	26.9
24.5	0.0	0.0
50.5	96.4	96.4
71.8	90.6	5.0
96.5	45.0	0.0
121.0	89.2	79.0
172.0	95.7	91.5
192.5	90.5	58.6
219.0	0.0	0.0
243.0	88.1	61.4
267.0	64.1	0.3
289.0	0.0	0.0
313.0	94.0	64.3
360.5	85.5	16.7
389.0	93.1	0.0
408.5	50.0	0.0

Female "Black Spot" (1980) - only two ovulations monitored.

Time from Start of Experiment (hours)	% Fertilisation	% Hatch
0.0	80.3	56.6
25.5	53.9	0.0
46.0	0.0	0.0
70.5	95.3	18.8
97.5	28.2	0.0
123.5	0.0	0.0
145.5	0.0	0.0

Female "XCX"

Time from Start of Experiment (hours)	Description of Eggs
0.0	good-fair
50.0	s.i./dimpled
76.0	very good
100.5	fair/s.i.
122.0	s.i./dimpled
146.0	s.i./dimpled
167.0	very good
218.0	s.i./dimpled
240.0	no eggs
265.5	good
309.0	s.i./dimpled
338.0	no eggs - spent

Female "VIV"

Time from Start of Experiment (hours)	% Fertilisation	% Hatch
0.0	76.1	0.0
24.0	30.0	0.0
48.0	98.5	94.8
69.5	81.2	0.0
92.5	57.4	0.0
114.5	12.9	9.7
119.0	98.8	97.0
142.5	80.2	0.0
167.0	0.0	0.0
187.0	0.0	0.0
210.0	94.5	88.9
260.5	0.0	0.0
284.5	93.1	88.0
307.0	74.5	0.8
331.5	40.0	0.0
356.5	0.0	0.0
377.8	98.2	85.7
402.5	69.5	0.0
427.0	20.0	0.0
458.5	96.4	96.0
498.8	79.3	0.0

Female "VCV" (1981)

Time from Start of Experiment (hours)	Description of Eggs
0.0	poor
24.5	s.i./dimpled
42.5	good
92.5	poor/s.i.
114.0	good
165.0	all sunken
170.0	all sunken
175.0	good
240.0	s.i./dimpled
250.0	good
260.0	poor

Female "CO"

Time from Start of Experiment (hours)	% Fertilisation	% Hatch
0.0	83.2	0.0
23.0	8.0	0.0
47.0	80.0	71.5
98.0	10.0	0.0
121.0	0.0	0.0
146.0	92.8	1.1
169.0	56.3	0.0
190.0	30.0	0.0
208.0	0.0	0.0
214.5	40.0	?
238.5	85.9	41.5
262.0	10.0	0.0
288.5	0.0	0.0

Female "VVI"

Time from Start of Experiment (hours)	% Fertilisation	% Hatch
0.0	0.0	0.0
22.5	96.5	95.5
48.0	86.1	0.0
69.0	0.0	0.0
95.0	92.6	53.6
117.0	0.0	0.0
143.5	99.3	95.6
165.0	84.5	17.1
192.5	0.0	0.0

	Description of Eggs
260.0	poor (approx. 30-40 % fertilisation)
267.0	s.i./dimpled
285.0	dimpled
311.5	dimpled
333.0	dimpled
358.0	good
382.0	s.i./dimpled
406.5	very good
454.5	fish spent

(This female appeared to "slip out of step" in her final ovulations, possibly due to stress because she had been heavily stripped earlier in the season).

Female "LPM"

Time from Start of Experiment (hours)	% Fertilisation	% Hatch
0.0	73.1	0.0
22.5	0.0	0.0
46.5	72.0	70.0
51.5	99.8	95.3

Female "LPM" (cont'd)

Time from Start of Experiment (hours)	% Fertilisation	% Hatch
69.5	82.2	25.4
99.0	10.0	0.0
125.0	0.0	0.0
147.5	100.0	80.0
168.5	61.4	0.0
190.0	0.0	0.0
216.0	45.0	42.3
218.5	96.9	93.7

Female "BWM" (1980) - only 2 ovulations monitored.

Time from Start of Experiment (hours)	% Fertilisation	% Hatch
0.0	95.7	78.1
23.0	78.2	0.0
46.5	0.0	0.0
68.0	0.0	0.0
91.5	98.0	82.6
149.0	0.0	0.0
165.5	0.0	0.0
190.0	53.0	0.0
212.5	0.0 spent	0.0

Female "Black Bar"

Time from Start of Experiment (hours)	Description of Eggs
0.0	good
21.5	poor
49.0	s.i./dimpled
142.0	good-fair
169.0	s.i./dimpled
192.5	dimpled
214.0	good
287.0	very good
360.5	s.i./dimpled
408.5	s.i./dimpled
454.0	s.i.
0.0	good-fair
26.3	s.i.
47.8	s.i./dimpled
96.3	fair/s.i.
120.3	poor/s.i.
170.3	good
266.8	s.i.
290.3	dimpled
312.3	very good
336.8	fair/poor

Female "BWM" (1981)- only 2 ovulations monitored.

Time from Start of Experiment (hours)	Description of Eggs
0.0	s.i./dimpled
22.0	good
69.3	s.i./dimpled
95.5	dimpled
117.0	very good (just ovulated)
165.5	s.i./dimpled
194.5	dimpled
239.5	all sunken
266.5	all sunken fish spent

Female "Black Fleck" (1981)

Time from Start of Experiment (hours)	Description of Eggs
0.0	good
27.0	s.i./dimpled
147.0	good/s.i.
169.0	s.i.
216.3	all sunken
242.5	good
264.0	fair
312.5	all sunken
336.5	all sunken
341.5	good (just ovulated)
386.5	fair
411.5	dimpled
435.0	all sunken
483.0	all sunken
506.5	no eggs - fish spent

Each of the percentage fertilisation and hatch numbers is a mean of duplicates. Plates XV to XVIII show the appearance of eggs defined as good, poor, spotted infertile (s.i.), dimpled and sunken and indicate their approximate ages.

APPENDIX IVa

Data from milt-viability experiments. (See Section IV(2) and Figs. 31a, b & c).

i). For data from experiment performed on 7-11-80, see Tables 18a & b.

ii). 10-12-80 - corresponding to Fig. 31b:

"WET" MILT:		"DRY" MILT:	
Age:	% Fertilisation:	Age:	% Fertilisation:
5 mins.	97.5	5 mins.	97.0
12 mins.	97.5	12 mins.	96.5
16 mins.	92.9	30 mins.	75.5
20 mins.	75.5	60 mins.	42.4
25 mins.	65.8		
30 mins.	54.5		
60 mins.	25.1		

(Fresh milt, used to fertilise the dry-stored eggs after 70 minutes from the start of the experiment, gave 77.2% fertilisation).

iii). 11-12-80 - corresponding to Fig. 31c:

"WET" MILT:		"DRY" MILT:	
Age:	% Fertilisation:	Age:	% Fertilisation:
5 mins.	89.7	2 mins.	96.0
12 mins.	87.0	10 mins.	92.4
30 mins.	58.2	15 mins.	90.4
40 mins.	27.4	39 mins.	87.4
70 mins.	11.1	58 mins.	59.3

(Fresh milt, used to fertilise the dry-stored eggs after 70 minutes from the start of the experiment, gave 75.9% fertilisation).

APPENDIX IVb

Data from egg-activation experiments. (See Section IV(3) and Figs. 32a, b, c & d.)

i). Female "XC" - corresponding to Fig. 32a. (22-6-80).

Time-lapse Between Water Contact And Insemination (mins).	% Fertilisation
2	73.2
5	48.7
10	17.2
15	20.2
20	11.2

(Freshly stripped eggs used to test milt-viability:
beginning of experiment - 70.7% fertilisation
end of experiment - 40.3% fertilisation).

(23-6-80 - i.e., one day over-ripe eggs from Female "XC")

Time-lapse Between Water Contact And Insemination (mins).	% Fertilisation
1	83.7
7.5	44.9
19.5	7.7

ii). Female "Black Bar" - corresponding to Fig. 32b. (27-11-80)

Time-lapse Between Water Contact And Insemination (mins).	% Fertilisation
1	98.7
3	98.8
5	98.8
10	96.9
15	86.2

(Dry-stored eggs fertilised with freshly stripped milt at the end of the experiment gave 97.5% fertilisation).

iii). Female "Black Fleck" - corresponding to Fig. 32c. (28-11-80)

Time-lapse Between Water Contact And Insemination (mins).	% Fertilisation
1	92.3
5	86.7
10	74.3
15	38.8
25	5.6
30	4.6

(Dry-stored eggs fertilised with freshly stripped milt at the end of the experiment gave 83.16% fertilisation.

iv). Experiment to investigate the effect of temperature on the rate of the water-activation reaction - corresponding to Fig. 32d.

14.5 - 16°C

Time-lapse Between Water Contact And Insemination (mins).	% Fertilisation	
3.0	95.3 96.0	95.65
7.0	96.9 95.9	96.40
15.5	74.7 79.6	77.15
21.5	76.5 67.3	71.90
28.0	72.7 74.2	73.45

11°C

Time-lapse Between Water Contact And Insemination (mins).	% Fertilisation	
2.0	94.2 96.9	95.55
6.0	94.3 97.4	95.90

Temperature Experiment (cont'd)

Time-lapse Between Water Contact And Insemination (mins).	% Fertilisation	
14.5 . . .	75.8 72.1	73.95
20.5 . . .	71.4 74.0	72.7
27.5 . . .	73.47 70.43	71.95

(Dry-stored eggs (kept at 11°C) fertilised with freshly stripped milt at the end of the experiment gave 91.5
88.7 } 90.10% fertilisation).