CHEMORECEPTION IN LARVAL HERRING AND PLAICE

A Thesis Presented for the Degree of Doctor of Philosophy

in the University of Stirling

By

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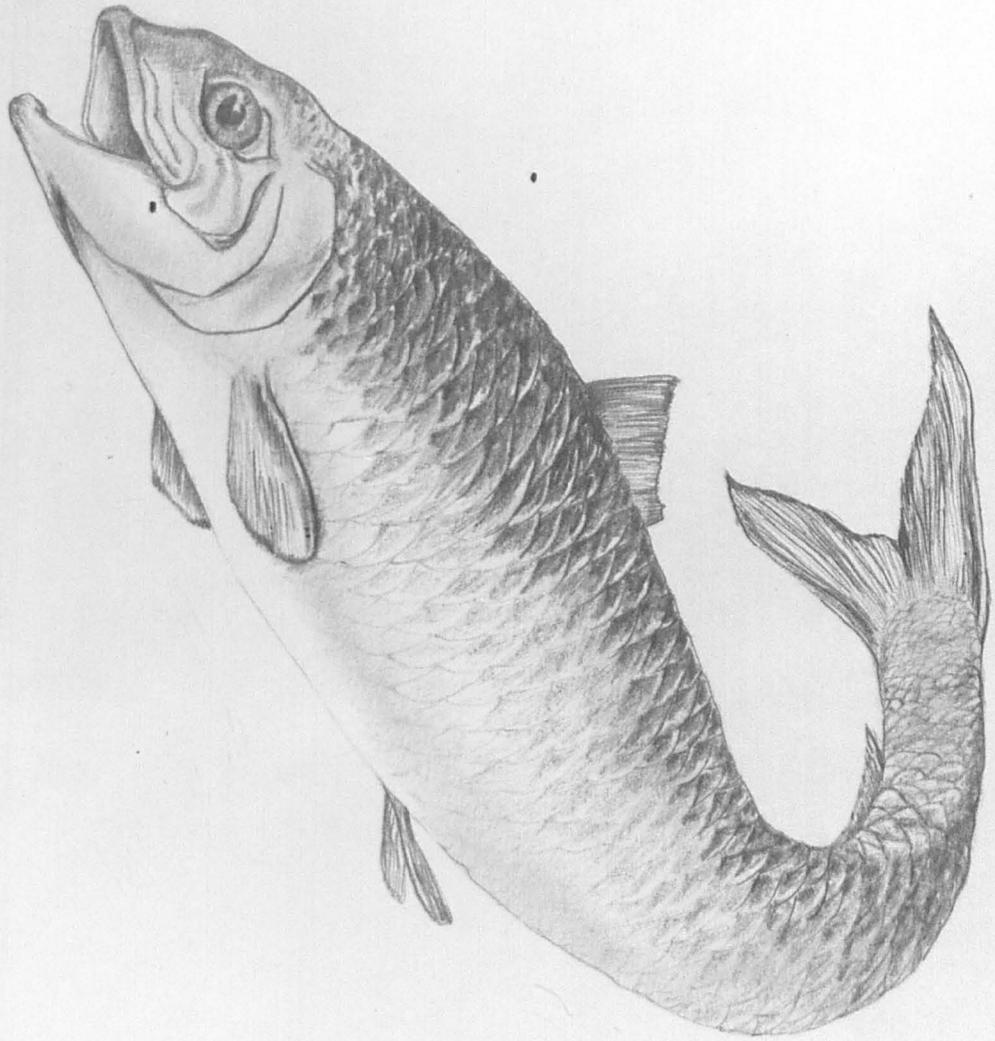
And it was only by deep knowledge that a man might come to
know the ways of the herring .... and if he had this he would
become known amongst the others as a lucky man.

Naomi Mitchison, 'The Big House'.

ACKNOWLEDGEMENTS

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INTRODUCTION

The fact that fish possess a sense of smell has been known for some time. Fabricus (1780) described how lampreys and sharks would follow rotting meat and Monro (1744) demonstrated that fish would react to a worm in the water and show adaptation to its odour. Bateson (1890) showed feeding responses in both elasmobranchs and teleosts to hidden food and juices squeezed through cloth. These reactions are present in both seeing and blind fish. Copeland (1912) and Parker (1914) showed that dogfish were able to localize hidden food by olfaction, thus proving that it could be a directional sense.

Initially the study of chemosense had been performed by observing the occurrence of a definite feeding reaction either as a result of detecting food odour or to extend the type of stimulus studied when the reaction had been conditioned to another stimulus (Göz, 1941; Teichmann, 1959). Conditioning is a long process and requires a suitably hardy species. In many cases where a stimulus not connected with feeding is used to condition a feeding reaction we may learn of sensory acuity in absolute terms but nothing of the natural use of the reaction. To examine more subtle reactions to stimuli, especially those not related to feeding, specialized apparatus had to be developed. This allowed workers to look at how a response would be used by the fish in nature.

As well as showing reactions to food and prey organisms fish have been shown to use olfaction in social behaviour both with other species and conspecifics of both sexes. Göz (1941) managed to condition a single, blinded, minnow Phoxinus phoxinus to show a feeding reaction to the odour of another fish species Ictalurus nebulosus not the prey of the minnow. This took many weeks using even the easily-trained minnow. Because of the difficulties and

limitations many workers have adopted more direct approaches,
Wrede (1932) found blinded minnows (a shoaling species) preferred
to visit a compartment in an aquarium where the odour of a
conspecific lingered rather than a control compartment. Hemmings
(1966a) used a more complex preference trough derived from that of
Shelford and Allee (1914) and found that the shoaling, freshwater
roach (Rutilus rutilus) showed a preference for the end which had
the odour of other roach. By analysis of movement in the trough
he showed that this preference was due to increased turning rate.
Investigating these pheromones in char, Døving, Nordeng and Oakley
(1974) used electrophysiological methods, recording electrical
impulses from the olfactory tract, and found the char was able to
identify racial differences in this social identifier. Døving,
Enger and Nordeng (1973) proposed a component of mucus to be the
pheromone.

Interest in amino acids as a possible stimulus to feeding in
marine organisms began when Steven (1959) found that glutamic acid
produced a feeding reaction in two species of tropical marine fish.
Case and Gwilliam (1961) found that a range of amino acids would
stimulate a blinded crab to feed when applied to the chelae. This
reaction was confirmed electrophysiologically on isolated dactyl
preparations, responses being obtained from the dactyl receptors to a
range of amino acids. Many workers including Hara et al (1973),
Hashimoto et al (1968), Haynes et al (1967) and Suzuki and Tucker
(1971) have found similar reactions from the olfactory nerves of
teleosts. The long, easily accessible olfactory tract in some
teleost species makes them ideal subjects for electrophysiological
investigation. The technique of monitoring nerve impulses enables
a large number of amino acids to be rapidly tested; the thresholds
obtained however may not be those which will stimulate a feeding

reaction and hardy species are needed. Most work on chemosense in teleosts has been performed on adult and juvenile fish; this is not surprising since rearing beyond the non-feeding yolk sac stage of many important marine species has only been successful in the last decade.

The histology and morphology of the development of the olfactory system has also received little study since Holm (1894) described this process in Salmo salar. He showed that in Salmo salar, which took 90 days from fertilization to hatch, there was no nervous connection between epithelium and brain at 60 days post fertilization but one was found at 83 days post fertilization. The olfactory nerve appeared as the groove closed. Attention has been drawn to this lack of knowledge by Hasler (1957), Johnson and Brown (1962) and Branson (1963). In many of the teleosts so far studied the olfactory system is undergoing development not only for the whole of larval life but beyond into juvenile development. Larval development is a valuable time to study this, and indeed any organ system, since it is changing in physical structure, increasing in complexity and possibly changing in acuity and function.

The role of a larva can be in many cases to give the early stages of an organism a different ecological niche from the adult, preventing intraspecific predation and competition for food. Therefore its senses may be used for different purposes to the adult, (for example the use of chemosense and touch in the settlement of Balanus nauplii (Crisp,1974) a system obviously of no use to the adult). In the case of a nektonic shoaling fish such as the herring with a planktonic non-shoaling larva there would be a possibility of larvae dispersing over a large area prior to onset of shoaling behaviour. There would obviously be some value in keeping larvae together in loose groups and it is likely chemosense may play some part in this. Aggregations of

planktonic marine organisms are a well documented phenomena (Barnes and Marshall, 1951; Weibe & Holland, 1968) from the longevity of these aggregations it seems unlikely that this is caused by physical boundaries of water parcels. It seems possible that chemosense acts to keep aggregations intact and in some organisms is retained in adult shoaling life, perhaps to maintain aggregations when the shoals disperse at night (Harden-Jones, 1962).

Feeding patterns in larvae and adults can also differ; for example herring and plaice larvae will snap at food organisms in the water column, taking selected individual prey; in the case of herring almost stalking. As adults, herring mainly feed by filtering, although evidence from other filter feeding fish suggest a proportion of particulate feeding will also occur depending on prey size and density (O'Connell, 1972), and plaice move along the sea bed eating epibenthic organisms, mollusc siphons and sedentary worms. Both modes of feeding require good vision. Newly hatched herring (Blaxter and Jones, 1967) and plaice (Blaxter, 1968a) have very different eyes from the adult, the eye developing throughout larval life. In Sardinops caerulea, Schwassmann (1965), the eye is a very rudimentary structure when feeding begins. Although the feeding act is visual, the volume which can be searched using sight alone is small (in herring 0.3-2.0 litres per hour and in plaice 0.1-1.8 litres per hour (Blaxter and Staines, 1971). It seems possible that olfaction could assist in the search for food either by directionally guiding the fish larva to a concentration of food organisms or restricting energy-requiring searching behaviour to periods when food organisms can be detected by presence of their odour. With these possibilities in mind the outline of study below was adopted.

OUTLINE OF STUDY

It was decided to take a byead view of those chemical senses
used by the fish in the natural environment and to examine, where
possible, the mode of action, acuity, and site of this sense.
Existing knowledge both in this laboratory and elsewhere (Marr,
1956; Farris, 1960) of the delicate nature and poor survival rate
of larval marine teleosts meant that since classical conditioning
methods are of a long term nature, relying on continued use of
individual animals, these were ruled out. It was therefore
decided that the best approach to the study would be to look for
manifestations of natural reactions, to natural stimuli, which could
be quantitatively recorded. Initial experiments had shown that a
range of substances produced an increase in total activity and to
some extent modified swimming behaviour. It was decided to adopt two
complementary approaches. First to quantify total movement of larvae
per unit time in a static body of water with a uniform, non-
directional stimulus, secondly to quantify any changes in type of
movement in a situation with water currents and a stimulus gradient.
After initial observations it was found that no easily recognizable
and specific consistent behavioural reaction was apparent as an
indicator of detection of stimulus and that whole animal movement
would have to be recorded. Turning behaviour and orientation to
current direction could then be analysed as indicators of detection of
and reaction to stimuli.

The basis of the chemical sense examined in this study was
thought to be olfaction; this having been shown (Parker, 1911) to
be the distance chemoreceptor in teleosts and known to function at
the low levels of test substances used. It was hoped to establish
for at least one stimulus that the olfactory system was in fact

mediating the response. To this end a histological study was devised to examine nervous connections between sensory epithelium and the olfactory lobes of the brain. This would confirm that a complete olfactory system was present from the time of hatching. Secondly this would reveal the position of the olfactory nerve to allow an ablation study to check for a continuing post-operative response.

The Atlantic herring Clupea harengus L. was chosen as the main subject for this study as reliable rearing techniques had been developed for it and owing to its economic importance a good deal of research has been carried out into its ecology to date, which would be useful in drawing conclusions from the results. In addition the plaice Pleuronectes platessa L. was also studied. Reliable techniques for rearing this fish in the laboratory also exist and it was thought that its taxonomic separation and different life history would make an interesting comparative study.

REARING METHODS

1. HERRING LARVAE

A. Incubation of eggs

Herring (Clupea harengus L.) eggs were obtained from 2 sources:

chiefly from fish provided by Dr J.H.S. Blaxter caught by trammel net

in February on the Ballantrae bank in the Firth of Clyde. These fish

are a spring spawning stock. In order to provide a year round supply

of larvae, eggs from autumn spawning stock were obtained in September

1975, when experimental techniques had been perfected, in this case

from the Manx fishery, on spawning grounds 4 miles to the west of the

Isle of Man. These fish were taken by pair trawling. In both cases

gonads were dissected from adult, 'running' fish removed which were from the

catch as soon as it was landed on deck, and transferred in conditions

of absolute dryness to small glass jars which were stored on ice in

vacuum flasks at 4-5 deg C. The gonads were brought back to the

laboratory within 12 hours when fertilization was carried out

according to the method of Blaxter (1968b). Glass plates were spread

in a rectangular tray filled with sea water to a depth of 10 cm.

Eggs were then dispersed with a dry spatula into the water where they

sank, becoming sticky and so adhering to the plates. An ideal density

of about $4/cm^2$ was aimed at and clumps of eggs which would die due to

lack of oxygen and so decay were removed. The plates were transferred

for 15 min to tanks of milt suspension from the gonads of 4 or 5

male fish. The plates were then rinsed in clean sea water and

transferred to 200 l circular black 'Alkathene' rearing tanks.

After an hour the degree of fertilization of the eggs from

different females could be established by examining plates under a

low power binocular microscope. In fertilized eggs a space can be

seen between chorion and egg contents. In order to provide

experimental animals over a longer period some were incubated and

reared in a 6.5 deg C constant temperature room in the spring, the
rest being kept at the aquarium temperature shown in Figure 1.
Plates were transferred to a clean rearing tank every other day
with a washing action on removal from the old tank, this having been
found to improve hatching success, removing detritus etc. from the
eggs. Care was taken to exclude any temperature shock of over
0.5 deg C. Rearing tanks were covered in black polythene during
incubation to exclude light.

Onset of hatching was dependent on the temperature and was at
12 days post fertilization in spring and 7 days post fertilization
in autumn. The ambient light consisted of 80 Watt fluorescent strip
lights controlled by a time switch which was set to come on after
dawn and go off before dusk to prevent a sudden change in light which
was thought to cause some larvae to swim up the sides of the rearing
tanks. The time of switching on and off was altered weekly with
changing day length.

B. Maintenance and feeding

The rearing tanks received fresh sea water input of 1-2 l/min.
The flow was directed to operate a slight circular current which was
found to keep larvae away from the sides of the rearing tank. The
outlet was covered in a removable 'Perspex' ring with a mesh filter
to prevent escape of larvae and food organisms; this was cleared
twice daily to prevent clogging. When feeding commenced the tank
surface was skimmed to remove bacterial scum and ongrown food
organisms each day, and the bottom cleaned of faecal material and
other deposits approximately twice weekly.

For the first 2-3 weeks of life herring larvae do not feed,
but metabolize their yolk reserves, however before this is
entirely depleted they will begin to take live food. Initially,

Fig. 1.   Weekly mean temperature in rearing tanks .

used in this study.

⊡ Spring 1974

△ Spring 1975

⊙ Autumn 1975

◇ Spring 1976

Upper abscissa   Spring months

Lower abscissa   Autumn months
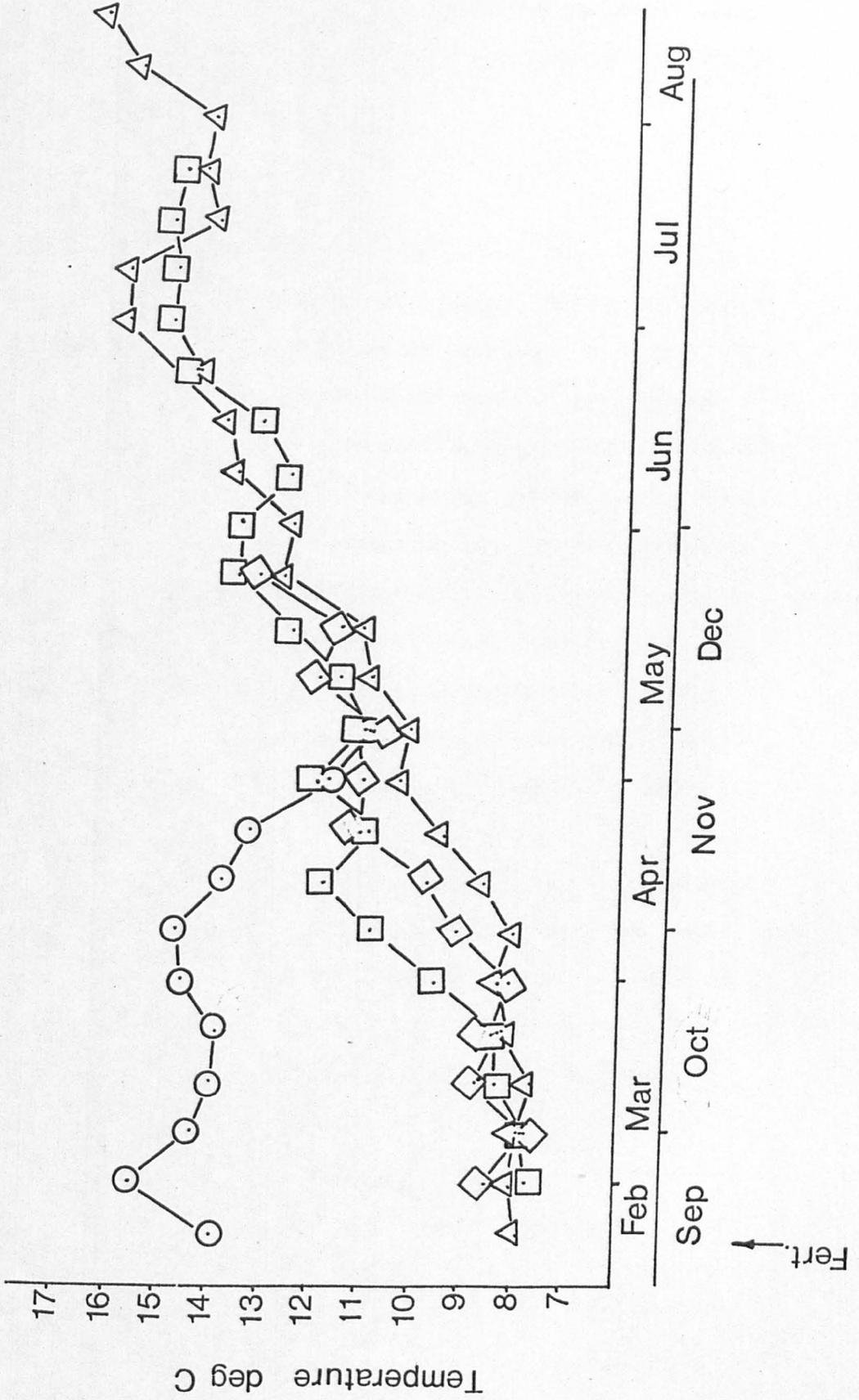
especially in the case of smaller autumn reared larvae, the maximum size of the prey that can be taken by the larvae governed the diet which was fed. The diets fed to experimental animals in spring and autumn are detailed below.

(i)   Spring.

Balanus nauplii was the first food given. These are easily obtained by crushing and sieving adult Balanus, during the spawning season when they contain nauplii in egg sacs prior to release. This was found to be a useful food source and there is some evidence of it being an important natural food source. Lebour (1916) reported that Balanus nauplii were frequently found in the guts of herring larvae during February. In two tow nettings it was the most common single food organism. The main disadvantage as a food item for reared larvae is that it is a relatively fast moving organism. Only about 20% of herring larvae had captured Balanus nauplii and although they were thought to be more nutritionally valuable than Artemia nauplii, the latter were usually added as soon as they could be taken to prevent losses by starvation.

Spring larvae would take the larger newly hatched Artemia nauplii from time of first feeding, even in the presence of excess natural food Balanus nauplii. The main reason seemed to be the lower level of activity in Artemia nauplii. Theilacker and Lasker (1974) in a study of predation by euphausid shrimps considered an Artemia nauplius to be a 'passive particle' and preferential feeding on Artemia was a function of its ease of capture. In the presence of both nauplii in the rearing tank it was noted that Artemia was taken to the exclusion of Balanus. As the size of herring larvae increased the Artemia nauplii were ongrown for 48 h at 26 deg C to deplete yolk reserves. Later they were kept for a further 24 h

Fig. 2. Development and diet changes of

herring larvae in Spring and Autumn.

Development stages are those of Doyle (1975).

Days Autumn Spring	Total Length(mm)	Diet Autumn Spring	Development Stage
6 –12	8	None None	Eggs
12 –25	10	Rotifers Balanus nauplii	1
–50	12		2
28	14		
–75	16	Artemia Artemia	3
–100	24		4
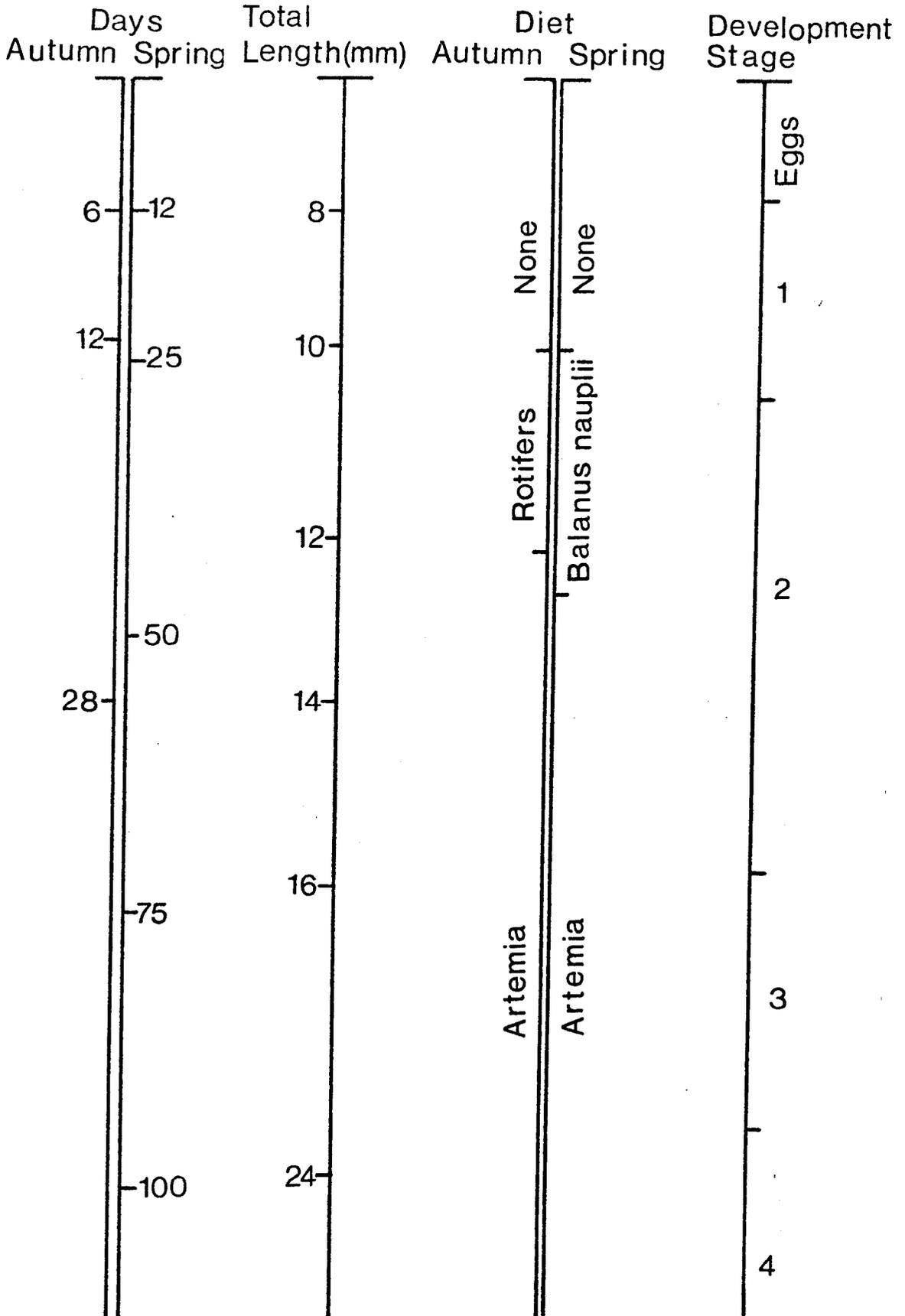
being fed on dried Spirulina, live yeast, and adding a vitamin
mixture to the water. The Artemia nauplius diet was supplemented
by addition of fresh live natural plankton which was sieved through
a mesh with holes 0.5 mm square, to exclude any possible predators.
Later in the season, from May onwards, Tigriopus sp. was added,
this harpacticoid copepod being obtained from small rock pools
near the laboratory.

(ii)   Autumn.

In the absence of Balanus nauplii, which are only obtainable
in the spring, rotifers were used as a first food. The rotifers,
Brachionus plicatilis, were obtained from Scottish Seafarms marine
fish hatchery at South Shian, Argyll, where they are used for
feeding larval flatfish. Rotifers were added at 2000-5000 per
litre of rearing tank per day for a period of 14 days they were
readily taken by the larvae. When Artemia nauplii were introduced
together with rotifers, both were taken for a period of three days
after which the rotifers were excluded from the diet. The diet was
again supplemented with natural plankton, this only formed a small
proportion of the diet fed, and experience with Balanus nauplii
and rotifers showed that it was/unlikely to be taken in any quantity
by the larvae in the presence of Artemia. Therefore it was added
some time before the addition of Artemia nauplii to increase the
chance if it being taken by the hungry larvae.

2.   JUVENILE HERRING

In addition 150 juvenile herring were kept during 1973-1974 for
experimentation. These were wild fish, caught in Dunstaffnage Bay
by seine net, and were kept in two 500 l circular fibreglass tanks.
They were fed on a diet of minced squid which they began to take

after one week, feeding became well established within two weeks.

3. PLAICE LARVAE

A. Incubation of eggs

Plaice (Pleuronectes platessa L.) were reared only in the spring

and eggs came from two sources. First, a supply of naturally

fertilized eggs were obtained from Dr A. Bowers of Liverpool

University, Department of Marine Biology, Port Erin, Isle of Man,

secondly a supply of stripped and artificially fertilized eggs

from Mr J. Dye of the White Fish Authority, Ardtoe. These were

obtained at different times during late March and early April to

give an extended supply of experimental fish. The plaice were

reared in much smaller numbers than herring and were kept in 20 1

black 'Alkathene' tanks until metamorphosis when they were transferred

to 200 1 tanks. Plaice eggs were transported as embryos close to

hatching in large vacuum flasks of sea water. After temperature

equalization they were transferred to the rearing tanks. The water

in the rearing tanks was raised in salinity as necessary to that of

the water the eggs were supplied in, this was done by addition of

'Analar' Sodium Chloride and kept the eggs at the surface where the

oxygen tension was higher. The antibiotics Sodium benzyl penicillin

and Streptomycin sulphate were added according to Shelbourne (1964)

to decrease the possibility of bacterial infection. Sodium benzyl

penicillin at 30 mg/l and Streptomycin sulphate at 5 mg/l

equivalent/l were added to the static tanks. After 3 days a trickle

flow of clean sea water was introduced, this was increased after

hatching.

B. Maintenance and feeding

Plaice were kept in the same part of the aquarium as the herring

Fig. 3. Development and diet of plaice larvae.

Development stages are those of Ryland (1966)

MM = Metamorphosis
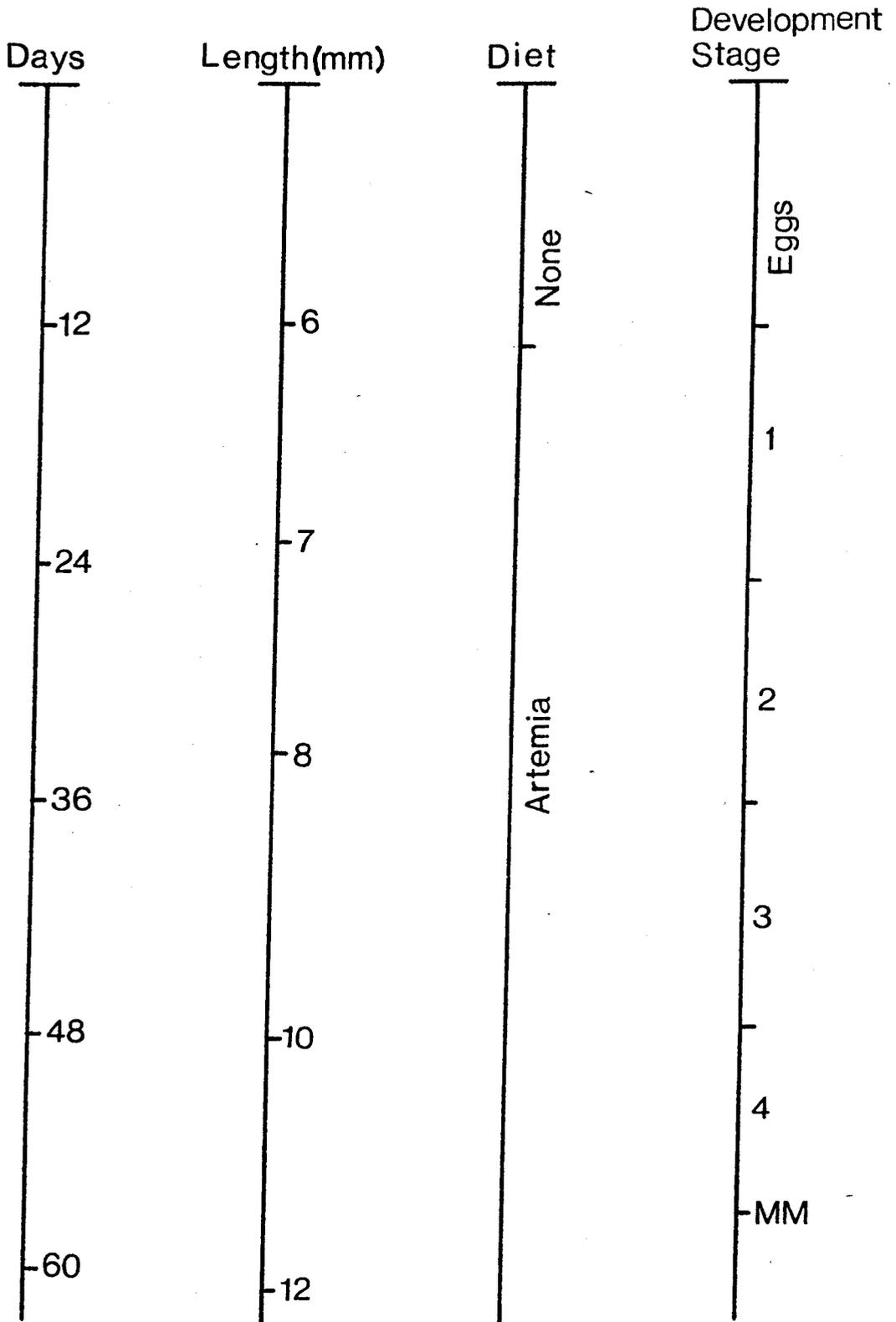
larvae and subject to the same ambient light and temperature
changes. A similar routine of tank hygiene was adopted.

A simpler feeding regime was adopted for the plaice since it
was not proposed to study any changes in chemosense with diet changes
and after the non-feeding yolk stage of 3-4 days the plaice larvae
would take newly hatched Artemia nauplii and continued to feed on
these until metamorphosis 50 days later.

EXPERIMENTAL METHODS

1. LABORATORY SEAWATER SUPPLY AND EXPERIMENTAL CONDITONS.

A. Seawater supply.

The laboratory seawater supply used in this study is described by Currie, Blaxter and Joyce (1976). It is a throughflow system drawing water from one side of the Dunstaffnage peninsular and discharging into Dunstaffnage Bay on the other. A partial recirculation is employed during low spring tides and during periods of maintenance or mains electricity supply failure. No experiments were carried out during these periods.

B. Experimental conditions.

All experiments were carried out in a 3.3 m x 2.2 m x 2.5 m high room at the Dunstaffnage laboratory. The room had all daylight blacked out and was cooled by an air-conditioning unit which kept the air temperature at $13^{\circ}C \pm 1^{\circ}C$. Seawater, except in static vessels, was at the same temperature as the general laboratory supply (see Fig 1).

2. DEVELOPMENT OF THE OLFACTORY SYSTEM IN HERRING LARVAE.

The study of this was carried out in two ways. First, in the case of the early larvae it was found impossible to elucidate the morphology by dissection so serial sections were cut of all stages up to stage 4 using the following procedure. Larvae were fixed in 4% formol saline buffered with hexamine, larvae being anaethetized first in MS 222 (Sandoz) to prevent distortion. They were dehydrated and embedded in 'Polywax' histological wax and sectioned transversely at 5-7 μm. The sections were mounted on glass slides and stained using the silver impregnation technique of Bodian (1936) detailed in Appendix IV. Additionally a sample at each stage was stained using haematoxylin and eosin as detailed in

Appendix III. Secondly for later larvae of stage 4 dissection under binocular microscope was employed, no staining being necessary.

From measurements taken from serial sections, a plan and elevation of larvae was made for each of the stages 1a, 1c, 2a, 2c and 3a. The measurements made from serial sections were corrected with measurements made from fixed whole larvae to correct for tissue compression during sectioning. Some sections are also shown as tissue diagrams to show points not apparent from the reconstructions. These were prepared by tracing from photographs taken of sections at points indicated on the reconstruction drawings. Four plates indicate the development of the olfactory nerve. Drawings of stage 4 a, b, c and d were made from measurements obtained during dissections.

3. ABLATION STUDY.

In order to confirm the olfactory nature of stimulus detection for at least one of the test substances, the olfactory nerves were severed so eliminating the olfactory system. The experiment was carried out as follows: Two groups of 10 larvae were used all being approximately 22 mm overall length. The larvae were anaethetized in 50 ppm MS222 and transferred to a petri dish the bottom of which was covered in a thin layer of agar. A groove was cut in the agar to position the fish and the dish was filled with 50% seawater. Mounted needles were sharpened by dipping a tungsten wire mounted in glass rod repeatedly into boiling sodium nitrate. Using these needles incisions were made in the region of the olfactory nerves. In the case of test animals the olfactory nerve was severed on both sides, however for control animals a sham operation consisted of cutting through the epithelium between the two nerves without

damaging nerves or sensory epithelium. All animals were then allowed to recover in 50% seawater (which is approximately isosmotic) for 2 days before experimentation. Water was subsequently changed at 2 day intervals. Artemia nauplii were present in the vessels and during this time some animals recommenced feeding. Up to commencement of experiments mortality was one from each group of 10 larvae. An examination of operated larvae 12 days after the operation showed no regeneration of the nerve to have taken place. Melanic 'stars' had developed in the epithelium of both operated and sham-operated larvae around the region of the incision, a normal reaction in the healing process.

Experiments were carried out using the thermistor apparatus described below and standard Artemia extract (1 gm/l) with seawater placebo. One larva per chamber was used and individual larvae were not used in experiments more than once per day.

4. DESCRIPTION OF BEHAVIOURAL TECHNIQUES.

A. Thermistor activity experiments

The use of balanced thermistor circuits to record activity in fish larvae is an established technique (Blaxter 1973) and the apparatus used in this study was derived from that used by Blaxter. Experimental chambers consisted of opaque white polypropylene beakers, (covered externally in black plastic) the sizes of which were 7 cm and 10 cm diameter and 4 cm depth, the size used being dependent on the size of the test animal. Two of these were placed side by side under a hardboard box, painted matt black (Fig 4). Light was provided by two 24v 5W bulbs, through diffusers, from above. The bulbs were in adjustable holders designed to throw a circle of light only onto the surface water of the chamber below. The light level in the test chamber was 5.0 lux at the surface of the water. A

Fig. 4.   Thermistor activity chamber showing details of
           illumination and delivery system for test
           substances.

           D.   Diffuser of white plastic.
           C.   Lightproof cover.
           DT.   Delivery tube for test substance or placebo.
           T.   Thermistor.
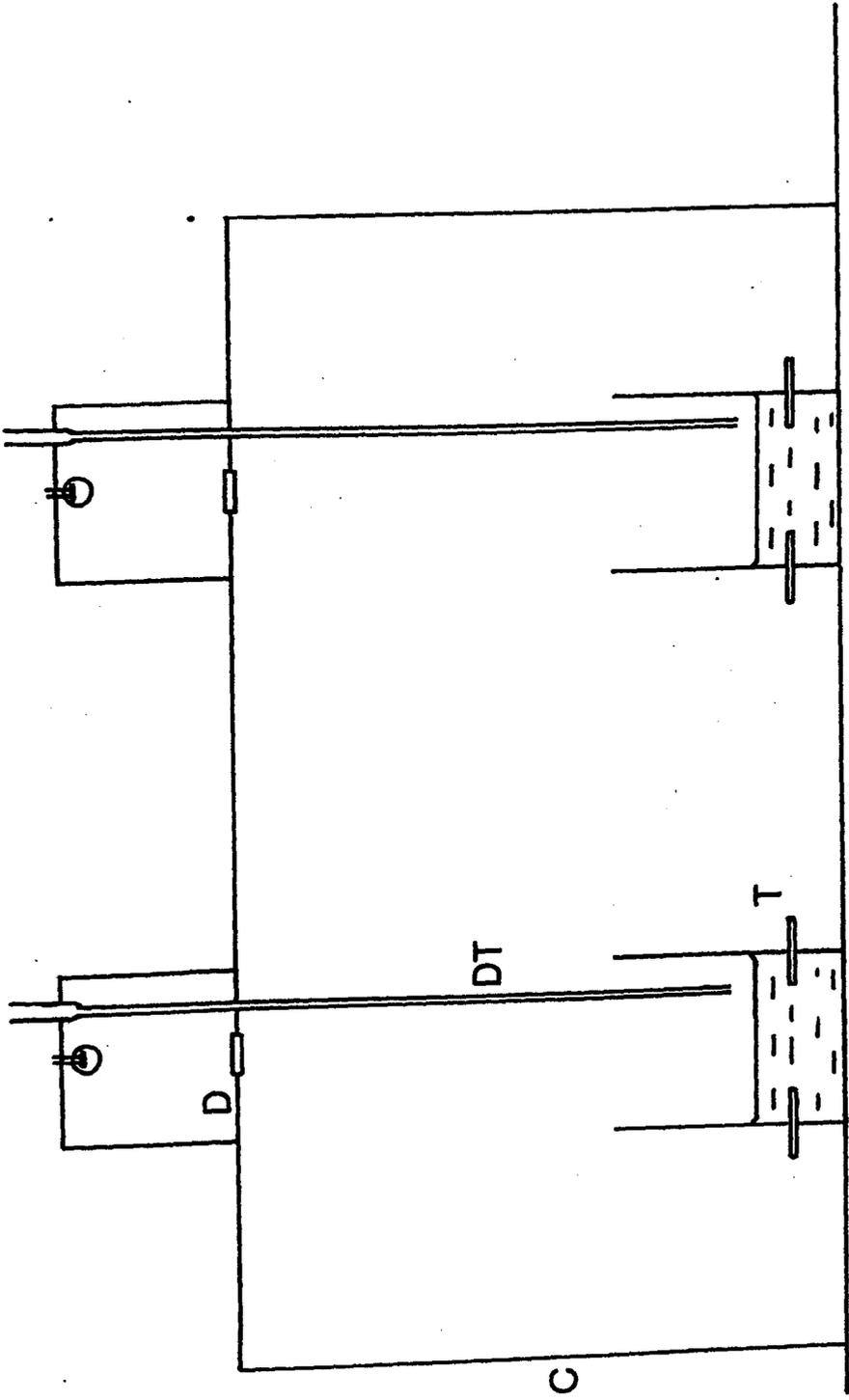
Fig. 5.   Circuit diagram of bridge circuit used in
          association with the thermistor activity
          chamber.

                    R   47 Kohm

                    P1   10 Kohm

                    P2   5 Kohm

                    OP   Output to pen recorder

Thermistors

90V + −

R

R

P1

P2

OP
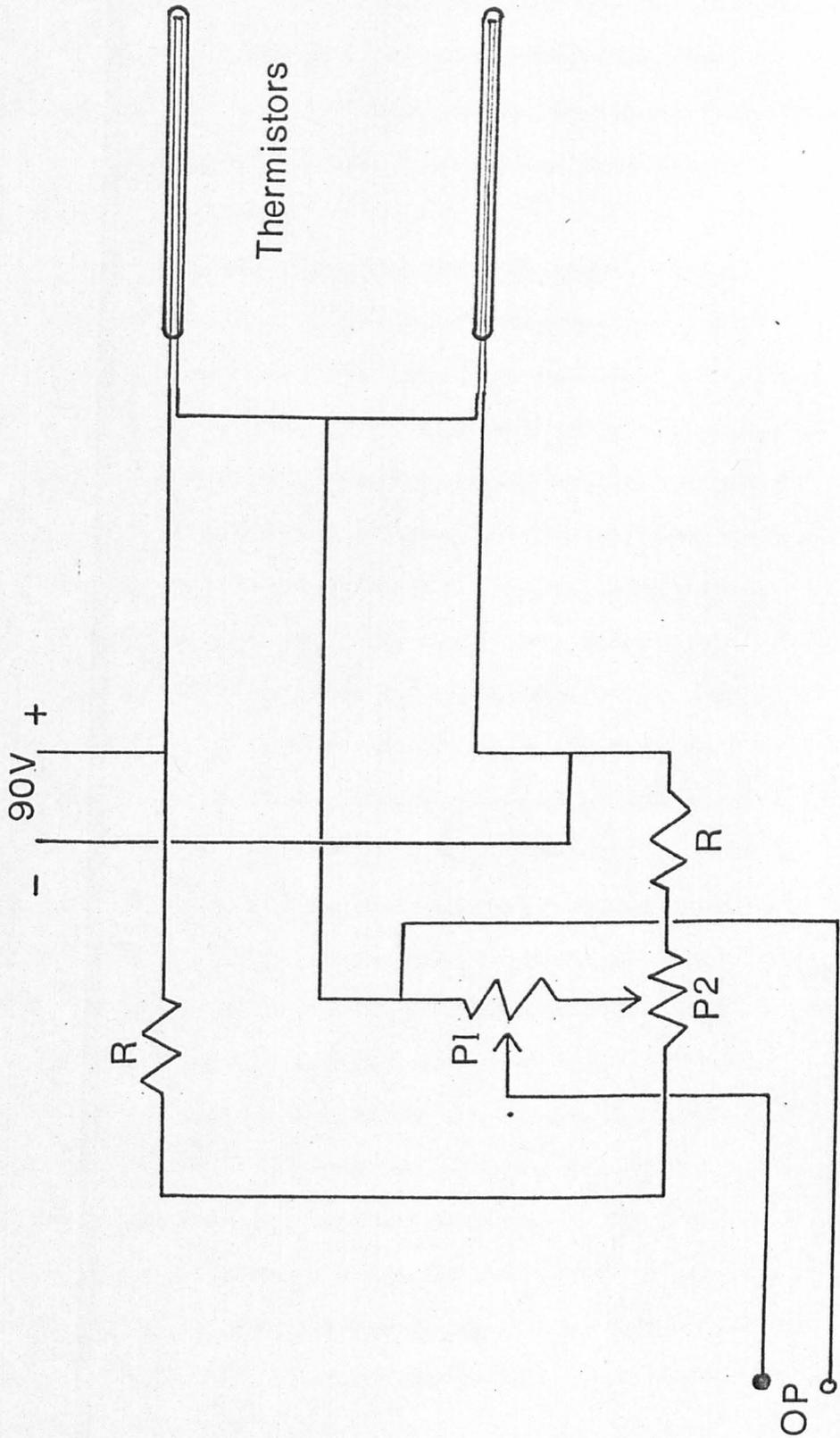
matched pair of ITT F15D thermistors were fixed into opposite sides
of the chamber and a polythene delivery tube for addition of test
substance and seawater placebos was introduced into the chambers
from above (Fig 4). In order to record the imbalance caused by
differential cooling as a larva swam past one thermistor the pair of
thermistors was connected to the bridge circuit shown in Fig 5.
Output was to a pen recorder.

The following experimental procedure was adopted for all
experiments. Animals were collected from the rearing tanks and
taken to the air conditioned room and, where applicable, sorted into
categories for the experiments, for example Yolk sac or Feeding, of
a similar size. Length measurements were obtained by taking the
animal into a glass tube with a millimetre scale, measurements being
to the nearest millimetre total length. Groups or single animals
as detailed below were placed, using a wide bore glass pipette, into
the test chambers which contained volumes of filtered seawater 100 ml
in the case of the 7 cm chambers and 200 ml in the 10 cm chambers.
The cover was placed over the chambers with the lighting on. The
experiment then started and ran for 30 min after which, with the
recorder switched off for 60 sec to avoid false readings a volume
of test substance or seawater, equivalent to 10% of the final volume
was added. Dye tests showed very rapid mixing to occur. The
recorder was restarted and the experiment continued for a further
30 min. Between trials the test chambers and delivery tubes were
rinsed in fresh seawater. In order to interpret the results,
visual observations were made of the fish moving in the chamber in
order to correlate pen recorder output with events in the chamber
at the different sensitivity settings used. The minimum height of
peak taken as an activity event was that which correlated with a
larva swimming between the thermistors set in opposite sides of the

chamber.

B.   Fluvarium experiments

Several approaches have been adopted by workers studying
chemosense to demonstrate behaviourally responses to the detection
of stimuli in the aquatic environment. Methods used have included
monitoring the occurrence of definitive behavioural sequences
related to feeding or other situations, also used has been different
apparatus producing areas of different levels of a chemical and
recording the percentage time spent in different sections. The
most basic, and often used, of these being the 'preference trough'
devised by Shelford and Allee (1913). This apparatus has the major
disadvantage that it produces a 180° change in current direction
at the interface of the two water bodies. Other devices have been
used to avoid this, those of McCleese (1970) and Ishio (1965) and
a very elaborate circular multiple choice apparatus of Kleerkoper
(1967).

For this study on small larvae it was decided to use a modification
of an apparatus used by Gamble(1971) to study oxygen tolerance in
amphipods. This was itself a much smaller adaptation of the original
fluvarium designed by Hoglünd (1951). The apparatus has the
advantage that rather than a choice situation it establishes a
semi-continuous gradient of a solute of 90° direction to current thus
separating rheotaxes and chemotaxes. The principle of the fluvarium
is that it provides an increasing concentration of a solute in an
experimental chamber by mixing two bodies of water, one of which
contains the solute the other of which acts to dilute it. The two
bodies of water enter the horizontally divided distribution chamber
(Fig 6) and leave it for a mixing chamber which is filled with
glass beads through two complementary sets of holes in the

Fig. 6. Constructional details of the fluvarium

DC   Distribution chamber

MC   Mixing chamber

EC   Experiment chamber

DP   Distributor plate

OB   Outlet baffle

55cm

DC DP   M.C   EC   OB

Glass beads

as   above
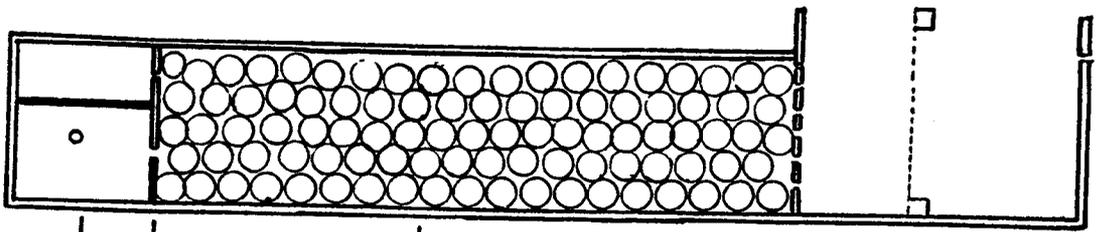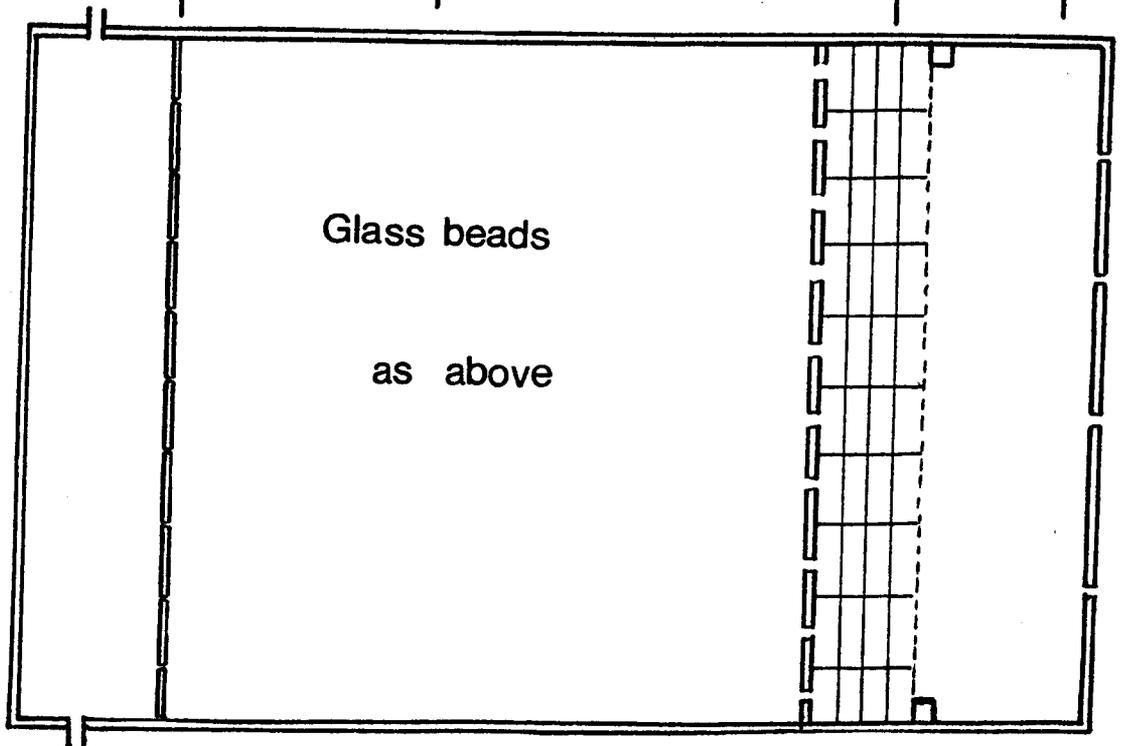
Fig. 7.   Diagram of distribution plate at 'downstream' end
           of distribution chamber showing position of holes.

           P.   Diagonal partition between upper and lower
                halves of distribution chamber.

           Details of hole sizes drilled into distribution
           plate are given in Appendix I.
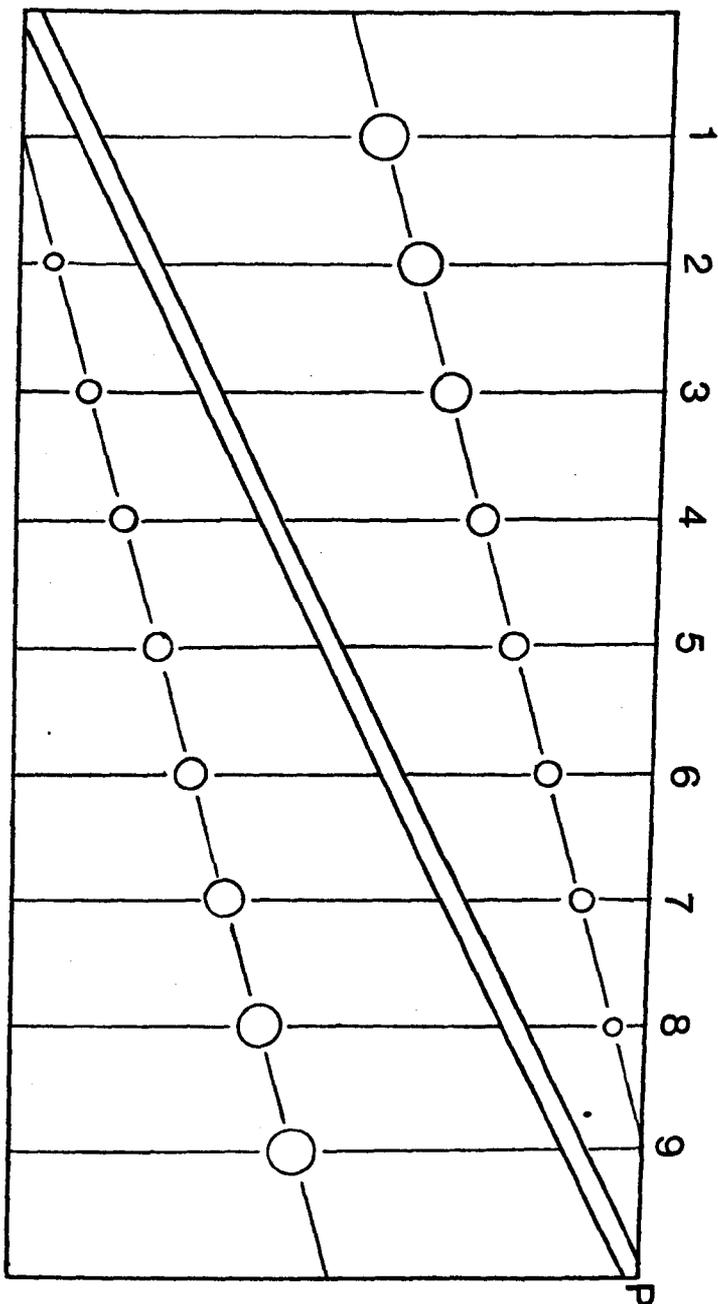
distribution plate this is shown in Fig 7. Hole sizes were chosen
on the basis of available drill sizes and on the assumption that
flow through a hole would approximate to its area. The sum of the
areas of the upper and lower holes was constant in all positions
to prevent areas of differential current which might have proven
a stimulus to test animals. In the middle position the upper and
lower holes had the same areas. Details of hole size are given in
Appendix 1. The fluvarium was constructed in 'Perspex' and
dimensions are given in the scale diagram (Fig 6). The roof of
the mixing chamber was removable for cleaning. The purpose of the
glass beads was to allow vertical but not horizontal mixing. The
outlet baffle ensured that the outlets did not affect current
direction of the gradient in the experimental chamber. An inflow
of filtered seawater was provided by a constant head tank made of
polythene and thence through polythene tubing to the fluvarium, a
through flow of 1 l/min being established. Addition of test
substances was by a small electrically-powered peristaltic pump
(Schuco minipump) with a flow rate of 5 ml/min. This could be
introduced to either of the paired inlets. The extra inflow formed
0.05% of the total and did not seem to affect the behaviour of the
fish. Lighting was provided by a single 60 Watt 240 Volt
incandescent pearl bulb reflected from the white wall of the room
this gave a light level in the test chamber of 5.0 Lux, comparable
with that in the thermistor chambers.

The following experimental procedure was adopted for all
experiments on free-swimming herring and plaice larvae. Some
modifications detailed below were required for post-metamorphic
plaice. Single larvae were placed in the test chamber towards the
centre and left for up to 1 min to settle down. At the commencement
of observations the position of the larva was noted in the grid and

Fig. 8.   Preference trough used for experiments on juvenile

herring in autumn 1973.

CH   constant head inlet system

IC   Inlet chamber to prevent turbulence

 B   Baffle of black plastic drilled with $\frac{1}{2}$" holes

to form an inlet chamber

EC   Experimental chamber

OS   Common outlet (by siphon)

 A   Aspirator providing test solutions

CH IC EC OS B A 1m
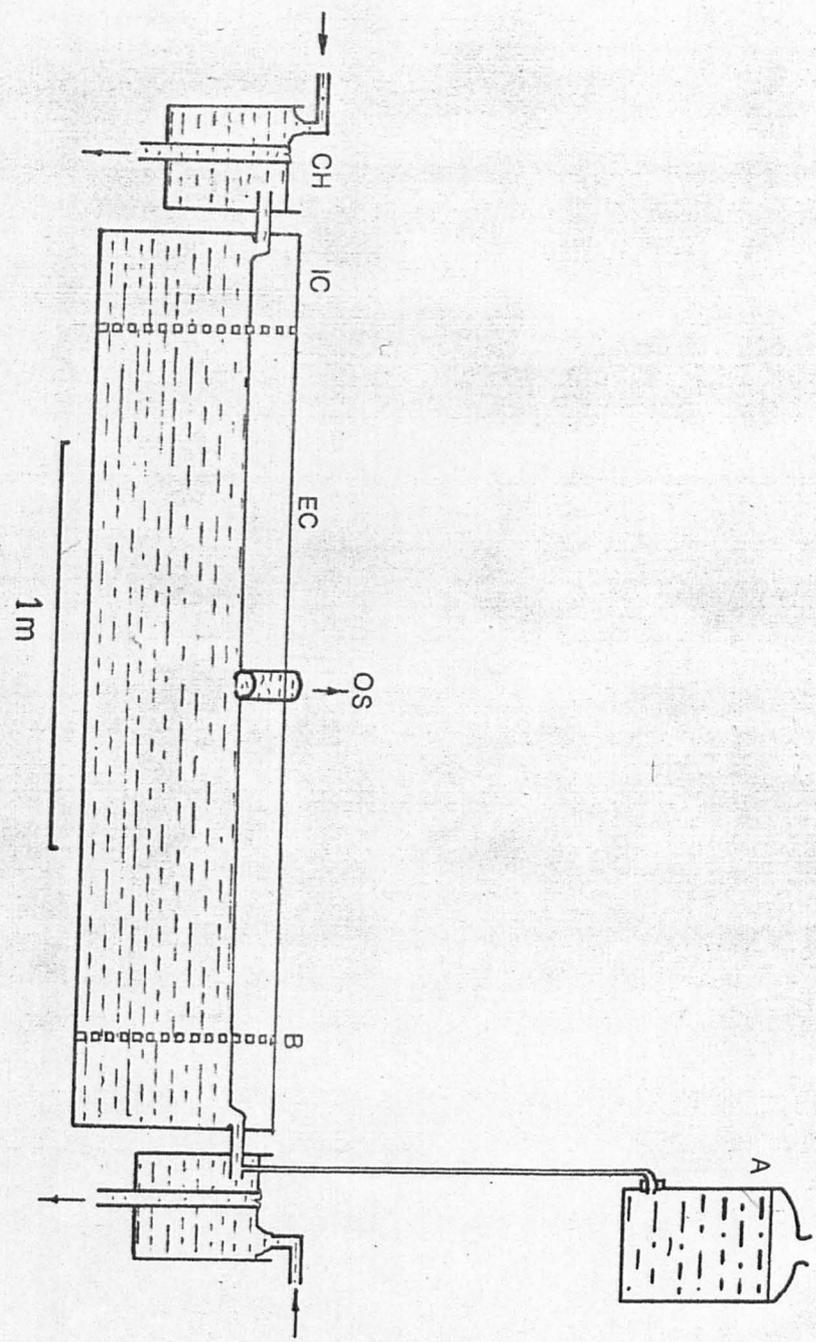
verbally recorded on a tape recorder. The position of the larva
was taken as the point directly between the eyes. At 10 sec
intervals for a total of 15 min further notes were made of the
fish's position. In addition notes were made of general behaviour.
The first 5 min of the experiment was a control period with no
addition to the seawater flow. After 5 min the test substance
reached the test chamber and over a period of 1 min built up in
concentration to produce a gradient shown by dye tests to be stable
for over an hour. The form of the gradient was examined by
spectroscopically testing samples taken from the chamber with an
addition of an acid fuchsin solution as 'test substance', details are
given in Appendix II. Observations in all experiments were continued
for a total of 10 min, giving a 5 min control period and 2, 5 min
test periods. The above procedure was modified slightly for the post-
metamorphic plaice which tended to spend long periods stationary on
the bottom of the chamber. Groups of five fish were introduced and
left to settle on the bottom. The experiment was started and
continuous notes taken of the elapsed time and distance of any
movements even if the animal did not leave the grid square it was in.
After 5 min control period a 5 min test period followed giving a
total time per experiment of 10 min.

C.   Preference trough experiments on juveniles.

In autumn 1973 a series of experiments were carried out on a
group of wild caught 0-group herring. These experiments used a
type of preference trough shown in Fig 8. It was constructed in
'Perspex'. The inflow at each end was 500 ml/min and included
an inflow of test substance from one of the ends of 2 l over the
20 min period. Three fish per experiment were used and put into the
presumed odourous end of the apparatus and left for 3 min before

beginning observations. The positions of the fish were noted at 15 sec intervals for a total of 15 min.

5. TEST SUBSTANCES

Experiments fell into two groups. First those using substances connected with feeding; either extracts of prey organisms (whole extracts or the dialysable fraction), water which had contained prey organisms (referred to as washings) or chemical substances (amino acids) found as part of food. Secondly those using intraspecific identifiers of unknown composition.

A. Feeding related substances

(i) Extracts

These were made up from the food organisms <u>Balanus</u> nauplii and <u>Artemia</u> nauplii prepared by subjecting live or frozen whole organisms to disintegration in a 'Polytron' ultrasonicator and filtering the residues through 'Whatman' GF/A glass fibre filter papers. Standardization was achieved by quickly filtering the live animals to a standard 'mass' of organisms as free from water as possible without leaving them out of water for too long. After weighing and dispersing the still live organisms in a suitable volume of water to give 10 g/l they were disintegrated and filtered free of residue to give a clear pink liquid which was kept at 4 deg C until dilution prior to use in experiments. Fresh extracts were kept no longer than 3 days but were usually made up daily as required. Frozen nauplii were used for experiments carried out using extracts of <u>Balanus</u> nauplii in the autumn and later in the spring when live nauplii were not available. Live <u>Balanus</u> nauplii were filtered free of water and kept at -15 deg C for up to eight months. For some experiments extracts were subjected to dialysis. 100 ml of extract was sealed

an envelope of 'Visking' tubing and put into a volume of 400 ml
of filtered seawater and left for 12 h and the dialysate used.

(ii)   Washings

For experiments using washings of live prey organisms a portion
of animals weighing 11.3 mg $\pm$ 0.7 mg was taken from the standard
free 'mass' mentioned above using a standard spatula tip and
dispersed into 500 ml of seawater. This did not cause any noticable
mortality of the animals. The container was left for 16 h,
including periods of light and darkness, the nauplii filtered off
and the filtrate used the same day for experiments.

(iii)   Amino acids

The amino acids used in this study were chosen as those
reported by Shepheard (1974), McCleese (1970), Suzuki and Tucker (1971),
Hara et al (1973) and Hashimota et al. (1968) to be effective as
stimuli on marine organisms and were:- alanine, proline, glycine, methionine,
cysteine, glutamic acid and aspartic acid. In all cases the
L-isomers were used as these are the naturally occurring forms and
those found by Case and Gwilliam (1961) to be the most stimulatory
on Carcinus maenas, and by Hara et al (1973) for teleosts.

Solutions of amino acids were made up as concentrated stocks
in filtered seawater at a standard concentration 10^{-3} Molar. This
was chosen as it was close to the solubility limits of cysteine in
seawater. Solutions were kept at 5 deg C and diluted as necessary.

B.   Intraspecific identifiers.

(i)   Larvae

A group of experiments was carried out using a standard
preparation of water which had contained herring larvae. This was

to look for a social i dentifier. In further experiments to examine the action and production of this substance the method of collection was altered. The standard method of production consisted of placing 10 larvae in 500 ml of filtered seawater for 16 h which included equal periods of light and dark. For initial purposes it was assumed that the social identifier was persistent and produced at a constant rate. Water containing this identifier was produced overnight for use the following day. A series of experiments were carried out using dilutions of the standard preparation with fresh seawater to find the threshold of reaction. This being the greatest dilution to give a statistically significant response. In order to test whether in fact the identifier was persistent the threshold was redetermined but this time the test substance was prepared by using one larva in 25 ml seawater for decreasing periods of time initially 30 min the larva being removed and the water used in the test immediately afterwards. As a further check to see if in a group of larvae production, per individual, of this substance was increased, the above experiment was repeated using 3 larvae in 75 ml seawater, instead of 1 larva in 25 ml seawater, to produce the test substance.

(ii) Juveniles

For experiments on juvenile herring the test stimulus was water from the stock tank, other than the one the experimental animals were removed from. This water was passed through synthetic filter wool to remove particles.

6. DETAILS OF EXPERIMENTS CARRIED OUT.

A. Juveniles autumn 1973.

Eight control experiments were carried out using plain seawater additive to determine if, in the absence of a presumed odourous additive, the fish showed a preference for one or the other half of the trough. Six trials were then carried out using an inflow additive of filtered water from the stock tank other than the one the test fish came from.

B. Larvae 1974-1976.

For the purposes of experimentation herring larvae were divided into three groups according to the feeding regimes which they pass through in larval life under our rearing system. These were:-

1. Yolk sac, taken as the non-feeding stage, corresponding to Doyles (1975) stages 1a - mid 1c, when feeding commences, although the yolk is not entirely used up.

2. Balanus (nauplius) feeding stage, corresponding to Doyles stage 2a to approximately late 2b or early 2c, whilst the larvae were fed on a natural diet presumed similar to that encountered in the sea.

3. Artemia (nauplius) feeding stage, this comprised of the rest of larval life to metamorphosis. During this stage the larvae were fed on a diet of Artemia nauplii. This would be totally beyond the experience of wild marine fish larvae. As this is a long stage it was subdivided to some extent into early and late as detailed below.

Plaice larvae as has been mentioned have a much shorter larval life than the herring and have an extremely short non-feeding stage. Therefore in the case of plaice, the test animals were only divided into pre- and post- metamorphic fish, this division not being related to any diet change.

After some initial experiments when it was decided to adopt the

above categories, it was also decided to select a group of test substances and conduct trials on each stage given above for the two species. It was intended to duplicate all experiments in both thermistor apparatus and using the fluvarium. Trials to find thresholds only used the thermistor apparatus. A list is given below of the experiments carried out. Where it was not possible to experiment on a particular feeding stage details are given.

Extracts	Balanus nauplii extract [1]
	Artemia nauplii extract
Washings	Balanus nauplii washing [2]
	Artemia nauplii washing
Amino Acids	Glycine
	Proline[3]
	Methionine
	Glutamic acid
	Cysteine
	Alanine
	Aspartic acid[3]
	Social Identifier[4] Standard preparation[5].

1. Also tried on later Artemia feeding stages.

2. Not available for Artemia feeding stages or plaice.

Key   3. Not tried on the short Balanus nauplii feeding stage.

4. Social Identifiers were only examined in the herring.

5. Using single test larvae.

For all experiments in the fluvarium ten trials per test substance in each stage were carried out. For the thermistor experiments, as mentioned below the results analysis method used meant that between 5 and 23 trials were carried out in individual experiments. Five larvae being used in each test chamber. In addition experiments using dialysate of _Artemia_ nauplius extract were carried out on early _Artemia_ feeding stage larvae and threshold experiments for reaction to _Artemia_ extract and glutamic acid were carried out on early _Artemia_ feeding stage larvae. Using the thermistor technique only, experiments (detailed above) were carried out into the nature of the social identifier.

7. ANALYSIS OF RESULTS.

A. Juveniles

Results were analysed using a two tailed Zm test (Langley, 1968) adopting the null hypothesis that the fish spent an equal amount of time in both halves of the trough.

B. Larvae

(i) Thermistor experiments

Using criteria detailed in the methods section the pen record spikes were divided into activity events and background noise. Results from pen recorder traces were counted up as events per half hour, before and after administering either a test substance or placebo. The result for test and control treatment were calculated by expressing the number of events after administration as a proportion of the number before, this compensated for motivational differences between larvae. In this way it was possible to compare test and control, if a greater activity increase occurred in the test chamber than in the control then this was regarded as a positive result, the converse as a negative

result. During the period when experiments were being carried out
the sign test was used as a running test as it is quick and easy
to apply and if a significant deviation from equal proportions of
positive and negative results of trials were to be reached then an
experiment could be halted before doing more trials than were
necessary. In order to make an estimation of where to stop an
experiment easy, a result diagram was prepared (Fig 9B); this was
from the chart (Fig 9A) which was adapted from Walker and
Lev (1953). A positive result caused a movement to the right and
down, a negative result only a movement to the right; significance
at the 5% level was achieved when the marker leaves the squares. This
test however makes no use of the quantifications of the difference
between test and control. An adaptation to take this into account
would make the test even more powerful. Such a test is the
Randomization test for matched pairs. This uses all the information
available in the data, taking into account the size and direction
of differences. Because of this it has a power-efficiency of 100%;
that is to say, for any set of data for which both tests are
applicable it is as powerful as Students 't' test, its parametric
equivalent. Experiments were later analysed with the Randomization
test to confirm the sign test results.

(ii)   Fluvarium experiments

    Analysis of movements in gradients produced by the fluvarium
was done completely by a series of programs in Fortran IV on an
1CL 4100 computer. Details of the programs are given in appendices
V-VII. All the trials were grouped according to species, feeding
stage and test stimulus for analysis. The movements of larvae in
the fluvarium were analysed with two aims: first to look for
changes in activity level related to presence of a stimulus. To

Fig. 9.

A. (Top): Maximum number of results in an experimental
   series allowed, where a greater increase in activity
   occurs in the control chamber than the test chamber, if
   the result is significantly different from random at the
   5% level with the Sign test.

B. (Bottom): A cumulative result chart which allows an
   experimental series to be discontinued when a significant
   result is reached. The dot is moved diagonally downwards
   and to the right for a positive (see text) result and
   horizontally to the right for a negative result. If a
   significant deviation from an even distribution of
   results (at the 5% level) is reached the dot leaves the
   squares.

   Shown is a series of 11 experiments with experiments 5 and
   7 giving negative results and all others positive results.

Number of Experiments	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Maximum number of negative results if a significant difference at 5% level	0	0	1	1	1	2	2	3	3	3	4	4	5	5	5	6

Experiment Number

this end the number of moves made was expressed as a percentage of the maximum possible number of changes of position, this was termed 'percentage time active'. Additionally the mean total distance moved in the 5 min periods of a trial were calculated and from these a mean distance moved in a 10 sec interval (step length) was calculated. The differences, if any, between control and test situations were then to be analysed for significance. In each trial the larva would act as its own control. This assumes that no changes in activity occurred after transfer into the fluvarium due solely to the length of time spent in the apparatus. Personal observations of activity levels in thermistor experiments had shown in later herring larvae a fall in number of activity events per unit time occurred with the passage of time. This was confirmed by Wales (pers. comm) who reported that in shallow chambers as distinct from deeper columns a decrease in activity with time occurred. As a result of this the two control experiments described above were carried out on late yolk sac larvae (stage 1c) to cover non feeding and Balanus nauplii feeding stages and on stage 3 larvae to cover the Artemia nauplii feeding stage.

The parameters 'percentage of time spent active' and 'total distance moved' were tested by a Zm test on the null hypothesis that no difference existed between the values found for the test periods of the control experiments and the population of control periods for the experiments using test stimuli.

An analysis for differences in behaviour in control periods between herring larvae of the three stages of feeding regime was carried out. The parameters of 'percentage time active' and 'total distance per trial' for the control periods of trials in each of the stages was compared with those of the other two.

which was calculated from them and the 'mean direction' and 'degree of dispersion'.   A Zm test was carried out on the null hypothesis of no difference existing between the stages. If as was originally hypothesised, in the absence of stimulation a constant level of activity was found, the experiments could be analysed by a Wilcoxons matched-pair signed-ranks test (Wilcoxon, 1949) on the null hypothesis of no significant difference existing between control and test periods in each trial for the parameters 'percentage of time active' and 'total distance moved per trial'. If, however, a difference was found due to the time spent in the fluvarium then the experiments would be analysed by a Mann-Whitney U test (Mann and Whitney, 1947) on the null hypothesis that the difference between control period and test periods was the same in the experiment using a test stimulus as in the control experiment.

Analysis of response of post-metamorphic plaice was carried out by comparing the total number of moves in control and test periods by a Wilcoxons matched-pair signed-ranks test.

Additionally from Gibson (1975 and Unpublished data) it seemed possible that the locomotor behaviour of young plaice could be divided into short 'shuffles' typical of searching the immediate area and longer moves of more than one body length. The proportions of the two sorts of move were compared by a Wilcoxons matched-pair signed-ranks test for any differences between test and control periods.

The second aim of the anlaysis of movement in the fluvarium was to see if a recognizable change in mode of swimming behaviour occurred as a response to the stimuli used either as a result of simply being in an environment of stimulus or as a result of sensing the gradient of concentration. Histograms of the

distribution of angles of turn were prepared and are presented in Appendix VIII. In order to quantify the distribution of these angles a vector addition as described in Appendix IX was carried out using the program shown in Appendix VII. This gives the mean direction of travel and a value, the resultant, which is simply related to the circular variance which gives the degree of cluster around the mean direction of the observed directions. Additionally the movement of a larva laterally in the fluvarium during a test period would cause it to experience a change in stimulus concentration, this might modify subsequent behaviour to allow the larva to localize the odour source. The relationship between consecutive moves was analysed in two ways using the program in Appendix V. Firstly it was thought possible that a larva might respond to an increase in concentration of a stimulus by orientating to swim against the current, where, in nature, one might expect the direction of the source of the stimulus to lie. Accordingly the number of turns made against and with the current after a move up or down the concentration gradient were listed.

These were analysed by the Chi^2 test on the null hypothesis that no difference in the preference of turn direction after experience of a change in concentration, between the test periods and the control period when there was no gradient. A second possibility would be to continue swimming in that direction and to a decrease to turn back, in the case of a negative stimulus the converse being true. Accordingly the number of times a larva turned back or continued on after a move up or down the concentration gradients were listed.

These were analysed by the Chi^2 test on the null hypothesis that no difference existed in preferred direction of travel after experiencing a change in concentration between test periods and the control period when there was no gradient.

<u>RESULTS</u>

1. <u>THE POSTEMBRYONIC DEVELOPMENT OF THE OLFACTORY SYSTEM IN</u>

    <u>HERRING LARVAE</u>.

    At the time of hatching the olfactory organ is present as
    an ectodermal plate, only very slightly indented, situated low
    down on the side of the larval head, in front of the eyes. This
    is shown in Fig 10. At this stage no olfactory nerve exists, the
    olfactory epithelium is in close proximity to the forebrain and
    some cells from the forebrain merge into the epithelium as is
    shown in Fig 11 (A) and plate 1. A similar stage is described in
    the embryonic development of <u>Salmo salar</u> by Holm (1894). No
    skeletal structures were seen in this area of the newly hatched
    larva (8 mm total length). In contrast Wells (1922) describes
    a developed chondrocranium in sea-caught herring larvae whose
    lengths he gives as 5.5, 6.5 and 7.8 mm. Wells (1922) is unlikely
    to have studied newly hatched larvae using sea-caught material and
    Holm (1894) and Peter (1901, 1911) both describe the development of
    cartilage in <u>Salmo salar</u> in this region to occur at time of hatching.

    Between stages 1a (newly hatched) and 1c (late yolk sac), the
    next stage examined, there has been considerable development of
    the head as can be seen by comparing Figs 10 and 12. Skeletal
    structures are clearly seen in the sections Fig 13, in particular
    the development of the palato-pterygoid cartilage and trabeculae
    cranii causes a dorsal and forward movement of the olfactory
    epithelium relative to the forebrain. To maintain a nervous
    connection between forebrain and sensory tissue an olfactory
    nerve develops, this is shown in Figs 12 and 13 (B) also in Plate 2.
    No details are given in the present study of the ontogeny of the
    olfactory nerve but Holm (1894) considered that it is not formed
    from the elongated cells of the forebrain found extended into the

sensory epithelium both in his 46 day embryo of <u>Salmo</u> and in the present study in <u>Clupea</u>. This view was supported by Disse (1897) who described the appearance of the olfactory nerve in vertebrates as bipolar ganglionic cells formed in the sensory epithelium and therefore ectodermal in origin. One extension of this nerve goes to the olfactory bulb and the other to the surface of the olfactory groove. The nervous connection in early stage 1c larvae shown in Fig 12 resembles the description of a 60 day <u>Salmo</u> embryo by Holm (1894) where the olfactory nerve has arisen to join the sensory tissue and the forebrain, now separated by a layer of mesoderm. In contrast to <u>Clupea</u> as shown in the present study this is prior to the appearance of the palato-pterygoid cartilage in <u>Salmo</u>.

By stage 2a the area of olfactory tissue shows a great increase in area and is situated on the flat area of the head in front of the eyes and forebrain (Fig 14). The further changes seen until stage 4 are a lengthening of the olfactory nerve and an increase in area both resulting from the development of the chondrocranium (Figs 16-19). The increase in area is not thought to produce a similar increase in acuity as it is largely due to increase in indifferent epithelium (Branson, 1975) but may do so by virtue of spacing the sensory cells so allowing a greater volume of water to be sensed. The olfactory plate is still seen as a flat plate-like structure only slightly indented. The innervation of the epithelium is shown in Plates 3 and 4. Throughout stage 4 the plate invaginates to form an olfactory capsule which begins to close by extension of two epithelial flaps from either side to form an internasal septum although this process is not complete at metamorphosis (Fig 20). This situation of post hatching closure of the olfactory groove has been re ported from <u>Lota lota</u> and <u>Esox</u>

(Sagemehl, 1884) and <u>Salmo</u> (Hoffmann, 1884). In some elasmobranchs these flaps do not fuse.(Dumeril, 1807).

In the late stage 4D larvae just prior to metamorphosis a fingerlike fold can be seen in the epithelium, this is the beginning of the lamellar structure seen in the developed organ (Fig 20D). The extension, in development, of the olfactory nerve in herring larvae is seen to a greater or lesser extent in most teleosts, being in response to change in head shape. The olfactory tract connecting the olfactory lobe with the rest of the forebrain is in <u>Clupea</u> contained within the forebrain itself, however in 3 families of teleosts (Ostariophysii, Anacanthini and Mormyrids) the olfactory nerve remains relatively short. The olfactory bulb in these families is at a distance from the forebrain being connected by a myelinated olfactory tract (Prosser 1961). Uchihashi (1953) published a list of relative lengths of tract and nerve in Japanese fish and has correlated this with their ecology. Doving (1967) showed that the tracts in <u>Lota</u> contained fast conducting fibres. Aronson and Kaplan (1968) suggest this may allow co-ordination of taste and olfaction as the 3 groups possessing these tracts have well developed central and peripheral taste systems.

Fig. 10.   Lateral and dorsal view of the head of a newly

hatched herring larva. The drawing of a stage

1a larva (8 mm total length) is drawn as being

transparent and was reconstructed from

measurements made from serial sections.

Correction for tissue compression in sectioning

was made from measurements taken from specimens

preserved whole in 4% Formol saline.

OE   Olfactory epithelium

Br. Brain

A,B,C and D indicate the position from which the

sections shown as Fig 11 A,B,C and D were taken.

Br.

OE

A B C D

100 μm
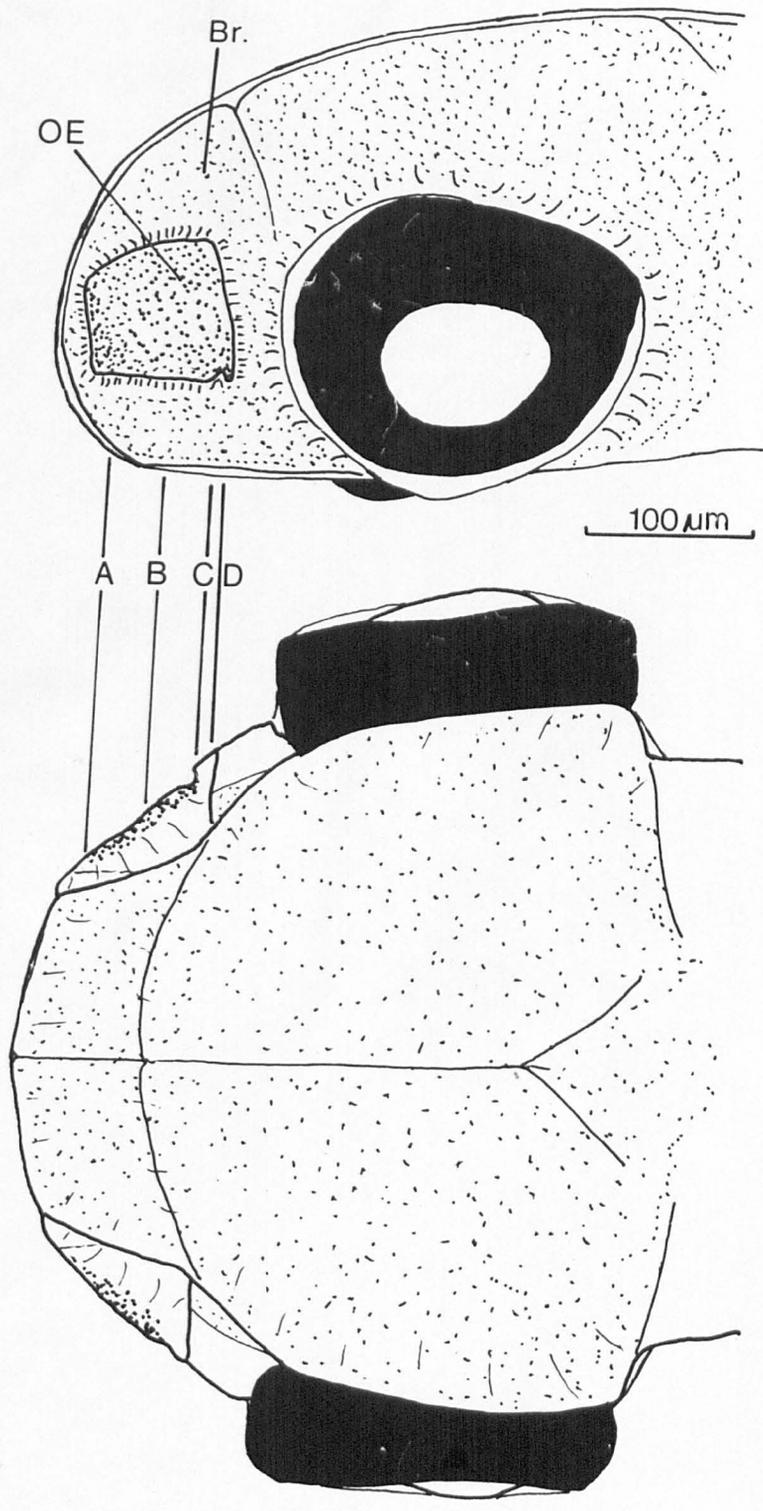
Fig. 11 (A,B,C and D).

      These are tissue diagrams drawn by tracing from

      photographic enlargements of the sections at

      positions A,B,C and D in Fig 10. No correction

      has therefore been made for tissue compression.

A

B

C

D

Brain

Olfactory epithelium
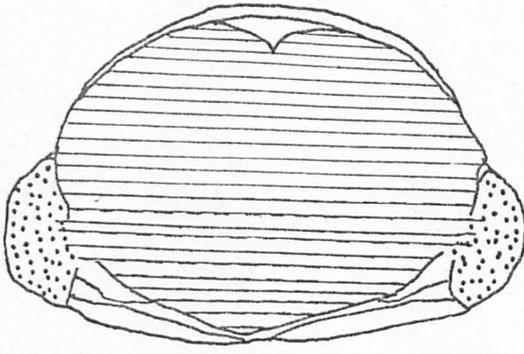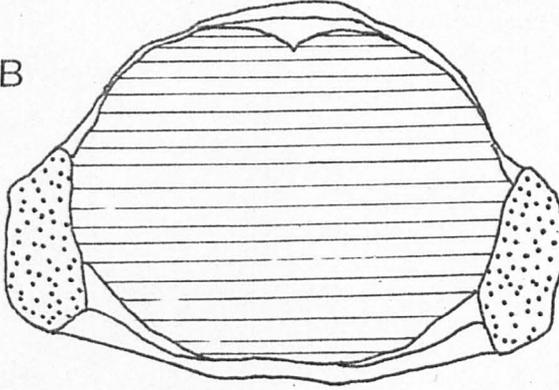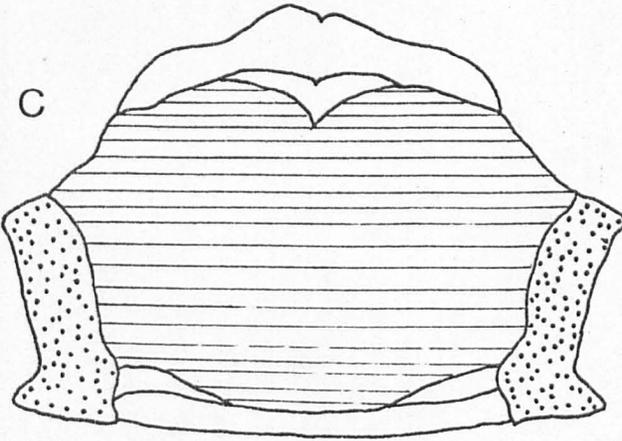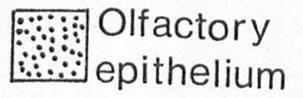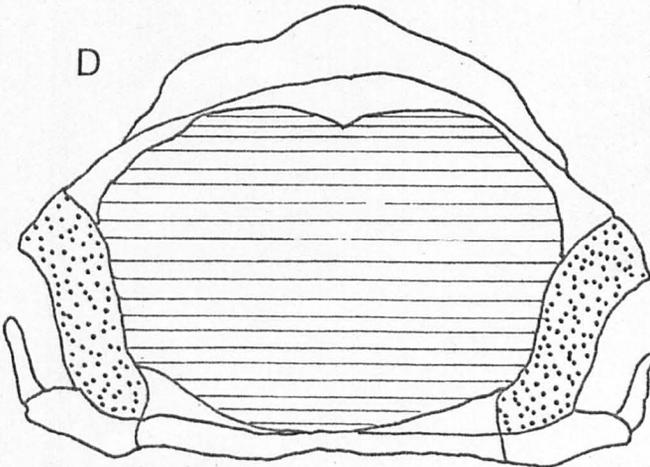
100μm

Fig. 12.   Lateral and dorsal view of the head of a stage

1c herring larva (10 mm total length) drawn as

transparent and reconstructed as in Fig. 10.

OE   Olfactory epithelium

Br.   Brain

A,B and C indicate the position from which the

sections shown as Fig 13 A,B and C were taken.
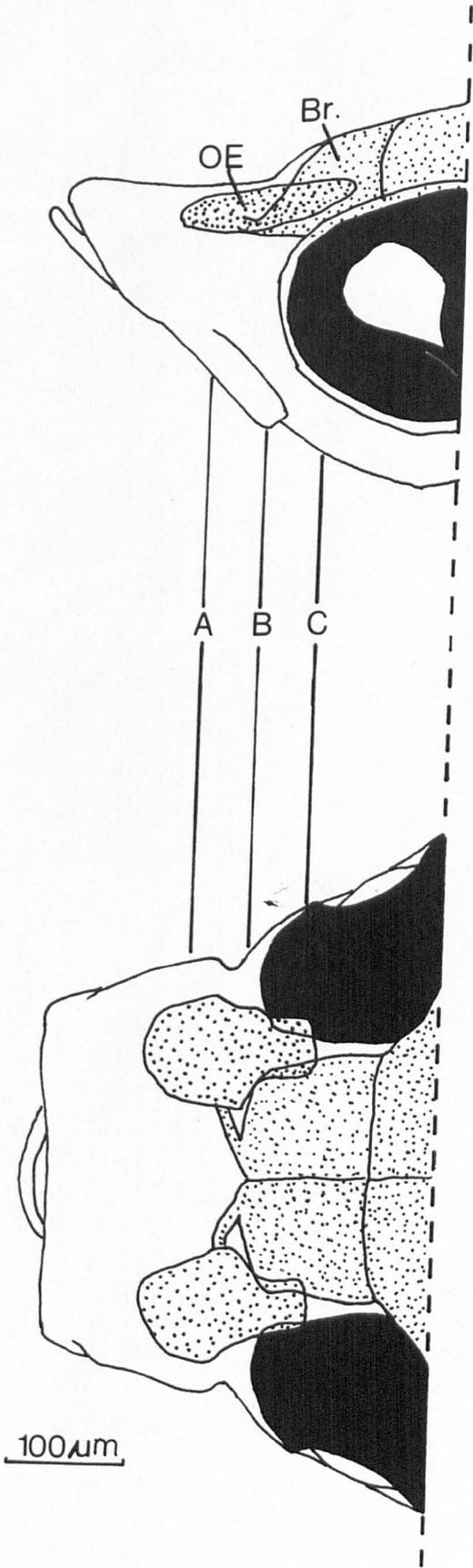
100μm

Fig. 13 (A,B,C).

Are tissue diagrams of sections from positions '

A, B and C in Fig 12.

A

B

C
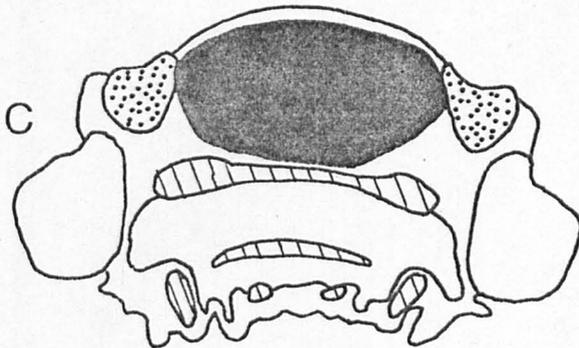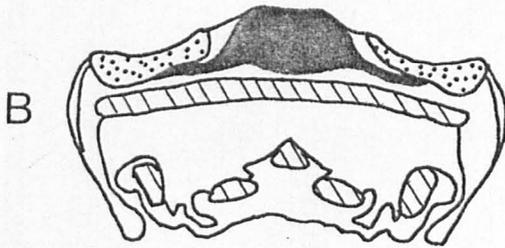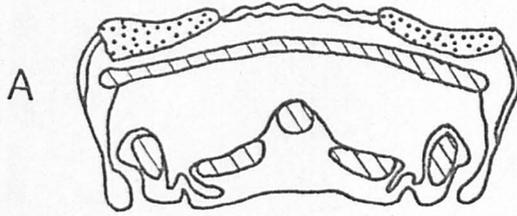
100 μm

Cartilage

Olfactory epithelium

Nervous tissue

Fig. 14.   Lateral and dorsal view of the head of a stage

2a herring larva (12 mm total length) drawn as

transparent and reconstructed as in Fig 10.

            OE   Olfactory epithelium

            OT   Olfactory nerve

            Br.   Brain

A, B and C indicate the position from which the

sections shown as Fig 15 A,B and C were taken.

OE   OT   Br.

A B C
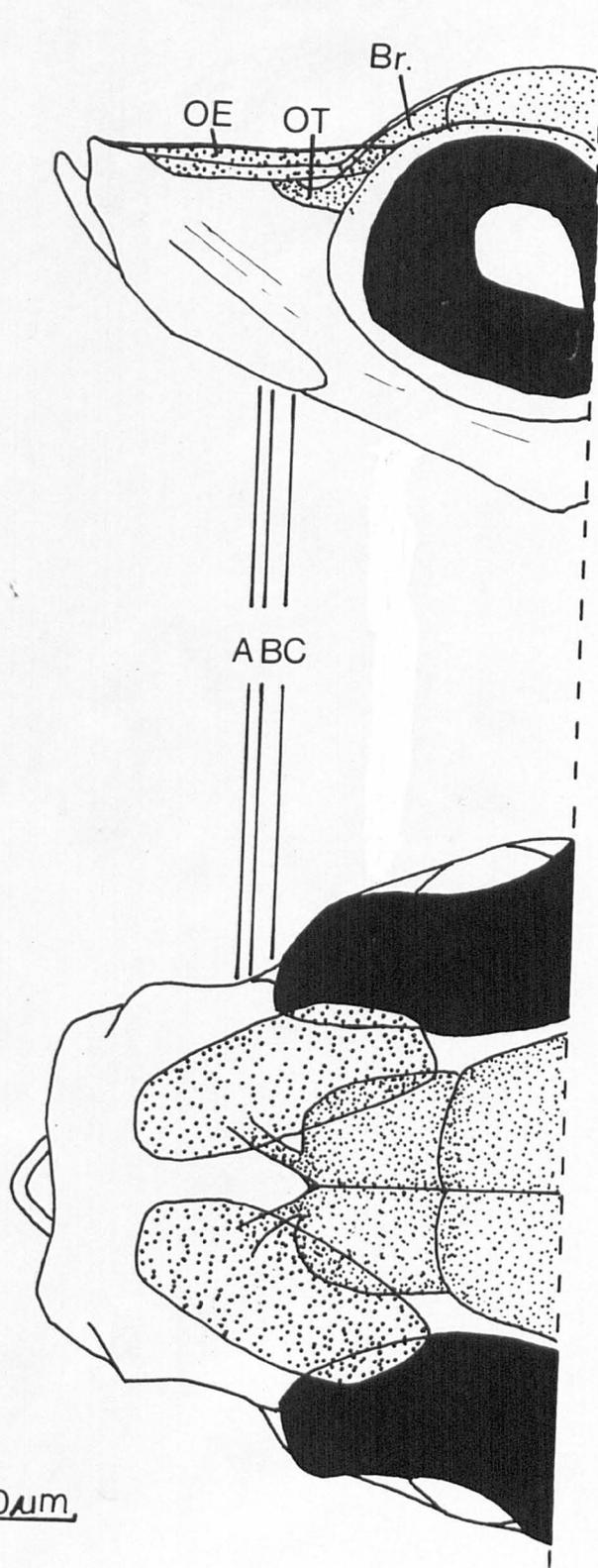
100μm

Fig. 15 (A,B and C).

Are tissue diagrams of sections from positions

A,B and C in Fig 14.

A

B

C
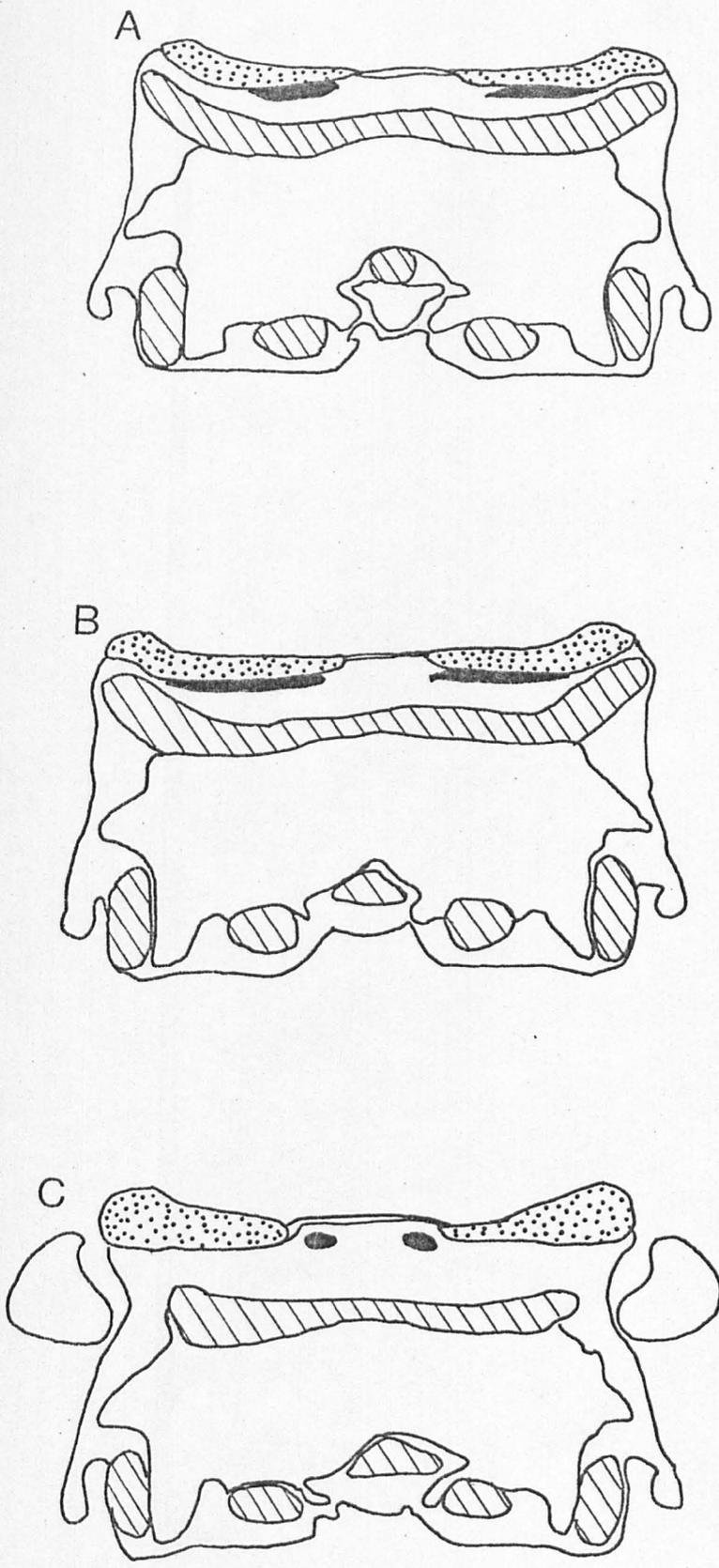
Cartilage

Olfactory
epithelium

Nervous
tissue

100μm

Fig. 16.   Lateral and dorsal view of the head of a stage

2c herring larva (15 mm total length) drawn as

transparent and reconstructed as in Fig 10.

            OE   Olfactory epithelium

            OT   Olfactory nerve

            Br.   Brain

A,B, C and D indicate the position from which the

sections shown as Fig 17 A,B,C and D were taken.
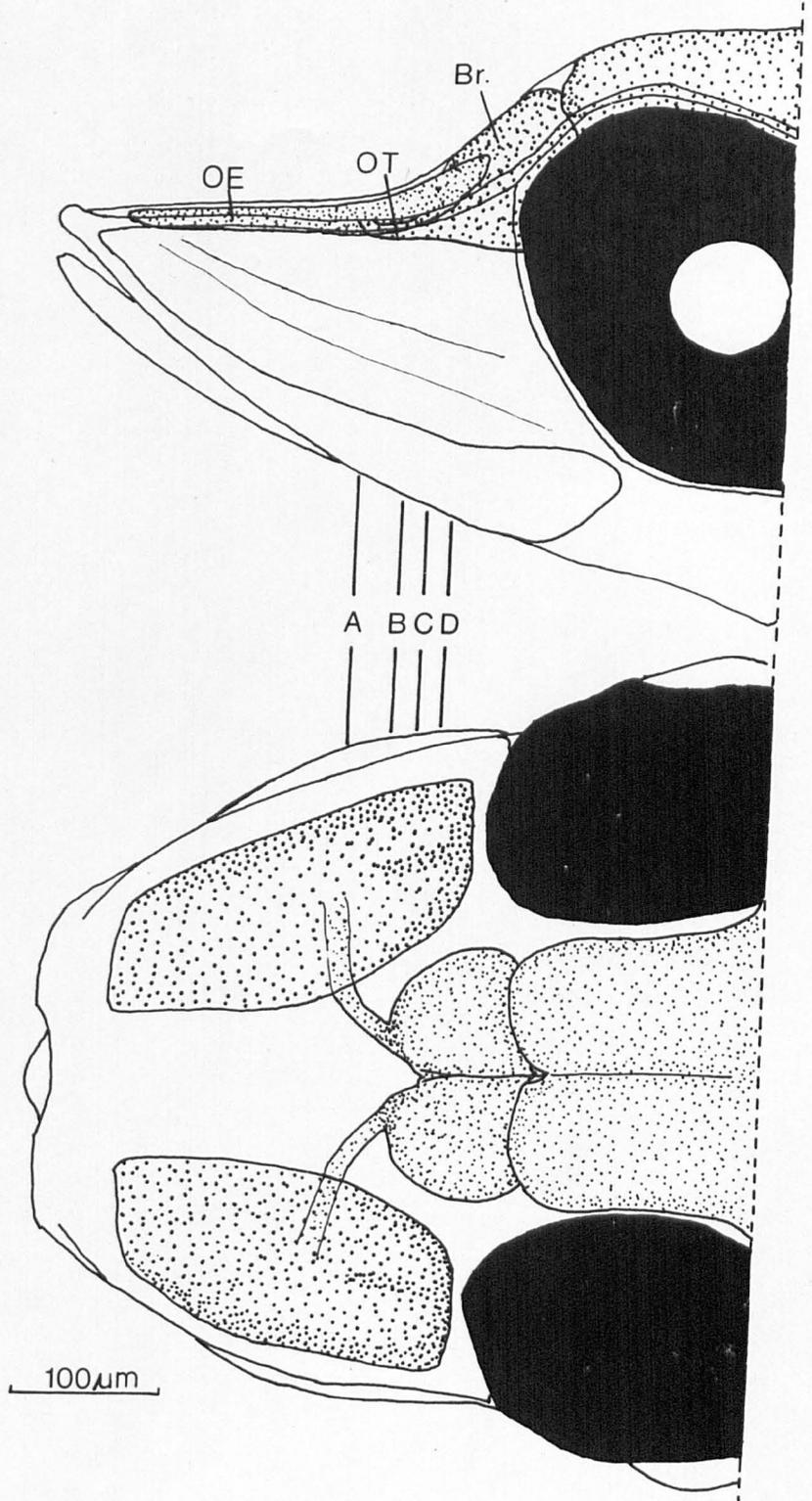
100μm

Fig 17 (A,B,C and D).

Are tissue diagrams of sections from positions

A, B, C and D in Fig 16.
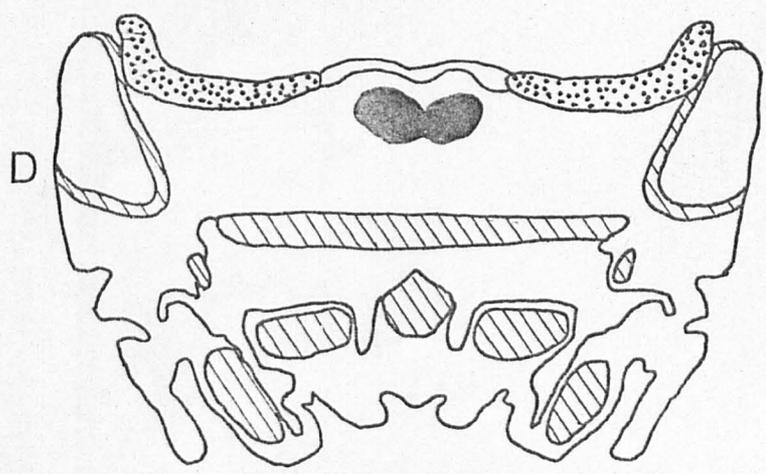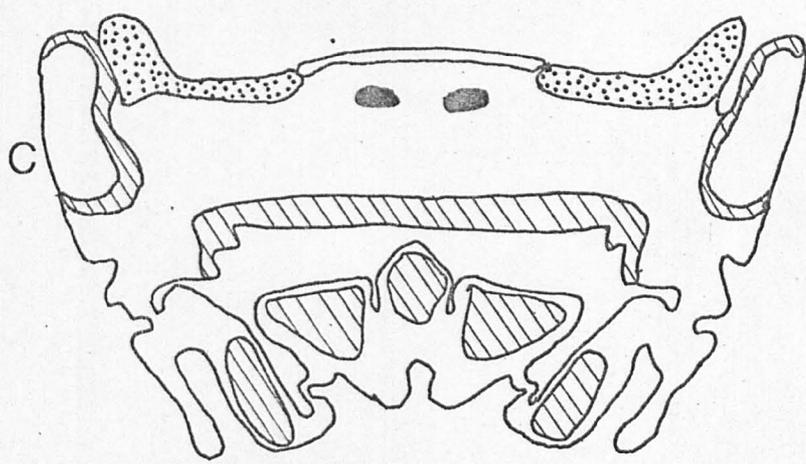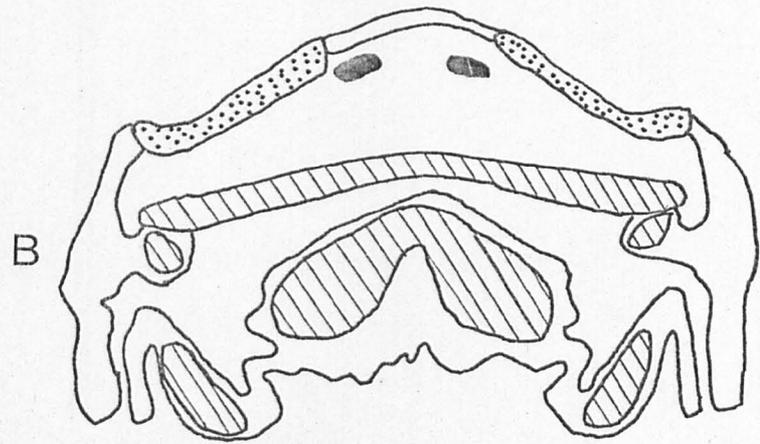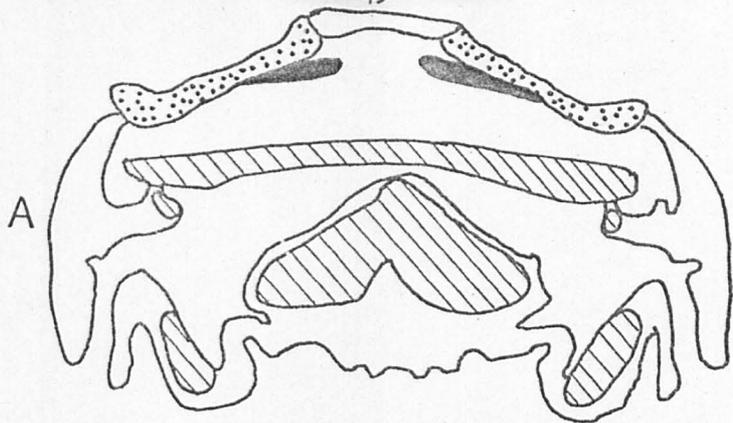
Cartilage

Olfactory epithelium

Nervous tissue

100μm

Fig. 18.   Lateral and dorsal view of the head of a stage 3a

herring larva (17 mm total length) drawn as

transparent and reconstructed as Fig 10.

OE   Olfactory epithelium

OT   Olfactory nerve

A,B,C,D and E indicate the position from which the sections shown

as Fig 19 A,B,C,D, and E were taken.

OE   OT

A B C   D E
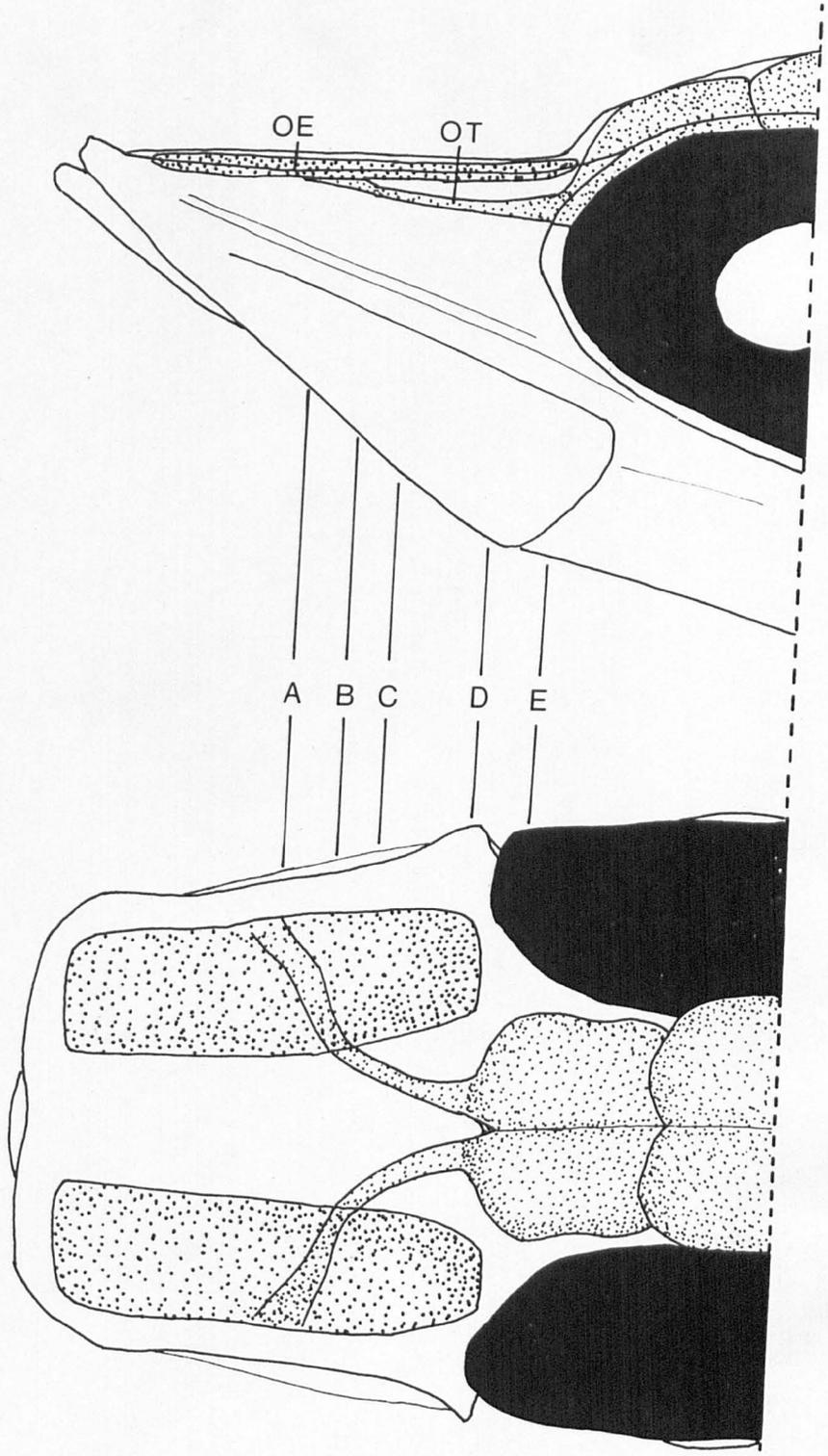
100μm

Fig. 19 (A,B,C,D and E)

Are tissue diagrams of sections from positions

A,B,C,D and E in Fig 18.

A

B

C

D

E

Cartilage

Olfactory epithelium

Nervous tissue

100μm
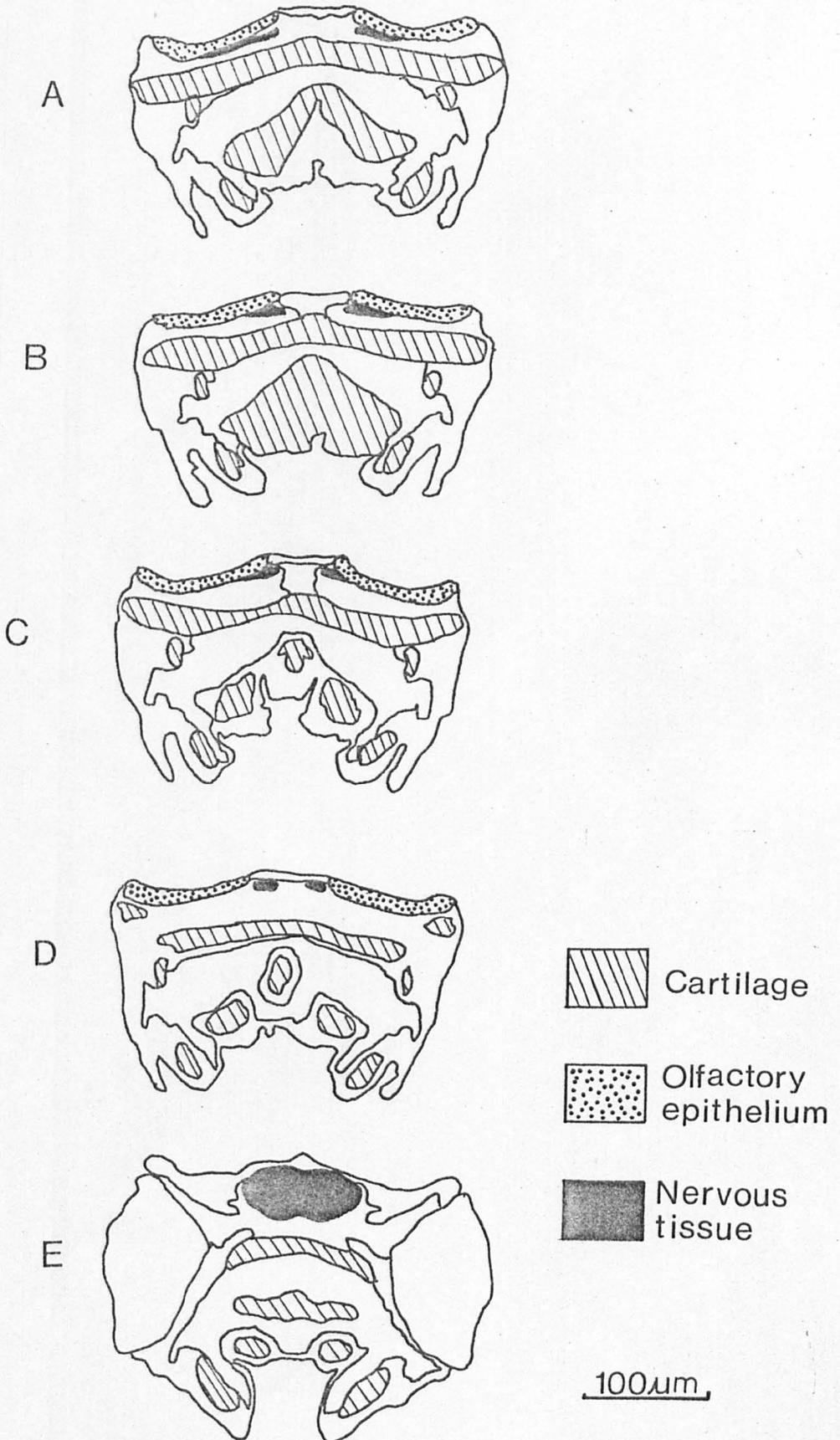
Fig. 20.

    A. Dorsal view of a stage 4a herring larva (23 mm total length)

    B. Dorsal view of a stage 4b herring larva (25 mm total length)

    C. Dorsal view of a stage 4c herring larva (30 mm total length)

    D. Dorsal view of a stage 4d herring larva (35 mm total length)

All are shown as transparent and were drawn from measurements

taken from whole specimens preserved in 4% formol saline.

    OE   Olfactory epithelium

    OC   Olfactory capsule

    OT   Olfactory nerve

    M   Melanophores, on the surface of the skin and

          brain.

A.

1mm

B.

OT

OE

1mm

C.

M

OT

OC

1mm

D.

OT

OC

M

1mm
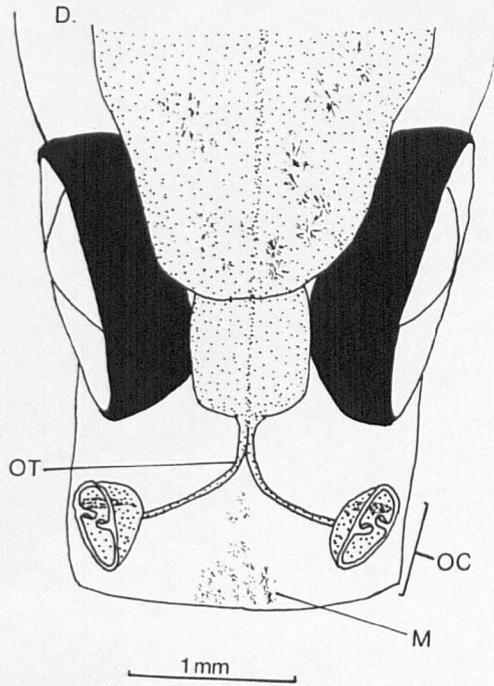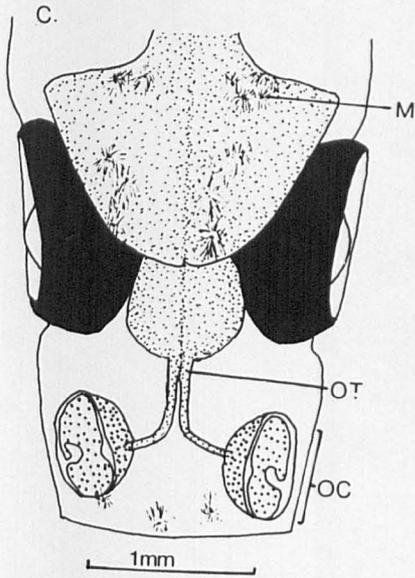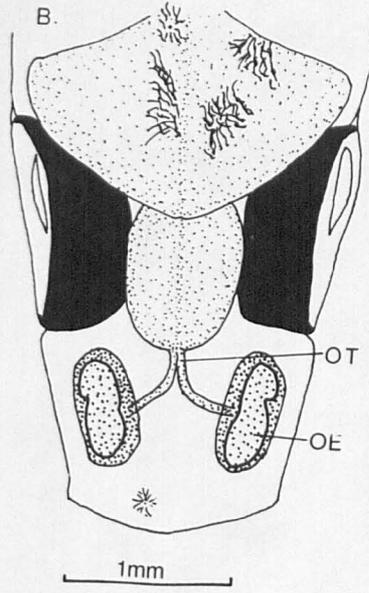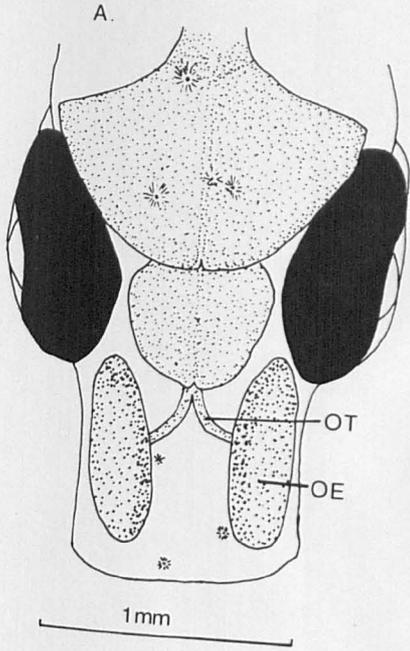
Plate 1.   Photomicrograph of the region in a newly
            hatched herring larva where cells from the
            forebrain extend into the olfactory epithelium.

Plate 2.   Photomicrograph of the olfactory nerve in a
            stage 1b larva. This is the earliest stage of
            those examined where a distinct olfactory nerve
            could be seen.

Plate 3.   Photomicrograph showing the relationship between
            the olfactory nerve and sensory epithelium.

Plate 4.   Photomicrograph showing the olfactory nerve
            entering the sensory epithelium.

            Plates 3 and 4 are taken from sections 21 μm
            apart cut from a stage 3a larva. Plate 4 being
            the anterior.

                            KEY

            B   Brain

            SE   Sensory epithelium

            ON   Olfactory nerve

            F   Area where cells of forebrain
                            extend into sensory epithelium.

            C   Cartilage

1

100 μm

50 μm

2
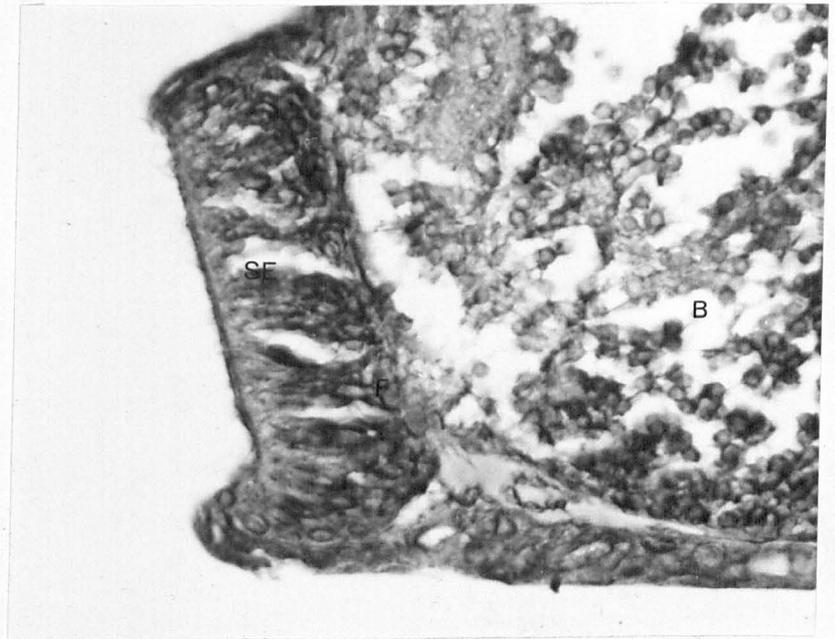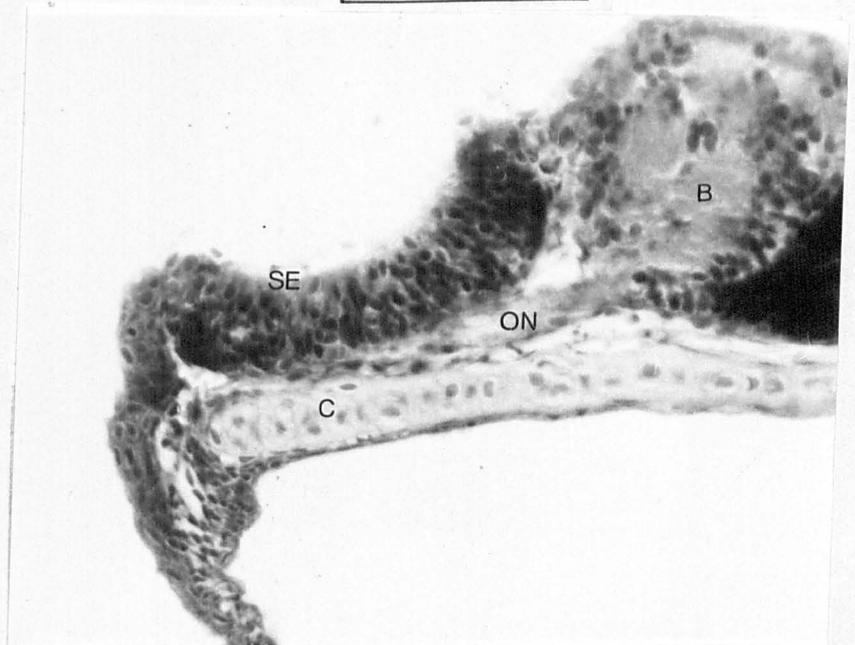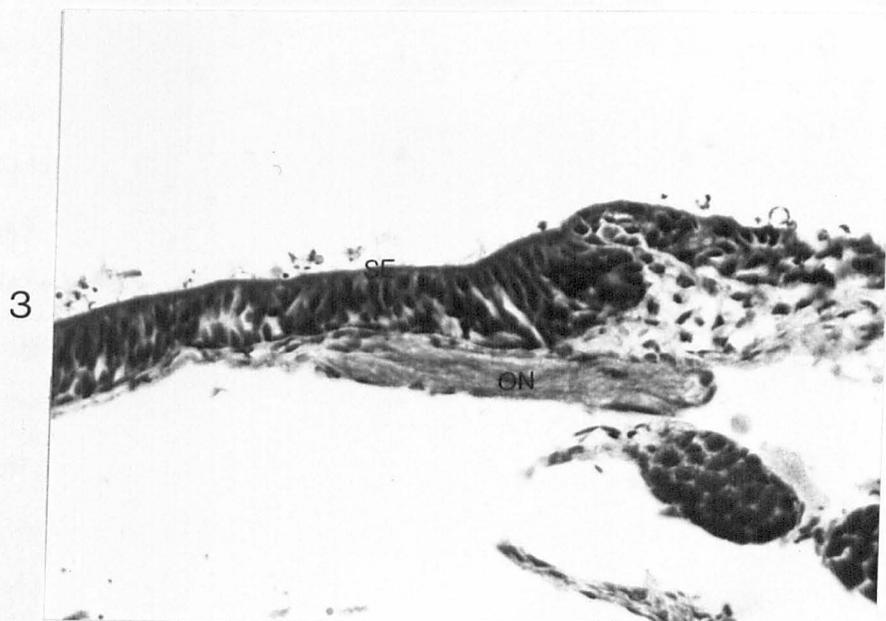
50 μm

2.   SURGICAL ABLATION OF THE OLFACTORY NERVE IN HERRING.

Five experiments were carried out on the sham-operated larvae to see if they had retained their reaction of increased activity to aqueous Artemia extract. An extract of 1 gm (wet weight)/l was used, this being over 10 times the threshold for reaction. A significant increase in activity was found after addition of extract compared to addition of seawater (p = 0.03). 8 similar experiments were carried out on the larvae with severed olfactory nerves to see if they had retained their reaction to the extract. No significant difference could be found between reactions to the addition of an extract of 1 gm (wet weight)/l and seawater (p = 0.53). It was therefore concluded that severing the olfactory nerve caused a loss of reaction to this stimulus and that detection of the stimulus was by olfaction alone.

3. GENERAL OBSERVATIONS OF LOCOMOTOR ACTIVITY.

As a result of watching total movements in the fluvarium it soon became obvious that one could distinguish several types of swimming behaviour as well as noting a change with development. An understanding of the various types of activity helped in interpret ing the results from fluvarium and activity chambers.

A. Herring.

On first hatching the larvae swam only feebly although they were able, when startled, to cover relatively large distances at greater speed. Most of the active time was spent in vertical movement. The larvae (Fig 21A) rose in a corkscrew action and sank at an angle, usually about 20° to the vertical, the loops of the spiral being up to 2-3 cms in diameter. Very little horizontal swimming was seen. The majority of time (particularly in shallow containers) was spent lying on the bottom. After a few days the corkscrew action in rising was no longer seen, but much of the time was still spent rising and sinking in the water column. Much more horizontal swimming was seen and less time was spent resting on the bottom. At the end of the yolk sac stage hardly any time was spent resting on the bottom (except in obviously moribund fish). The time was spent alternately swimming slowly by body movement described below and drifting without any swimming action. This drifting inevitably brought larvae in the experimental chamber of the fluvarium into contact with the mesh screen at its downstream end. Most of the time was spent near the surface; and swimming, even if far below the surface, was now usually in a horizontal plane.

After feeding became established a new pattern of activity was seen to develop and differences in swimming behaviour were seen.

Fig. 21.   Locomotor patterns of larval herring.

See text for explanation.

A.

B.

C.
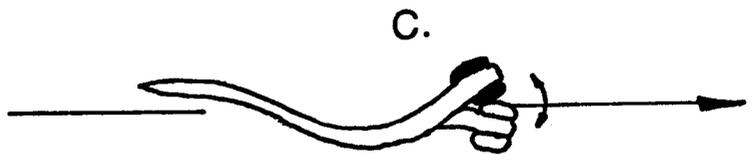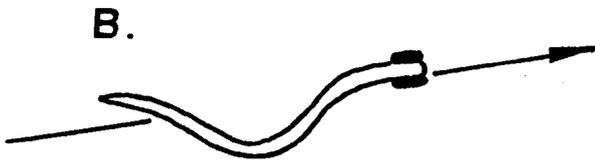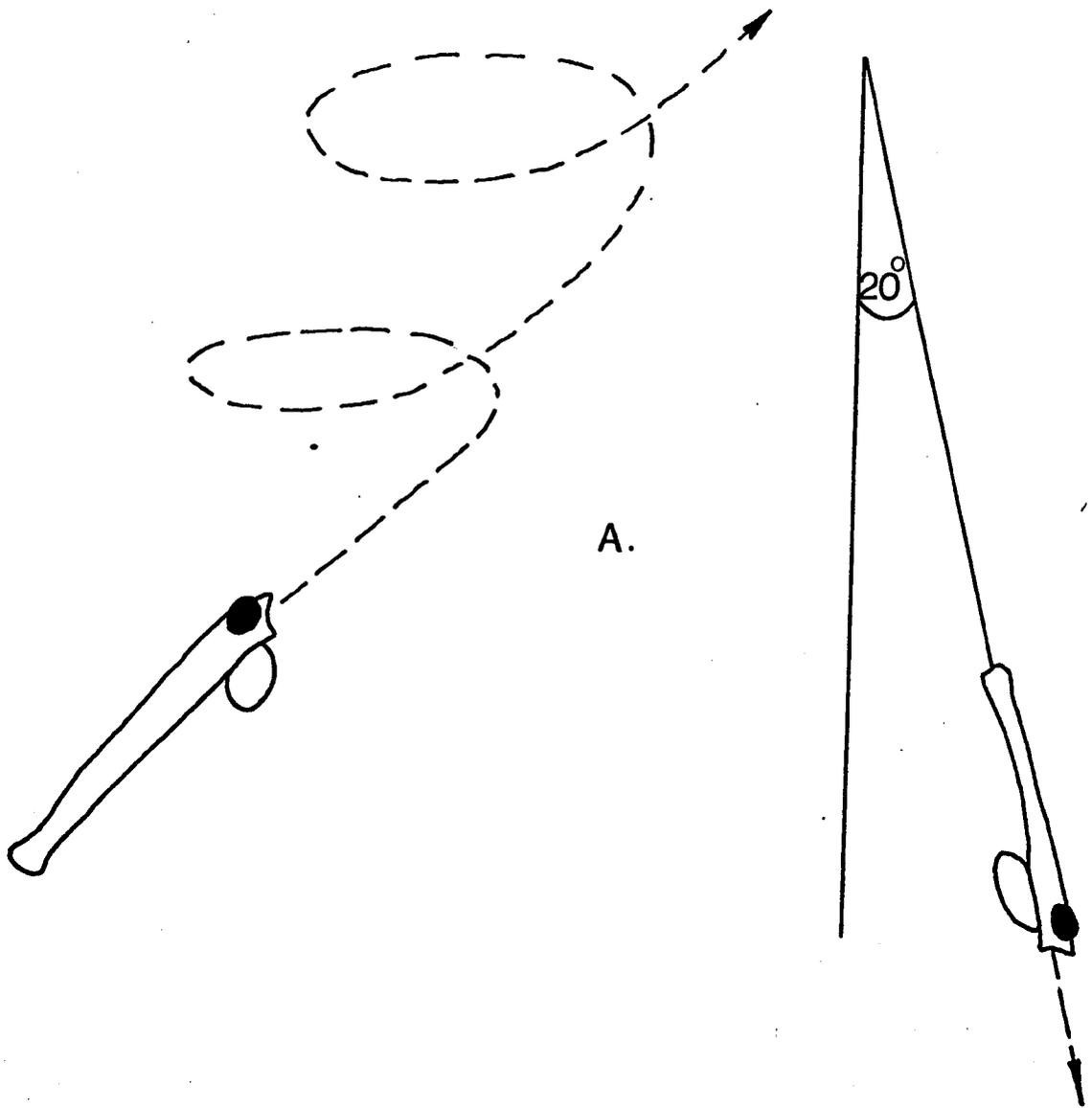
Some of these differences were thought to be related to motivational differences as a result of some of the substances being tested. The larvae still alternately swam and rested. Resting was at several levels in the water column and was characterized by a complete absence of swimming action although resting was not the only cause of a larva staying in one position. Larvae occasionally rested on the surface where they could remain in one position, not necessarily at the mesh screen. This was presumably due to surface tension effects. Lower in the water if swimming action ceased larvae were inevitably carried into the mesh screen by the current. Coming into contact with this screen did not usually provoke an escape reaction and the larvae could remain against the mesh for up to several minutes. On many occasions the larvae could be seen actively swimming into the mesh screen, with the current, this would be kept up for up to 1 min. This behaviour was seen less frequently as the larvae grew older. Similarly larvae would also swim into the 'Perspex' forming the upstream end of the chamber. It was rare for the larvae to swim into the wall at the end of the chamber, that is, perpendicular to current flow, and in this case it was almost always into a corner of the chamber.

Swimming tended to be in the form of 'forays' from the stationary phases (either resting or swimming into the side) and was of three types. The usual form of swimming was a slow form, the body moving like a snake through the water (Fig 21B). This action could be kept up for some time. The usual action was occasionally modified to a slower but essentially similar action where the head was moved from side to side, thought to allow a greater area to be searched visually (Fig 21c). This modified behaviour was usually only seen when chemical stimuli were present, but by no means on

all such occasions. A third sort of swimming was obviously an escape reaction. Using this a larva could swim quickly several hundred mm in 1-2 sec. This was usually only seen on introduction of the larva or during attempts to remove it from the apparatus.

Observations of juvenile herring had shown that in addition to turning by swimming in a circle they could turn very rapidly through 180 degrees by body flexion. This was never seen in larvae. Although the rate of tail beat, when the larva was swimming into the sides of the chamber was too high to gain an accurate measurement visually a subjective opinion was adopted that the rate of tail beating increased when some test substances were introduced. But again this was not seen in all cases.

B. Plaice.

The swimming action of plaice larvae contrasts strongly with that of herring larvae. The plaice used its pectoral fins to swim and so was able to swim backwards for short distances. For the first days after hatching the larvae stay at the surface, probably as a result of the buoyancy of the yolk sac. After 2-3 days the plaice began feeding and were able to swim in mid-water but remained mainly at the surface, particularly at the sides of the experimental chamber. As with the herring larvae the current in the fluvarium meant that unless actively swimming the larvae were inevitably swept into the mesh screen. The plaice larvae tended to move along the mesh screen when against it but also spent some time resting, when they showed no movement of the pectoral fins. Periods of swimming as with the herring larvae tended to be in the form of 'forays' from a resting state and were typically of short duration. An 'escape reaction' was also seen which was similar to that of the herring larva but of shorter duration. This pattern of swimming

continued until the onset of eye migration when body undulations begin to play an increasingly greater part in swimming. Swimming was still in a typically roundfish mode but periods of swimming on the side were seen. Despite the fact that the right eye had not yet migrated the larvae tended to spend more and more time on the bottom of the experimental chamber. When eye migration was complete the metamorphosed larvae spent most of their time resting motionless on the bottom showing occasional short darts of a few millimetres or longer movements. The plaice very rarely rose into the water column and on these occasions swam on their side in a typical flatfish manner.

4. REACTIONS TO FEEDING-RELATED STIMULI.

A. Thermistor experiments

(i) Herring

Before discussing the results mention must be made of the anomalies in Table 1 caused by the experimental method. As was mentioned in the methods section the number of replicates carried out was the minimum number necessary to achieve significance for the results at the 5% level using the sign test. The further application of the randomization test was both to quantify the probability and elucidate some results which approached significance using the sign test and perhaps by using more of the data allow the null hypothesis to be rejected. In the event no experiments, which did not allow the null hypothesis to be rejected using the sign test, allowed this using the other test applied. In two cases however using more of the information meant the null hypothesis could not be rejected by the second test. As in these cases the number of replicates was limited to when significance was reached on the sign test, it is very likely more replicates would allow significance on the more powerful test. Looking at the case of glycine a reaction is proven at earlier and later stages than that which the anomalous result appears. In view of this it was decided to reject the null hypothesis on both anomalous occasions.

Results presented in Table 1 show that from the earliest stages herring larvae show a response to the prey organism, nauplii of _Balanus balanoides_, this response being seen to both intact organisms and to an extract. At this stage the larvae have not experienced, even as eggs, any prey organisms in the rearing tank such that they might learn to associate the odour with the prey. One is therefore drawn to the conclusion that this is an innate reaction. The existence of such an innate reaction is not

TABLE 1

ANALYSIS OF RESULTS OF HERRING LARVAE THERMISTOR ACTIVITY EXPERIMENTS

Test stimulus	Feeding regime	Yolk sac stage		Balanus feeding stage		Artemia feeding stage		Rotifer feeding stage	
		Sign test	P	Sign test	P	Sign test	P	Sign test	P
1. Balanus nauplii washing		+	0.094	+	0.030				
2. Balanus nauplii extract		+	0.006	+	0.010	−	0.370		
3. Artemia nauplii washing		−	0.310	−	0.190	−	0.340		
4. Artemia nauplii extract		−	0.250	−	0.370	+	0.023		
5. Rotifer extract		−	0.690					−	0.50
6. Glutamic acid 10^{-3} M		−	0.230	+	0.046	+	0.015		
7. Aspartic acid 10^{-3} M		−	0.300			+	0.016		
8. Glycine 10^{-3} M		+	0.030	+	0.180	+	0.016		
9. Methionine 10^{-3} M		−	0.100	−	0.660	+	0.016		
10. Alanine 10^{-3}M		−	0.720	−	0.960	+	0.010		
11. Proline 10^{-3}M		+	0.010			+	0.016		
12. Cysteine 10^{-3} M		−	0.640	−	0.630	−	0.860		

Shown above are the results of a sign test at the 5% rejection level and the probability (P) of no significant difference between control and test calculated by the randomization test. + indicates null hypothesis is rejected, − indicates null hypothesis is not rejected.

From the results in Table 1 it will be noted that in non-feeding larvae there is a reaction to washings and extracts of Balanus but not to those of rotifers or Artemia, although a reaction to an Artemia extract but not a washing, develops after Artemia has formed part of the diet. Reactions are also found to two amino acids in the non-feeding larvae and the range of amino acids responded to increases in both of the subsequent feeding regimes.

surprising for two reasons. First as has been mentioned <u>Balanus</u> nauplii are used as food in the rearing regime because it is known they form an important food source both for larval (Lebour, 1916) and adult herring in the Clyde (Marshall, Nicholls and Orr, 1938). Secondly, a selective advantage would be conferred on a larva if it could restrict expenditure of energy, which is derived from a limited supply of yolk, to such times as it can tell from chemoreception that prey organisms are available. This would tend to maximize the number of prey taken per unit of energy expended. Further evidence as to the innate nature of the reaction to <u>Balanus</u> nauplii is shown in the finding that the reaction is also present in the autumn-reared larvae, living at a time outside the <u>Balanus</u> spawning season.

It is only after feeding has become established on <u>Artemia</u> nauplii that a reaction is seen to an <u>Artemia</u> extract. It is thought that this must arise by the larva associating the odour of released body fluids with the feeding act. The strength of this conditioning is seen in the finding that no response could be shown to a <u>Balanus</u> extract in an experiment performed 50 days after feeding on <u>Artemia</u> had commenced. At no stage was it possible to show a reaction to <u>Artemia</u> washing; nor was it possible to show a reaction to Rotifer extract in the autumn reared larvae. Rotifers were only part of the diet for 10-12 days, possibly not long enough to develop a reaction to a new diet. In the non-feeding yolk sac stage reactions are seen to two of the seven amino acids tested (glycine and proline), after feeding has begun the range is increased to include glutamic acis and after the diet change to <u>Artemia</u> responses are found to methionine, alanine and aspartic acid although reaction to the latter may also occur in the <u>Balanus</u> feeding stage. This progressive stepwise increase in range of response to amino acids could be a

result of development of acuity of the sensory system or as a result of the fish conditioning itself to the scent of further amino acids as the changes in diet occur. The first possibility seems unlikely as in view of the ability of the earliest larvae to distinguish between extracts of Balanus and Artemia nauplii it would seem the sensory system was functionally very precise at   this stage. The second possibility seems worth pursuing further. The free amino acid composition is known for Balanus balanoides (see Barnes and Blackstock, 1976) and for Artemia salina (see Emerson, 1967) and a comparison of both studies is given in Appendix X. It is interesting to note that the two amino acids proline and glycine which produced increased activity in the yolk sac stage herring larvae together form nearly 40% of the free amino acid in Balanus and are present at considerably higher levels than in Artemia. In the case of proline almost an order of magnitude difference exists. This provides indirect evidence of the role of amino acids in producing a response to tissue extracts and could help to explain the lack of response to Artemia extract in yolk-sac larvae.

Experiments were carried out into threshold of response to Artemia extract which was found to be 11-15 mg/l (dry weight), 75-100 mg/l (wet wt) (Table 2). Comparing this with other workers Steven (1959) found a response at 0.1 mg/l of mollusc muscle extract (wet wt) in Hepsitia and McBride et al. (1962) found a threshold of response in juvenile pacific salmon to be 0.13 mg/l wet weight for zooplankton 0.4 mg/l for Artemia, this incidentally also shows a difference in sensitivity to two extracts. From these results over an order of magnitude exists between the thresholds found by Steven (1959) and McBride et al (1962) and those found in this study. Both these studies were on metamorphosed fish however and used presence of a definite behavioural observation as a positive

TABLE 2

THRESHOLDS OF RESPONSE OF HERRING LARVAE IN THERMISTOR ACTIVITY EXPERIMENTS.

Stimulus	Concentration	P
Artemia nauplii extract	0.010	0.72
	0.050	0.81
	0.075	0.65
	0.1	0.02
Dialysed Artemia nauplii extract	0.2	0.02
	0.02	0.05
Glutamic acid	10^{-7}	0.41
	10^{-6}	0.75
	5×10^{-6}	0.03
	10^{-4}	0.01

Shown above is the probability (P) of no difference between controls and tests calculated by the randomization test. Concentrations of extracts and dialysed extracts are given in terms of a standardized wet weight (g) of whole organisms per litre and for glutamic acid as Molar.

Thresholds therefore are:

Artemia nauplii extract	0.075 - 0.10 g/l
Dialysed Artemia nauplii extract	0.02 g/l
Glutamic acid	$10^{-6} - 5 \times 10^{-6}$ M

A conversion factor was obtained to convert standardized wet weights to dry weights.

This factor is 0.15

So in terms of dry weight the threshold values are:

Artemia nauplii extract	0.011 - 0.015 g/l
Dialysed Artemia nauplii extract	0.003 g/l

reaction, it may well be a more sensitive method than that used in this study. It may also be possible that acuity increases with development. This was discussed above. Experiments into the threshold of response of dialysed _Artemia_ extract found a value of 20 mg/l this means that the dialysed fraction is at least as effective as the whole extract. This fraction would contain substances with a molecular weight of less than ca. 14,000, information received from the manufacturer places a limit of 12,000-14,000 on the maximum size of molecule able to pass through the membrane. In the case of _Artemia_ nauplii extract for which thresholds were obtained the dialysate had a lower threshold than the whole extract, this could be because some substance in the whole extract either masked the active constituents or acted as depressants of feeding behaviour in antagonism to the active fraction.

(ii) Plaice

The parallel comparative study on plaice was of necessity less extensive than that on herring larvae owing to the much shorter larval life of the plaice. The results of thermistor activity experiments in Table 3 show a reaction by plaice larvae to _Balanus_ extract, a prey which they had not experienced, but not to _Artemia_ extract, their sole food supply. This is unusual and in marked contrast to herring which showed a reaction to _Artemia_ extract soon after feeding on _Artemia_ nauplii began. In comparison to herring larvae (see Table 1) a smaller range of amino acids produce a response; only aspartic acid and methionine of the six tested (Table 3). Again no reaction was found to cysteine. The lack of response to _Artemia_ extract could be for one of several reasons. First, the very different patterns of swimming behaviour in plaice and herring larvae may mean that a change in swimming behaviour in response to detection of a stimulus

TABLE 3

ANALYSIS OF RESULTS OF PLAICE LARVAE THERMISTOR ACTIVITY EXPERIMENTS

TEST STIMULI	SIGN TEST	P
1. Balanus nauplii extract	+	0.03
2. Artemia nauplii washing	−	0.37
3. Artemia nauplii extract	−	0.41
4. Glutamic acid 10^{-3} M	−	0.41
5. Aspartic acid 10^{-3} M	+	0.03
6. Methionine 10^{-3} M	+	0.03
7. Alanine 10^{-3} M	−	0.22
8. Proline 10^{-3} M	−	0.16
9. Cysteine 10^{-3} M	−	0.97

Shown above are the results of a sign test at the 5% level and the probability (P) of no significance difference calculated by the randomization test. + indicates null hypothesis is rejected, − indicates null hypothesis is not rejected.

is less readily detected by the thermistor monitoring system; this seems unlikely in view of the fact that some responses were obtained. It is possible that only much stronger responses are being detected by the system compared with herring. Secondly, as is discussed more fully below it is possible that plaice, to a greater extent than herring, require secondary reinforcement (for example visual cues) to initiate a response to an olfactory stimulus. It is also possible that the larval plaice olfactory system is less developed, less sensitive or has a poorer range than that of the herring larvae. The absence of a reaction at any stage in herring and plaice to cysteine is in contrast to its effectiveness stimulus to catfish (Bardach, Todd and Crickner, 1967; Suzuki and Tucker, 1971) and various salmonids (Hara, Law and Hobden, 1973). Cysteine is, however, the least soluble in seawater of those amino acids tested in this study 10^{-3}M being close to its solubility limit in seawater. This may give a clue as to why it may be of little value as an olfactory cue to marine teleosts. Blackstock (pers. comm.) also noted that it is quickly altered chemically in extracts and may have a short life outside living tissue. As with herring no response was found to Artemia nauplii washing, if as is suggested above reactions to Artemia nauplii are acquired by self-conditioning then the continual presence of Artemia nauplii in the water would mitigate against acquisition of a reaction to a washing due to the process of adaptation, Steven (1959) found the reaction to zooplankton washing was lost after a period in a system where such a washing formed the seawater supply. Also if washing was continually present rather than as the extract, released in the feeding act then no conditioning could take place.

B.   Fluvarium experiments (changes in activity level).

   (i)   Herring

   As has been mentioned fluvarium experiment results were to be analysed in two ways. First to test for changes in activity level which might explain the results of thermistor experiments and second to examine the effect of a move, where a larva experiences change in the stimulus concentration around it, on subsequent behaviour. The most obvious explanation of increased activity as recorded using the thermistor technique would be that an increased percentage of time was spent in active movement and either as a direct consequence or as a result of an increased swimming speed a greater distance was covered during periods of presence of a stimulus. To this end an analysis for significant differences between control and test in the parameters 'percentage of time active' and 'mean total distance moved' per trial was undertaken. No use was made of the mean step length as the differences between test and control were less than the minimum discernible move (12 mm). The results are presented in Table 4. Analysis of these parameters for differences in behaviour due to development (Table 5) showed that the <u>Balanus</u> feeding stage larvae are similar to the yolk-sac larvae, both of these being significantly less active than the later <u>Artemia</u> feeding stage larvae.

   Before discussing these results of activity level parameters we must consider the difference in behaviour in the absence of stimuli between early and later herring larvae due to time spent in the fluvarium. From Table 4 a fall off of activity with time occurred in control experiment 2 on later larvae compared with the steady level in control experiment 1 on early larvae. This fall off was found to be statistically very significant ($P < 0.01$), Table 6. As mentioned above this decrease in activity seems to be connected with depth of water in an apparatus rather than due to any restriction in the

TABLE 4

PARAMETERS DESCRIBING ACTIVITY LEVELS OF HERRING LARVAE IN A

FLUVARIUM GRADIENT OF FEEDING RELATED STIMULUS

Stage of larvae and test stimulus	N	Time active (% of total in experiment)			Mean step length (mm)			Mean total distance per trial (mm)		
		C	T1	T2	C	T1	T2	C	T1	T2
YOLK SAC STAGE										
Balanus nauplii washing	12	30	32	26	31	27	29	212	264	275
Balanus nauplii extract	13	27	19	10	21	27	24	134	172	80
Artemia nauplii washing	12	33	29	24	27	25	25	223	236	196
Artemia nauplii extract	10	30	32	29	31	23	31	211	239	273
Glutamic acid	13	41	24	20	24	20	25	210	154	171
Aspartic acid	10	30	16	21	23	26	24	156	126	168
Glycine	10	37	23	21	23	25	25	215	162	181
Methionine	10	33	31	21	25	29	24	177	205	173
Alanine	10	38	32	23	22	19	21	223	179	155
Proline	11	40	32	26	21	25	22	224	228	177
Cysteine	10	35	33	37	20	21	19	177	223	245
CONTROL EXPERIMENT 1	11	29	35	27	24	33	24	226	248	199
BALANUS FEEDING STAGE										
Balanus nauplii washing	10	26	23	19	20	21	23	130	139	149
Balanus nauplii extract	13	37	30	22	28	19	32	222	219	192
Dialysed Balanus nauplii extract	6	38	32	32	34	31	27	344	266	301
Artemia nauplii washing	9	33	30	22	23	22	25	165	188	192
Artemia nauplii extract	10	29	28	25	22	22	23	174	174	169
Glycine	10	40	26	24	20	18	23	236	175	182

TABLE 4 cont.

Stage of larvae and test stimulus	N	Time active (% of total in experiment)			Mean step length (mm)			Mean total distance per trial (mm)		
		C	T1	T2	C	T1	T2	C	T1	T2
ARTEMIA FEEDING STAGE										
Artemia nauplii washing	18	46	41	39	35	35	33	435	442	402
Artemia nauplii extract	23	48	47	44	30	23	26	390	350	382
Glutamic acid	10	47	47	41	29	26	24	339	344	306
Aspartic acid	10	38	29	26	26	21	21	253	179	174
Glycine	10	41	36	26	22	20	30	231	210	251
Methionine	10	45	37	36	24	20	23	309	237	275
Alanine	10	51	49	38	27	22	26	341	348	314
Proline	10	43	43	39	25	22	26	260	320	304
Cysteine	10	46	44	36	21	21	26	232	277	268
CONTROL EXPERIMENT 2	10	42	28	23	26	25	20	280	194	150

Shown above are the number of trials (N); the percentage of time spent in moving; the mean distance in mm travelled per move (step length) and the mean total distance travelled in the control period (c) and the first and second test periods (T1 and T2) by a single larva. The results of an analysis for a difference between these periods is presented in Table 7.

TABLE 5

ANALYSIS FOR CHANGES IN HERRING LARVA ACTIVITY LEVEL AND MODE
RELATED TO DEVELOPMENT.

A. Mean values and standard deviation of parameters

PARAMETER	Yolk sac stage		Balanus feeding stage		Artemia feeding stage	
	M	SD	M	SD	M	SD
% Time active	34.0	4.6	33.8	5.5	44.7	3.8
Mean step length (mm)	24.1	3.8	24.5	5.5	26.5	4.1
Total distance/trial	199	31	202	76	317	68
Mean direction	+6.5	15.1	-6.6	28.8	+11.8	16.0
Degree of dispersion	.99722	.00095	.99653	.00084	.99794	.00069

Shown above are the means (M) and their standard deviations (SD) of
the control periods of all trials on the three stages.

From Table 5A it can be seen that the parameter mean direction has
a high standard deviation and the parameters degree of dispersion and
mean step length show great homogeneity so no conclusions can be drawn
from these parameters. There is however differences between the
stages with respect to the parameters percentage time acive and total
distance moved per trial, suggesting a similarity between the yolk sac
and Balanus feeding stage larvae and a difference between these
larvae and the Artemia feeding stage larvae.

TABLE 5 cont.

B.   Analysis for differences between stages

PARAMETER	Compare yolk sac and Balanus feeding stage		Compare yolk sac and Artemia feeding stage		Compare Balanus feeding and Artemia feeding stage	
	Z	P	Z	P	Z	P
% Time active	0.41	>.10	6.09	< .002	6.48	< .002
Total distance/trial	0.24	>.10	12.04	< .002	4.14	< .002

Shown above are the result (Z) of Zm tests and the associated probability of no significant difference in the parameters of percentage time active and total distance per trial between the three stages. This shows the similarity of control period behaviour in yolk sac and Balanus feeding stages and the difference between both these stages and the later Artemia feeding stage to be statistically significant.

TABLE 6

ANALYSIS FOR CHANGES IN HERRING LARVA ACTIVITY LEVEL RELATED
TO LENGTH OF TIME SPENT IN THE FLUVARIUM.

| PARAMETER | Control experiment 1 | | | | Control experiment 2 | | | |
| | Test 1 | | Test 2 | | Test 1 | | Test 2 | |
	W	P	W	P	W	P	W	P
% time active	16.5	>0.05	15	>0.05	0	<0.01	3	0.01
Total distance/trial	31.5	>0.05	29	>0.05	2	< 0.01	2	<0.01

Shown above are the results (W) of a Wilcoxons matched-pairs signed-ranks
test and the associated probability (P) of the values of the parameters
in the first and second test periods not being significantly different
from their control periods.

This shows that in the case of control experiment 1 on stage 1c larvae
(Yolk sac and Balanus feeding stage) the null hypothesis cannot be rejected
and therefore it is concluded that there is no change in either of the
parameters due to length of time spent in the fluvarium. In the case of
control experiment 2 on Artemia feeding larvae the null hypothesis can be
rejected with 99% confidence and a new hypothesis proposed that there is a
real change in activity pattern due to length of time spent in the
fluvarium. This change is a decrease both in time spent in moving and the
total distance covered per trial.

This result means that only the results for yolk sac and Balanus feeding
stage can be analysed by the Wilcoxons matched-pairs signed-ranks test on the
null hypothesis of no significant difference between periods of the trials
existing. Results of this analysis are given in Table 7A.

Results for the Artemia feeding stage where analysed by comparison with
the control by the Mann-Witney U test as detailed in the methods section,
results of this analysis are given in Table 7B.

horizontal plane. Herring larvae are known to undergo vertical migrations in the laboratory (Blaxter, 1973). This is largely a function of light intensity, although evidence of some rhythmicity exists. The migratory tendency increases with age. No direct evidence exists but it is considered possible that light intensity will govern the position of the larva in the water column, this being maintained by varying the proportion of swimming and resting activity, a larva having a preferred depth at a given light intensity. As response to light does change with age it is entirely possible that at a given light intensity a later herring larva might prefer a lower position in the water column than an early larva, and in fact at a greater depth than the experimental chamber allows. So despite being more active overall than early larvae the later larvae increasingly spend relatively more time in a 'resting' mode in order to achieve the preferred depth by sinking. This possibility could easily be investigated by observing larvae of different ages under several constant light conditions in a water column and quantifying the time spent swimming and sinking and to test for evidence of a preferred position. The results of percentage activity and mean total distance per trial can now be examined to see if they can explain the activity increases seen in thermistor experiments. Results of an analysis are presented in Tables 7A and 7B.

Of the four substances found to cause increased activity of yolk sac herring in thermistor activity experiments (Table 1), Balanus nauplii extract and glycine both cause a significant decrease in the percentage of time active and in the case of glycine a significant decrease in distance moved. The marked decrease in distance moved in the second test period with Balanus nauplii extract did not prove to be significant. No difference could be found between control and test periods for Balanus nauplii washing and the decrease in

percentage activity and distance moved with a proline stimulus (Table 4) did not prove significant. Additionally glutamic acid which had not been shown to produce a reaction by the thermistor technique is shown to have produced a marked decrease in percentage time spent active. In the case of Balanus feeding stage herring larvae, no significant differences either in percentage time spent active or distance moved were found.

It would seem therefore that the thermistor technique is a more sensitive tool in recording any change in activity as a result of olfactory stimulation and that we have what at first appears to be an anomalous result. It has been found that those substances shown to produce increased activity in thermistor activity experiments which do produce significant changes in the parameters in fluvarium experiments show a decrease rather than an increase in activity parameters. A possible explanation for this is that if the response to detection of a cue was to change the mode of behaviour to the 'searching' mode which is essentially slower, less distance would be covered and as the analysis of percentage time active reflects the number of times the larva has changed its grid position, the slower moving larvae would register as less active. This is despite the fact that a greater area is being searched. Conversely the more exaggerated body movements of the 'searching' motion could create thermal imbalances to a greater degree than 'normal' swimming and thus create a significant increase in activity units on the pen record.

The marked and very significant decrease in percentage activity to glutamic acid (Table 7A) is in contrast to the inability to show a reaction by the thermistor technique. One possible explanation is that despite every effort to co-ordinate thermistor and fluvarium experiments, the thermistor experiments on yolk sac herring with

glutamic acid were carried out on autumn-spawned Manx stock whilst

fluvarium experiments were carried out on spring-spawned Clyde stock.

Although no differences were envisaged between these stocks it is

possible in view of the different first diets the two stocks might

encounter that such a difference in innate reactions might occur.

This is however only one possible explanation and the question is

discussed further below. From the results of experiments on Artemia

feeding stage herring larvae presented in Table 4 significant

increases (in comparison with the decreases in control experiment 2)

in percentage time active are found to Artemia nauplii extract,

glutamic acid, alanine, proline and unexpectedly cysteine (which had
                    recorded
not produced a/reaction using the thermistor technique) Table 6B.

All of these except alanine also show an increase in total distance

(compared with control experiment 2) in one or other test period.

Artemia nauplii washing also produces a significant increase in the

distance moved in the second test period, which is difficult to explain.

In view of the absence of any other evidence of a significant

behavioural change due to this stimulus this may be a spurious result

and will not be discussed further. The presence of a response to

cysteine is also not readily explained, unlike the anomalous reaction

to glutamic acid seen in yolk sac larvae both thermistor and

fluvarium were carried out in the same 2 day period. The results of

an increased activity in the case of Artemia feeding stage herring

larvae is in contrast to the significant decreases in activity

parameters seen in yolk sac stage herring larvae. It must be noted

however that results from the earlier larvae which had no tendency

to lowering of activity levels purely as a result of time were

analysed by comparison of test and control. The results from later

larvae were analysed by comparison with the control experiment 2

which has been discussed above and demonstrates the tendency to

TABLE 7

THE EFFECT OF TEST STIMULI ON HERRING LARVA ACTIVITY LEVEL PARAMETERS.

A. Yolk sac and Balanus feeding stages.

TEST STIMULUS	% TIME ACTIVE				TOTAL DISTANCE				CRITICAL VALUE of W
	Test 1		Test 2		Test 1		Test 2		
	W	P	W	P	W	P	W	P	
YOLK SAC STAGE									
Balanus nauplii washing	30	>.05	26	>.05	26	>.05	15	>.05	14
Balanus nauplii extract	7	.01	3	<.01	20	>.05	22	>.05	17
Artemia nauplii washing	46	>.05	20	>.05	20	>.05	19	>.05	14
Artemia nauplii extract	20	>.05	20	>.05	25	>.05	21	>.05	8
Glutamic acid	7	<.01	4	<.01	27	>.05	25	>.05	17
Aspartic acid	9	>.05	18	>.05	15	>.05	30	>.05	8
Glycine	6	<.05	9	>.05	6	<.05	16	>.05	8
Methionine	14	>.05	8	.05	27	>.05	24	>.05	8
Alanine	17	>.05	9	>.05	10	>.05	9	>.05	8
Proline	20	>.05	10	>.05	13	>.05	16	>.05	8
Cysteine	20	>.05	22	>.05	13	>.05	17	>.05	8
BALANUS FEEDING STAGE									
Balanus nauplii washing	23	>.05	16	>.05	25	>.05	29	>.05	8
Balanus nauplii extract	32	>.05	26	>.05	46	>.05	24	>.05	17
Dialysed Balanus nauplii extract	7	>.05	7	>.05	10	>.05	9	>.05	0
Artemia nauplii washing	16	>.05	17	>.05	19	>.05	24	>.05	6
Artemia nauplii extract	28	>.05	21	>.05	26	>.05	20	>.05	8
Glycine	15	>.05	9	>.05	22	>.05	26	>.05	8

Shown above are the results (W) of Wilcoxons matched-pairs signed rank tests, the associated probability (P) of no significant difference between control and test periods in percentage of time active and total distance moved and the critical value of W for rejection of the null hypothesis at the 5% level.

From this table it can be seen that there are relatively few significant differences due to introduction of test stimuli, when compared with those stimuli found to cause increased activity using the thermistor method of activity recording. Reference to Table 4 shows that in those cases where a significant difference is found a significant decrease in 'percentage time active' and 'distance moved' occurs in response to the test stimulus.

Table 7 cont.

B. Artemia feeding stage.

TEST STIMULUS	% TIME ACTIVE				TOTAL DISTANCE				CRITICAL VALUE of U
	Test 1		Test 2		Test 1		Test 2		
	U	P	U	P	U	P	U	P	
Artemia nauplius washing	68	>.05	69	>.05	60	>.05	41	<.05	55
Artemia nauplius extract	62	<.05	63	<.05	75	>.05	56	<.05	73
Glutamic acid	21	<.05	30	>.05	24	<.05	35	>.05	27
Aspartic acid	45	>.05	41	>.05	42	>.05	37	>.05	27
Glycine	38	>.05	35	>.05	40	>.05	29	>.05	27
Methionine	31	>.05	64	>.05	41	>.05	34	>.05	27
Alanine	21	<.05	42	>.05	50	>.05	36	>.05	27
Proline	12	<.05	21	<.05	14	<.05	20	<.05	27
Cysteine	24	<.05	33	>.05	15	<.05	17	<.05	27

Shown are the results (U) of a Mann-Whitney U test and the associated probability (P) of no significant difference in the relationship between control and test periods of trials in the conctrol experiment and experiments using test stimuli. Also shown is the critical value of U for rejection of the null hypothesis at the 5% level.

As with the results in Table 7A not all of the stimuli producing an activity change using the thermistor technique (Table 1) show a

significant difference in percentage of time active and total distance moved. Of those showing a significant difference reference to Table 4 shows that it is an increase in activity compared with control experiment 2 but a decrease compared with the trial control period, the same result shown in Table 7A for yolk sac and <u>Balanus</u> stage larvae.

TABLE 8

PARAMETERS DESCRIBING ACTIVITY LEVELS OF PLAICE IN A FLUVARIUM GRADIENT OF FEEDING-RELATED STIMULUS.

Test stimulus	N	Time active (% of total in experiment)			Mean step length (mm)			Mean total distance per trial (mm)		
		C	T1	T2	C	T1	T2	C	T1	T2
<u>Balanus</u> nauplii extract	11	36	35	29	45	40	29	394	409	281
<u>Artemia</u> nauplii washing	4	35	34	28	26	29	42	151	268	290
<u>Artemia</u> nauplii extract	8	39	37	35	15	10	23	273	320	396
Glutamic acid	10	57	53	49	36	40	34	556	625	524
Aspartic acid	10	34	27	24	25	34	26	245	257	221
Glycine	10	39	33	28	25	25	28	287	249	210
Methionine	10	41	38	34	31	27	29	334	304	314
Alanine	10	43	32	19	31	28	28	310	268	181
Proline	10	43	35	32	30	30	27	329	383	236
Cysteine	10	38	31	22	31	29	24	212	253	176

Shown are the number of trials (N); the percentage of time spent in moving; the mean distance travelled per move (step length) and the mean total distance travelled in the control period (C) and the first and second test periods (T1 and T2).

TABLE 9

THE EFFECT OF TEST STIMULI ON PLAICE LARVA ACTIVITY LEVEL PARAMETERS.

TEST STIMULUS	% TIME ACTIVE				TOTAL DISTANCE				CRITICAL VALUE of W
	Test 1		Test 2		Test 1		Test 2		
	W	P	W	P	W	P	W	P	
Balanus nauplii extract	22	>.05	21	>.05	27	>.05	25	>.05	11
Artemia nauplii washing	5	>.05	3	>.05	1	>.05	3	>.05	−
Artemia nauplii extract	13	>.05	17	>.05	7	>.05	9	>.05	4
Glutamic acid	21	>.05	16	>.05	18	>.05	18	>.05	8
Aspartic acid	12	>.05	14	>.05	25	>.05	27	>.05	8
Glycine	13	>.05	15	>.05	18	>.05	14	>.05	8
Methionine	20	>.05	13	>.05	21	>.05	16	>.05	8
Alanine	10	>.05	6	.05	7	>.05	9	>.05	6
Proline	17	>.05	19	>.05	28	>.05	14	>.05	8
Cysteine	7	<.05	7	<.05	25	>.05	20	>.05	8

Shown are the results (W) of Wilcoxon matched-pair signed-rank test, the associated probability (P) of no significant difference between control and test periods and the critical value of W for rejection of the null hypothesis at the 5% level.

Significant decreases in percentage time active are found in experiments using cysteine and alanine as stimuli.

decreased activity with time in the absence of a stimulus.
Examination of the relationship between trial control and test
periods in Table 4 showed a relative decrease in distance moved.
This would be compatable with an increasing proportion of 'searching'
swimming in stimulated larva, similar to the situation in yolk sac
larvae; as against the normal tendency of later herring larvae in
the absence of stimulation to show an increasing proportion of
resting behaviour in the test chambers as demonstrated in control
experiment 2.

(ii)   Plaice

Examining the parameters 'percentage time active' and 'distance
moved' in the case of plaice larvae (Tables 8 and 9), only in the
case of cysteine and alanine is any significant difference between
test and control periods seen, where a significant decrease in the
'percentage of time active' was found. Again neither cysteine nor
alanine were   shown to produce a reaction using the thermistor
technique. From Table 8 it can be seen that as with herring larvae
the increased activity recorded by the thermistor method was not
shown to be as a result of discernible increases in levels of
existing locomotor behaviour. In the case of plaice larvae no
behavioural reactions in response to stimuli were observed in this
study. Wyatt (1972) showed differences in proportions of resting
and swimming behaviour in response to prey density but does not
further categorise swimming behaviour as can be done for herring.
If such differences in proportions of swimming and resting behaviour
were shown in response to a purely olfactory stimulus it is thought
that these would have been demonstrated in this study.

From experiments on both herring and plaice the incompatability
of results from thermistor and fluvarium experiments reinforces the

earlier suggestion that the fluvarium technique as used in this study is a less sensitive technique than the thermistor technique for the measurement of changes in the activity level. The factors causing this are probably, first that the minimum step length discernible was 12 mm. This also meant that no attempt was made to draw conclusions from the differences found in mean step length. Second and perhaps more important that changed mode of behaviour rather than straight-forward changes in level of existing activity patterns were the outward signs of stimulus detection. It is possible though from the results of Wyatt (1972) that increased time spent in motion (as distinct from increased distance moved) may be a feature of the behavioural response in plaice larvae.

C. Fluvarium experiments (influence of concentration gradient on behaviour).

Having investigated the method in which detection of stimulus is shown behaviourally the results from the fluvarium experiments can now be further examined to investigate if herring or plaice larvae are able to localize an area of a stimulus by olfaction alone. Many workers have implicated alteration in turning behaviour in the localization of odour sources. The angles of turn in control and test periods were accordingly arranged in histograms of relative frequency. Appendix VIII. The statistics of mean direction and degree of dispersion also presented in Appendix VIII were found to be of little value in view of the remarkable variation and homogeneity respectively. If the histograms of angles of turn are studied by inspection this conclusion is strengthened and no clear picture emerges because of the variation which seems to be as great between controls as between these and test periods. As has been briefly mentioned with respect to distance moved, the lack of any clear

result may be due to the lack of precision in the recording of position. The limitation is that for any larva the angle of turn can only be expressed as one of a maximum of 30 categories. With the additional limitation of the normal step-length this is reduced to probably no more than 14 categories for most moves. In this way any subtle difference in angle of turn would be overlooked in this analysis.

Results were examined for the occurrence of any of the possible methods of orientation in a gradient of odour. Of these the simplest would be tropotaxis. This requires that an organism with a dual sensory system can perceive spatial differences in odour concentration. Reaction to this is either to turn directly to the right or left or to vary the proportion of right and left turns made from a nominally equal starting point. This is termed osmotropotaxis and is seen in many invertebrates (Wigglesworth and Gillett, 1934). Parker (1914) described 'circus' movements in unilaterally anosmic dogfish (Mustelis canis), but the experimental condition did not affect its ability to locate an odour source. Osmotropotaxis has been criticized (Von Buddenbrock, 1952) as a mechanism in weak gradients because of the closeness of paired organs. But it has not been totally rejected as an orientation method in a stagnant water body, when close to an odour source. In view of the closely paired and open olfactory organs in larval fish (as described for herring in the present study) it seems unlikely that it plays any part in larval fish orientation to an odour source. Results in Appendix VIII of turning behaviour also tend to refute this possibility.

A second possibility is perception of temporal differences in odour concentration by moving organisms (Phobotaxis). Thus an odour source could be localized by continuing in the increasing concentration direction and turning in response to a fall in

concentration gradient. This is a behaviour pattern common in invertebrates (Barrows, 1907). From the data presented for all stages of herring,Table 10 and plaice,Table 12 larvae it must be concluded that phototaxis does not take place.

Use of an olfactory cue as a releaser for positive rheotaxis is a more attractive hypothesis, this was shown by Copeland (1918) in 2 species of aquatic snail and confirmed by Henshel (1932). Kleerkoper (1967) demonstrated the phenomena in an elasmobranch (Diplodus sarqus). Results from the present study did not show any evidence in either herring,Table 11 or plaice,Table 13 for a positive rheotaxis as a result of moving into an area of increasing odour concentration. It is inherent in the design of the fluvarium that current flow be kept to a minimum so, it is possible that the current flow of c.10 cm/min in the experimental chamber was below that necessary to stimulate rheotactic behaviour in response to an olfactory cue (if such a response exists). From the literature, Bishai (1962) showed from the earliest stages herring larvae orientated themselves against a current of 35 cm/min, the lowest value studied and Arnold (1969) showed that plaice larvae would orientate to a current of greater than 60 cm/min from 24-48 h post-hatching; this was the lowest value he studied. Ryland (1963) also showed orientation to currents in plaice larvae against using higher current speeds (60-267 cm/min) than the present study. No values for theshold of current speed to induce rheotactic behaviour could be found for larval telosts in the literature but Wilson (1973) found orientation to a current in the absence of chemical stimulation at speeds of 44 cm/min by herring larvae and 65 cm/min by plaice larvae in a fluvarium device. Arnold (1969) shows the loss of rheotactic behaviour in dim light of an unspecified intensity (although below 2.5 lux). From the text it appears to have been very

TABLE 10

ANALYSIS OF HERRING LARVA MOVEMENT FOR TURNING BEHAVIOUR LIKELY TO BRING A LARVA INTO AN AREA OF MAXIMAL CONCENTRATION OF FEEDING-RELATED STIMULUS.

TEST STIMULI	N	CONTROL				TEST 1						TEST 2					
		MO		LO		MO			LO			MO			LO		
		F	B	F	B	F	B	S	F	B	S	F	B	S	F	B	S
YOLK SAC STAGE																	
Balanus nauplii washing	12	26	22	7	16	39	29		12	24		54	28		16	24	
Balanus nauplii extract	13	26	21	13	14	15	14	*	5	20		3	8		10	13	
Artemia nauplii washing	12	28	21	11	36	41	27		11	27		39	20		9	32	
Artemia nauplii extract	10	23	16	6	18	27	15		18	35		26	14		20	35	
Glutamic acid 10^{-3} M	13	27	24	20	37	19	11		25	37		24	12		24	31	
Aspartic acid 10^{-3} M	10	21	14	10	18	22	13		3	10		21	16		10	15	
Glycine 10^{-3} M	10	36	26	7	20	28	16		3	22		33	20		9	11	
Methionine 10^{-3} M	10	21	13	10	23	36	22		9	26		32	8		5	26	
Alanine 10^{-3} M	10	40	21	15	26	45	27		8	15		30	27		11	10	
Proline 10^{-3} M	10	40	20	8	26	41	20		11	40		29	21		26	23	*
Cysteine 10^{-3} M	10	28	23	10	18	36	26		45	27		8	15		30	27	
CONTROL EXPERIMENT 1	11	28	14	20	31	25	18		25	38		20	12		25	38	
BALANUS FEEDING STAGE																	
Balanus nauplii washing	10	17	14	13	10	30	18		6	14		26	15		10	16	
Balanus nauplii extract	13	35	12	12	33	38	19		16	43		42	20		10	24	
Dialysed Balanus nauplii extract	6	23	14	5	16	27	13		3	12		34	11		3	15	
Artemia nauplii washing	10	19	13	17	10	13	29	*	20	6		27	15		15	16	
Glycine 10^{-3} M	10	24	7	25	32	17	5		22	34		18	6		13	46	

TABLE 10 cont.

TEST STIMULI	N	CONTROL				TEST 1						TEST 2					
		MO		LO		MO			LO			MO			LO		
		F	B	F	B	F	B	S	F	B	S	F	B	S	F	B	S
ARTEMIA FEEDING STAGE																	
Artemia nauplii washing	18	51	30	39	78	67	26		36	76		56	30		40	84	
Artemia nauplii extract	23	72	41	58	111	87	48		88	126		86	61		83	120	
Glutamic acid 10^{-3} M	10	47	28	14	26	61	29		27	24		48	29		28	28	
Aspartic acid 10^{-3} M	10	40	15	8	25	31	33	*	13	25		16	14		20	37	
Glycine 10^{-3} M	10	25	15	26	34	37	28		10	33	*	45	23		19	31	
Methionine 10^{-3} M	10	48	22	18	22	41	29		15	25		38	32		24	23	
Alanine 10^{-3} M	10	45	23	16	43	41	25		35	47		27	24		27	45	
Proline 10^{-3} M	10	22	23	25	24	31	21		42	34		29	34		35	28	
Cysteine 10^{-3} M	10	49	31	15	19	46	37		22	27		45	29		18	29	
CONTROL EXPERIMENT 2	10	42	13	21	26	31	18		18	32		28	19		15	33	

Shown are the number of times a larva continued in the same direction (F) or turned back on itself (B) after a move towards the more odourous(MO) or less odourous(LO) end of the experimental chamber. Values are totals for the control and test periods of all trials in an experiment with a specific stimulus. Also shown is the number of trials (N) and in column S, asterisks which indicate rejection of the null hypothesis of no difference in the ratio of moves backward or forward between control and test periods at the 5% level, calculated by the Chi^2 test.

Such differences as are found to occur are not considered to be sufficient evidence that a larva could alter turning behaviour to cause it to reach an area of high or low stimulus concentration.

TABLE 11

ANALYSIS OF HERRING LARVA MOVEMENTS FOR RHEOTAXIS RELEASE DUE TO CHANGE IN FEEDING-RELATED STIMULUS CONCENTRATION.

TEST STIMULI	N	CONTROL				TEST 1				TEST 2				Chi^2_1	Chi^2_2
		MO		LO		MO		LO		MO		LO			
		U	D	U	D	U	D	U	D	U	D	U	D		
YOLK SAC STAGE															
Balanus nauplii washing	12	13	7	2	7	18	16	9	7	17	17	7	9	4.65	4.82
Balanus nauplii extract	13	14	11	13	9	13	7	6	8	6	7	8	7	1.71	0.58
Artemia nauplii washing	12	15	11	8	13	19	12	8	7	11	15	8	4	2.94	3.74
Artemia nauplii extract	10	15	7	4	4	7	9	16	17	6	12	9	10	2.88	4.95
Glutamic acid 10^{-3} M	13	14	9	15	12	9	14	11	12	8	5	10	9	2.51	0.59
Aspartic acid 10^{-3} M	10	11	9	9	7	5	9	2	2	9	12	12	8	1.59	1.36
Glycine 10^{-3} M	10	18	17	7	8	14	8	3	6	10	4	2	7	2.62	2.59
Methionine 10^{-3} M	10	11	7	7	7	17	19	7	9	14	6	4	4	1.25	1.76
Alanine 10^{-3} M	10	19	18	11	14	24	27	5	9	17	21	6	9	1.07	0.71
Proline 10^{-3} M	10	15	13	5	10	15	16	8	1	13	13	5	14	7.19	4.52
Cysteine 10^{-3} M	10	18	14	9	9	20	21	7	8	28	29	10	10	0.55	0.45
CONTROL EXPERIMENT 1	11	18	18	11	13	27	25	12	16	23	21	9	9	0.70	0.26
BALANUS FEEDING STAGE															
Balanus nauplii washing	10	11	13	3	7	14	14	4	6	10	11	6	6	1.29	1.09
Balanus nauplii extract	13	7	12	5	7	14	11	10	10	14	23	6	8	1.80	0.82
Dialysed Balanus nauplii extract	6	4	8	3	5	9	11	2	3	9	7	3	1	0.45	2.99
Artemia nauplii washing	10	8	12	6	6	15	18	5	7	10	15	7	8	0.36	0.49
Artemia nauplii extract	10	11	9	9	9	8	7	14	16	11	11	11	5	0.39	1.63
Glycine 10^{-3} M	10	6	8	18	16	4	8	12	20	3	6	13	13	2.21	1.30

TABLE 11 cont.

	N	CONTROL MO		CONTROL LO		TEST 1 MO		TEST 1 LO		TEST 2 MO		TEST 2 LO		Chi^2_1	Chi^2_2
		U	D	U	D	U	D	U	D	U	D	U	D		
ARTEMIA FEEDING STAGE															
Artemia nauplii washing	18	10	12	17	25	9	17	27	30	12	23	29	27	1.35	2.95
Artemia nauplii washing	23	19	16	36	45	32	44	57	63	42	43	50	60	1.61	1.25
Glutamic acid 10^{-3} M	10	15	15	6	8	23	21	10	14	24	22	10	8	0.90	1.52
Aspartic acid 10^{-3} M	10	10	9	4	6	13	13	12	13	5	5	23	20	0.44	0.61
Glycine 10^{-3} M	10	12	9	14	16	24	20	7	15	16	17	14	14	3.76	0.57
Methionine 10^{-3} M	10	12	17	6	4	18	19	9	15	21	18	12	13	1.81	1.52
Alanine 10^{-3} M	10	15	11	11	13	17	16	22	21	7	14	12	13	0.71	2.79
Proline 10^{-3} M	10	7	14	12	9	15	6	17	18	15	16	7	17	6.49	4.75
Cysteine 10^{-3} M	10	22	35	12	4	26	27	14	16	13	17	14	14	3.76	0.57
CONTROL EXPERIMENT 2	10	9	9	12	13	11	10	12	15	13	11	14	16	0.32	0.33

Shown are the number of turns made by larvae against and with the direction of water flow (termed U and D respectively) after a move towards the more odourous or less odourous end of the experimental chamber (termed MO and LO respectively). Values are totals for the control and test periods of all trials in an experiment with a specific stimulus. Also shown is the number of trials (N) and Chi^2_1 and Chi^2_2 the results of Chi^2 tests for a significant difference in the relative values between control and first test periods and control and second test periods respectively. In no case does the value of Chi^2 exceed the control value for rejection of the null hypothesis at the 5% level. This suggests no release of rheotactic behaviour occurs as a result of moving in the concentration gradient.

TABLE 12

ANALYSIS OF PLAICE LARVA MOVEMENTS FOR TURNING BEHAVIOUR LIKELY TO BRING A LARVA INTO AN AREA OF MAXIMAL CONCENTRATION OF FEEDING-RELATED STIMULUS.

TEST STIMULI	N	CONTROL MO		LO		TEST 1 MO			LO			TEST 2 MO			LO		
		F	B	F	B	F	B	S	F	B	S	F	B	S	F	B	S
Balanus nauplii extract	11	30	18	16	28	36	18		18	22		21	21		24	40	
Artemia nauplii washing	4	11	10	3	9	18	8		4	11		13	9		3	13	
Artemia nauplii extract	8	26	14	10	24	32	17		15	24		30	24		8	38	
Glutamic acid 10^{-3} M	10	56	34	7	50	56	34		10	59		62	29		20	44	*
Aspartic acid 10^{-3} M	10	22	10	10	34	23	4		20	35		33	6		11	31	
Glycine 10^{-3} M	10	30	16	11	33	38	10		16	34		20	16		16	32	
Methionine 10^{-3} M	10	41	16	10	30	37	22		11	34		42	13		11	46	
Alanine 10^{-3} M	10	33	10	15	33	30	10		17	39		15	11		16	23	
Proline 10^{-3} M	10	40	14	12	41	30	22		13	40		30	23		25	28	*
Cysteine 10^{-3} M	10	29	7	9	28	29	9		19	37		20	16	*	12	27	

Shown are the number of times a larva continued in the same direction (F) or turned back on itself (B) after a move towards the more odourous(MO) or less odourous(LO) end of the experimental chamber. Values are totals for the control and test periods of all trials in an experiment with the specific stimulus. Also shown is the number of trials (N) and in column S asterisks which indicate rejection of the null hypothesis of no difference in the ratio of moves backward or forward between control and test periods at the 5% level calculated by the Chi^2 test.

Such differences as are found to occur are not considered to be sufficient evidence that a larva could alter turning behaviour to cause it to reach or remain in an area of high or low stimulus concentration.

TABLE 13

ANALYSIS OF PLAICE LARVA MOVEMENTS FOR RHEOTAXIS RELEASE DUE TO CHANGE IN FEEDING-RELATED STIMULUS CONCENTRATION.

TEST STIMULI	N	CONTROL				TEST 1				TEST 2				Chi^2_1	Chi^2_2
		MO		LO		MO		LO		MO		LO			
		U	D	U	D	U	D	U	D	U	D	U	D		
Balanus nauplii extract	11	12	11	6	10	6	10	13	10	7	8	18	17	2.24	1.04
Artemia nauplii washing	8	7	7	3	5	7	6	1	2	5	5	3	2	1.35	0.67
Artemia nauplii extract	4	11	5	8	8	10	9	9	7	13	15	10	6	1.37	2.58
Glutamic acid 10^{-3} M	10	12	12	4	6	11	10	9	12	21	17	7	12	0.68	2.04
Aspartic acid 10^{-3} M	10	10	3	7	10	1	4	12	8	8	4	6	8	6.50	5.34
Glycine 10^{-3} M	10	10	7	6	6	14	12	8	5	10	11	11	15	0.44	1.14
Methionine 10^{-3} M	10	7	6	7	3	13	11	7	2	7	9	9	6	2.15	1.88
Alanine 10^{-3} M	10	9	8	9	3	6	7	12	13	1	4	9	10	2.82	4.77
Proline 10^{-3} M	10	6	4	6	8	13	7	6	11	15	18	18	22	3.94	0.86
Cysteine 10^{-3} M	10	1	5	9	4	5	10	13	11	8	13	8	7	6.36	5.70

Shown are the numbers of turns made by larvae against and with the direction of water flow (termed U and D respectively), after a move towards the more odourous and less odourous end of the experimental chamber (termed MO and LO respectively) in the control and test periods of all trials in an experiment. Also shown is the number of trials (N) and Chi^2_1 and Chi^2_2 being the results of Chi^2 tests for a significant difference in the relative values between the control and first test period and control and second test period respectively. In no case does the value of Chi^2 exceed the critical value for rejection of the null hypothesis at the 5% level. This suggests that rheotactic behaviour is not released as a result of movement in the concentration gradient.

dim requiring the observer to dark-adapt his eyesight for 30 min. As the light levels in this study were higher (5.0 lux) and required no dark-adaption of the observer, it was considered that this phenomena played little part in the absence of any consistent rheotaxis in control situations. In conclusion the absence of a release of rheotaxis by an odour gradient could be ascribed to either lack of response or low level of current flow. Further experiments at slightly below a threshold level of current flow for rheotaxis in the absence of chemical stimuli might elucidate this point. In view of the absence of any specific response to the gradient in this study it is possible that the gradient produced is not sharp enough to elicit changes. No information from other studies into natural stimuli is available in the literature. Other users of the fluvarium technique have used it to investigate irritants, pollutants or anoxic conditions (Hoglünd, 1951; Markström, 1959; Wilson, 1973). The overall levels in the chamber were comparable with those detectable by activity response using the thermistor technique. Without conditioning experiments which are thought to be impracticable on these larvae the minimum incremental increase detectable cannot be established.

D. Fluvarium experiments on post-metamorphic plaice.

From the results of fluvarium experiments on post-metamorphic plaice no significance can be attached to differences between control and test period either in respect of total numbers of moves, Table·14 or changes in the proportions of 'long' and 'short' moves, Table 15. Further investigations by Gibson (In press) have shown that the arbitrarily divided 'long' and 'short' moves thought to be related to feeding behaviour are in fact merely part of a continuous logarithmic distribution of length of move and although related to

TABLE 14

ACTIVITY OF POST-METAMORPHIC PLAICE IN A FLUVARIUM GRADIENT OF

FEEDING RELATED STIMULUS.

TEST STIMULUS		MEAN TOTAL MOVES				CRITICAL VALUE OF W
	N	CONTROL	TEST	W	P	
Artemia nauplii extract	14	2	3	57	$> .05$	21
Artemia nauplii extract*	19	3	4	85	$> .05$	46
Dialysed Artemia nauplii extract	24	3	2	139	$> .05$	81
Glutamic acid 10^{-3}M	16	2	2	51	$> .05$	30
Aspartic acid 10^{-3}M	29	2	2	169	$> .05$	124
Methionine 10^{-3}M	19	4	2	52	$> .05$	46
Proline 10^{-3}M	24	3	3	105	$> .05$	81
Alanine 10^{-3}M	15	2	3	52	$> .05$	25
Cysteine 10^{-3}M	17	3	2	45	$> .05$	35
Amino acid mixture 10^{-3}M	18	7	6	45	$> .05$	40

*Test fish not fed for 5 days prior to trial

Shown are the number of trials (N), the mean values to the nearest
integer of numbers of moves made by fish in control and test periods,
the results (W) of Wilcoxons matched-pair signed-ranks tests, the
associated probability (P) of no significant difference between control
and test and the critical value of W for rejection of the null
hypothesis at the 5% level.

TABLE 15

CHANGES IN LENGTH OF MOVES BY POST-METAMORPHIC PLAICE IN A
FLUVARIUM GRADIENT OF FEEDING-RELATED STIMULUS.

TEST STIMULUS	N	W	P	CRITICAL VALUE OF W
Artemia nauplii extract	7	14	>.05	2
Artemia nauplii extract*	16	63	>.05	30
Dialysed Artemia nauplii extract	14	54	>.05	21
Glutamic acid 10^{-3}M	9	19	>.05	6
Aspartic acid 10^{-3}M	20	49	<.05	52
Methionine 10^{-3}M	16	46	>.05	30
Proline 10^{-3}M	12	55	>.05	35
Alanine 10^{-3}M	12	34	>.05	14
Cysteine 10^{-3}M	12	23	>.05	14
Amino acid mixture 10^{-3}M	17	40	>.05	35

* Test fish not fed for 5 days prior to trial.

Shown are the number of trials (N), the results (W) of Wilcoxons
matched-pairs signed-ranks tests, the associated probability (P) of
no significant difference in the proportions of 'short'(fish remains
in grid sector) and 'long' (fish moves from grid sector) movements
made by fish between control and test and the critical value of W for
rejection of the null hypothesis at the 5% level.

                    this is
feeding behaviour, not in any simple way. The lack of any

significant results is therefore not unexpected.

   None of the techniques used in this study, which were

developed for active organisms are applicable to late and post-

metamorphic larvae. In order to investigate olfaction in these

plaice it would be more apt to look for specific behavioural

reactions of sequences associated with feeding, a difficult task

in such small organisms and outside the scope of this study.

5. REACTIONS OF HERRING TO A SOCIAL IDENTIFIER

A. Thermistor experiments

(i) Presence of a response in larvae

Results of an experiment using the thermistor activity technique to establish the presence of the identifier in larvae at an early and late stage of development. Test stimulus of 10 ml standard preparation per 100 ml

10 trials using 10 mm yolk sac stage larvae P = 0.040

6 trials using 16-18 mm stage 3 larvae   P = 0.006

Shown above is the probability (P) of no significant difference between control and test calculated by the randomization test.

(ii) Presence of a response in juveniles

Results of an experiment using the preference trough technique to establish the presence of the identifier in post-metamorphic juvenile herring.

CONTROL EXPERIMENT – NO STIMULUS ADDED

7 trials using groups of 3, 7 cm fish Z=0.03 P > 0.10

TEST EXPERIMENT – STIMULUS OF HOLDING TANK WATER

6 trials using groups of 3, 7 cm fish Z=1.99 P < 0.05

Shown above is the result (Z) of a Zm test and the associated probability that the fish spent an equal amount of time in the test and control halves of the trough. From these results it can be seen that a chemically induced intraspecific response exists throughout larval life and is also present after metamorphosis.

(iii)   Threshold of response in larvae

TABLE 16

RESULTS OF A GROUP OF EXPERIMENTS USING THE THERMISTOR ACTIVITY

TECHNIQUE TO FIND THE THRESHOLD OF REACTION TO THE SOCIAL IDENTIFIER

IN 16-18 mm STAGE 3 LARVAE.

Test stimulus of 10 ml per 100 ml

METHOD 1 By dilution of standard			METHOD 2 1 larva in 50 ml for increasing lengths of time			METHOD 3 3 larvae in 75 ml for increasing lengths of time		
Dilution	sign test	P	Time (min)	sign test	P	Time (min)	sign test	P
x 0	+	0.01	30	–	0.23	10	–	0.21
x 10	+	0.04	60	–	0.30	15	+	0.03
x 15	+	0.05	90	–	0.28	30	+	0.04
x 20	–	0.15	120	+	0.15			
≡ 2 larvae/l/h			≡ 4 larvae/l/h			≡ 1 larva/l/h		

Shown above are the results of a sign test at the 5% rejection level and

the probability (P) of no significant difference between control and

test calculated by the randomization test.

From the results presented in this study, of thresholds of

response in Table 16 it is possible to infer the relative production

of the 'social identifier'. This will be the inverse of the number

of larvae/l/h. Examining the differences between the standard and

alternative methods of preparation we find the following. First

comparing methods one and two. In method 2 a single larva has the

same volume of water per larva as the standard preparation and the

washing, produced totally in light over a short time period, and used

immediately after collection, is less effective than the standard preparation. This suggests several possibilities. There may be differential production of the identifier in light and dark, the substance(s) may age in some way and so increase in effectiveness, or the larvae may produce more of the substance(s) directly or indirectly as a result of interaction with other larvae. From the threshold for the identifier produced by the third method it was hoped to show which factor or which combination of these factors was responsible for the difference. Methods two and three differed only in that method 2 used one larvae in 50 ml and method 3 used 3 larvae in 75 ml. From the results we find that for method 3 the threshold was lower; a half that of the standard and a quarter that of method 2 was found. This tends to counter the possibility of ageing effects and differential production in light and dark and support a hypothesis that a group of larvae produce more than they would as individuals. As the stocking density in method 3 is twice that in the standard preparation this also suggests that production is inversely related to volume of water per larva (directly related to degree of crowding).

Crowding effects can be considered in two possible ways. First, assuming the substance is of a uniform nature (no individual variation in composition), the increased production in groups and by crowding is simply a result either of fish actively producing more substance as a reaction to population size or, if the substance is contained in some natural metabolite or secretion then increased production could be caused by increased activity within a group. This could easily be tested by determining if groups of larvae are more active than the sum of their activities as individuals, and secondly by seeing whether production of the identifier is increased when a single larva is subjected to

TABLE 17

PARAMETERS DESCRIBING MOVEMENTS OF HERRING LARVAE IN A FLUVARIUM
GRADIENT OF SOCIAL IDENTIFIER.

STAGE OF LARVA	N	TIME ACTIVE (% OF TOTAL IN EXPERIMENT)			MEAN STEP LENGTH (mm)			MEAN TOTAL DISTANCE PER TRIAL (mm)		
		C	T1	T2	C	T1	T2	C	T1	T2
Yolk sac larvae (length 10 mm)	8	35	28	25	23	24	27	177	157	199
Stage 3 larvae (length 16-18 mm)	10	43	55	53	43	48	44	487	862	645

Shown are the number of trials (N); the percentage of time pent in moving, the mean distance travelled per move (step length) and the mean total distance travelled; in the control period (C) and the first and second periods (T1 and T2). Application of a Wilcoxons matched-pair signed-ranks test showed no significant difference in the parameters of time active or mean total distance per trial between control and test periods in yolk sac larvae. For stage 3 larvae significant increases compared with control experiment 2 in time active and mean total distance per trial were found by a Mann-Witney U test. Except for the increase in time active between control and second test period, the values in test periods were also significantly higher than the trial control values at the 5% level calculated by the Wilcoxons matched-pair signed-ranks test.

TABLE 18

ANGULAR STATISTICS QUANTIFYING THE DISTRIBUTION OF ANGLES OF

TURN OF HERRING LARVAE IN A FLUVARIUM GRADIENT OF SOCIAL

IDENTIFIER.

	CONTROL		TEST 1		TEST 2	
	$\bar{X}o$	So	$\bar{X}o$	So	$\bar{X}o$	So
Control experiment 1	-5.2	.99858	4.8	.99701	9.6	.99605
Yolk sac larvae	38.4	.99570	-17.2	.99597	11.4	.99365
Control experiment 2	18.9	.99724	-2.8	.99630	-4.8	.99521
Stage 3 larvae	25.4	.99882	34.4	.99950	-16.5	.99944

Shown are the mean direction ($\bar{X}o$) and the degree of dispersion (So)

of angles of turn about this value in the control and test periods,

as with experiments using feeding related stimuli(Appendix VIII),

no differences in these statistics not the histograms of angles of

turn were found to be due to presence of stimulus.

Fig. 22. Distribution of angles of turn of herring larvae

in a fluvarium gradient of social identifier in

control and test situations.

Test stimuli and stage of larvae

1. Control experiment, Stage 1c larvae

2. Social identifier, Yolk sac larvae

3. Control experiment, Stage 3 larvae

4. Social identifier, Stage 3 larvae

Angle of Turn
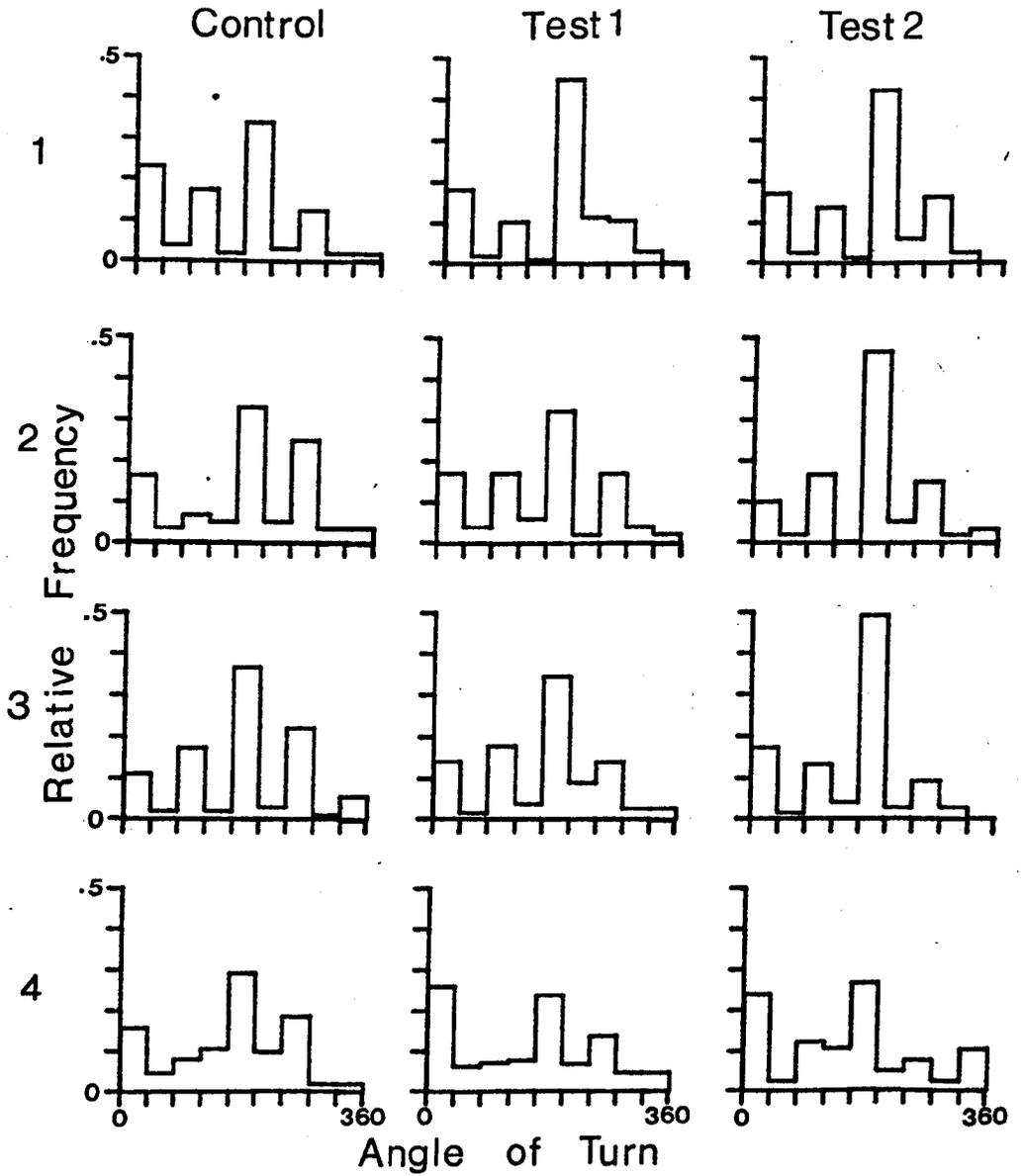
mechanical stimulation to increased activity. An alternative
explanation of the increased effectiveness of the washings of
groups of larvae is suggested by Göz (1941) who found that
minnows could distinguish between individuals of the same species
by olfaction. It is possible that the odours of individual herring
larvae are different and act synergistically, this being
independent of the crowding effect. Synergistic effects of amino
acids reported by Hashimoto et al (1968) have been discussed already.
From this study we can therefore conclude that the substance(s) are
produced continually by the larvae and are both effective immediately
and are relatively long-lived, (up to 24 h at 14 deg C and probably
longer), and that production is enhanced both in groups of larvae
and by crowding.

The only clear conclusion that can be drawn from studies of
the movements of larvae in gradients of the social identifier is
that significant increases both in percentage time spent active
and distance covered occur in stage 3 larvae (Table 17). No
distinct behavioural reactions (cf. searching behaviour) were noted.
These increases are increases compared with the trial control as
well as in comparison with control experiment 2 showing the
response to be different in nature to that shown in response to
feeding related stimuli, Table 4. It will be appreciated that
an increase in swimming activity and distance covered would be a
more useful response in a larva searching for an aggregation of
larvae than searching a smaller area more thoroughly as for small
prey items, the response seen to feeding related stimuli. No
conclusion could be drawn from the differences between the
values in control and test situations of the mean direction and
degree of dispersion of angles of turn (Table 18), nor from
differences in histograms of angles of turn (Fig 22). No

TABLE 19

ANALYSIS OF HERRING LARVA MOVEMENTS FOR TURNING BEHAVIOUR LIKELY
TO BRING A LARVA INTO AN AREA OF MAXIMAL CONCENTRATION OF SOCIAL
IDENTIFIER.

	N	CONTROL				TEST 1				TEST 2				Chi^2_1	Chi^2_2
		MO		LO		MO		LO		MO		LO			
		U	D	U	D	U	D	U	D	U	D	U	D		
Yolk sac larvae (length 10 mm)	8	11	15	6	4	11	15	5	5	11	8	2	3	1.14	1.69
Stage 3 larvae (length 16–18 mm)	10	14	13	3	7	10	8	12	8	16	12	9	8	2.54	2.21

Shown are the number of turns made by larvae against (U) and with (D)
the water flow after a move towards the more odourous (MO) or less
odourous (LO) end of the experimental chamber. Values are totals for the
control and test periods of all trials. Also shown is the number of
trails (N) and Chi^2_1 and Chi^2_2 tests for a significant difference in
the relative values between control and first test periods and control
and second test periods respectively. In no case is the critical value
of Chi^2 exceeded for rejection of the null hypothesis at the 5% level.

TABLE 20

ANALYSIS OF HERRING LARVA MOVEMENTS FOR RHEOTAXIS RELEASE DUE TO CHANGE IN SOCIAL IDENTIFIER CONCENTRATION.

		CONTROL				TEST 1						TEST 2					
		MO		LO		MO			LO			MO			LO		
	N	F	B	F	B	F	B	S	F	B	S	F	B	S	F	B	S
Yolk sac larvae (length 10 mm)	8	31	18	9	11	26	13		8	13		26	12		6	12	
Stage 3 larvae (length 16-18 mm)	10	40	22	11	34	51	27		27	68		63	26		18	46	

Shown are the number of times a larva continued in the same direction (F) or turned back on itself (B) after a move towards the more odourous (MO) or less odourous (LO) end of the experimental chamber. Values are totals for the control and test periods of all trials. Also shown is the number of trials (N) and in column S an asterisk to indicate rejection of the null hypothesis of no difference in the ratio of moves backward or forward between control and test periods at the 5% level calculated by the Chi^2 test. In no case is the critical value of Chi^2 exceeded for rejection of the null hypothesis at the 5% level. From the results of Tables 20 and 21 it is apparent that herring larvae cannot localize an area of high concentration of social identifier by phobotaxis or a release of positive rheotaxis.

evidence of any preference in direction of turn after a move up
or down the odour gradient could be found (Table 19) nor any
evidence for release of rheotactic behaviour (Table 20).
Therefore as with feeding-related stimuli it is not thought that
herring larvae are able to orientate to the source of a release
of social identifier.

DISCUSSION

1. THE SENSORY BASIS OF THE RESPONSE

The results of experiments involving larvae with severed olfactory nerves confirmed that the olfactory system was mediating the responses found. The same conclusion has been reached by many workers since Parker (1911) first demonstrated the loss of a reaction to meat juices by experimentally anosmic Fundulus heteroclitus. More recently direct evidence from electrophysiological investigation of the olfactory system innervation has shown a reaction to tissue extracts (Adrian and Ludwig, 1938), amino acids (Doving, 1966) and intraspecific odours (Doving, Enger and Nordeng, 1973).

The role of other chemosenses in distance perception of stimuli has not been well studied, although many workers have assumed rather than shown olfaction to be the sense studied in their research. Bardach, Todd and Crickner (1967) were able to show that experimentally blinded and anosmic examples of 2 species of bullhead of the genus Ictalurus were able to react to and localize the release point of liver extracts and 0.01 M cysteine hydrochloride at a distance of at least 25 body lengths (5.5 m). The bullhead forms a special example being well endowed with taste buds over the head and general body, especially on barbels which seemed to play some part in orientation to the source.

Chemoreception by free neuromasts associated with the lateral line of fish and amphibians has also been shown by Katsuki and Onada (1970) who investigated various elasmobranch and teleost species electrophysiologically. These receptors were found to be sensitive to a range of inorganic and organic anions including glutamate. Katsuki (1971) suggested these receptors formed the evolutionary predecessors of taste buds in higher vertebrates. Onada and Katsuki (1972) showed electrophysiological and

behavioural responses of the toad <u>Xenopus</u> <u>laevis</u> to liver extracts.
The free neuromasts described by Iwai (1963) in newly hatched
<u>Blennius</u> <u>yatabei</u> and other species (Iwai, 1965; 1972) are also
present in newly hatched herring larvae (Dempsey and Hickey, Unpubl.).

Taste and the general chemical sense of free neuromasts
undoubtedly can be used as distance (as distinct from contact)
chemoreceptors, but it seems unlikely that they play a significant
role in distance chemoreception in the majority of teleost species,
particularly in the case of the larval herring and plaice
investigated in this study. Certainly in the case of <u>Artemia</u> extract
and probably the other stimuli examined they do not initiate the
reaction noted in intact fish.

2.   REACTIONS TO FEEDING-RELATED STIMULI

It was seen in the results of the thermistor experiments that an
innate reaction to <u>Balanus</u> nauplii exists in herring larvae and the
selective advantages provided by such a reaction were discussed.
This reaction to <u>Balanus</u> nauplii and other feeding-related stimuli
merely shows a detection of the stimulus and a response to it by an
increase in activity it does not presume the use of chemoreception
in localizing prey. From the uniform non-directional stimulus in a
static tank all that can be deduced is that the reaction of increased
activity which takes place would tend to be beneficial to the larva
in allowing it to search a greater area when, by chemoreception,
food was known to be present. Localization of food patches by
olfaction could not be studied by this method, nor in such a
uniform system and without direct observation, could locomotor
movements be analysed to see if they would tend to cause a larva to
remain in such a patch of food odour. Such localization of patches
of live prey organisms by groups of anchovy larvae (<u>Engraulis</u> <u>mordax</u>)

has been reported by Hunter and Thomas (1974).

The fluvarium experiments allow investigation of the possible methods by which the larvae might react to olfactory stimuli. From the gross analysis of observations of herring and plaice locomotor patterns in the fluvarium it was seen that no aggregation in the more concentrated area of stimulus in the experimental chamber occurred, nor was any specific feeding activity such as the formation of the clupeid larval 'S' prior to striking a prey item (Blaxter, 1965). The presence of the slower, 'searching' form of swimming in herring larvae in many periods during which test stimuli were present was noted. This would correspond with the 'slow meandering' swimming behaviour reported from herring larvae by Rosenthal and Hempel (1970). In this mode of swimming the area searched was calculated to be 3-8 l/h compared with an average value of 1.5 l/h for larvae swimming in the 'normal' fashion (Blaxter, 1966). Rosenthal and Hempel (1970) give data for the percentage time spent in this searching movement by larvae in rearing tanks with live food present. This was often the predominant swimming mode. The percentage increased with age, while the frequency and duration of these phases was found to be positively related to the food supply. In the present study observations were made on single organisms, only olfactory cues being present in the absence of live prey. No quantification of the proportion of the 'searching' behaviour of total activity was made but it was thought to be much less extensive than 'normal' swimming particularly in control periods when it was rarely seen. Changes in behaviour of this sort could provide an explanation of the activity increases found using the thermistor technique, if the new behaviour pattern produced more 'activity spikes' than normal swimming as was discussed earlier.

Tail beat frequency changes in 'normal' swimming by herring larvae were also found to be possibly related to presence of stimuli, increasing during test periods. As no quantification was attempted this must remain conjecture but if true would seem to be in contrast to the adaptive value for a larva to remain in an area of plankton. The absence of a secondary visual cue in an area of olfactory stimulus might cause a larva to continue swimming at a greater rate in order to see a prey before beginning a search. Teichmann and Teichmann (1959) reported that in 3 species of elasmobranchs a period of non-specific activity preceded a search in response to an olfactory cue. Both parameters would warrant a further study using ciné camera recording techniques to attempt to discover if a true correlation between occurrence and olfactory stimulation does exist.

The stimulus to increased activity by prey extracts and washings in adult teleosts and other groups of organisms has long been established. In many cases a definite feeding reaction could be produced, this has been shown for teleosts by Steven (1959). Pawson (1977) showed an increase in swimming activity in whiting (Merlangius merlangus) and cod (Gadus morhua) due to detection of Arenicola extracts. These fish also showed searching behaviour and occasionally a feeding response by biting the inlet pipe. Carr, Gondeck and Delanoy (1976) showed a biting reaction to the source of a flow of separate extracts of seven marine species (covering four phyla) in the pinfish Lagodon rhomboides. This response was consistent enough to form a bioassay technique. From these results and those of other workers it can be seen that the responses produced by an extract of prey can vary in intensity between species from merely awareness to a definite feeding act. This may be related to the importance of chemoreception in the

feeding act in nature. Fish with poor vision from turbid waters or who rely for other reasons on chemoreception in feeding are more likely to show a definite feeding response than more visual feeders. The level of nervous organization may also be important, Fuzessery and Childress (1975) reported that several species of crustacea will grab and carry out a feeding action on a suspected 'prey' when stimulated by amino acids, in the absence of visual cues. It seems likely that higher organisms would need some secondary reinforcement.

It was also seen in the present study that an early herring larva reacts to a natural food but not to Artemia nauplii which it would not meet in nature. The specificity of the response is perhaps unusual as many workers have found a response to a wide range of tissue extracts, including some which could not form part of the natural diet, although in some cases may have been part of an experimental diet (Steven, 1959). Carr, Gondeck and Delanoy (1976) were able to show that differences in degree of response occurs to different tissue extracts in pinfish but these fish did not show a significant difference in response to extracts of two species of Penaeid shrimp. Although the biochemical composition of tissue is generally speaking very similar, Raymont, Morris, Ferguson and Raymont (1975), many small differences do exist. These can be detected by analysis and more importantly by chemoreceptors. Larman and Gabbott (1975) were able to show that settling Balanus balanoides cyprinids were able to distinguish between old sites of adults of their own species and those of Elminius modestus and even Balanus balanus in the same genus by means of touch chemoreceptors. Differences in biochemical composition between B. balanoides nauplii and A. salina nauplii do exist and were discussed above. The herring larvae, once feeding on Artemia nauplii, quickly began

to show a reaction to _Artemia_ extracts, this would seem to be a
result of self-conditioning the larvae relating to body fluids
released in the feeding act. The fact that herring are reacting
to a specific prey extract odour rather than a general tissue
extract odour was also shown in the finding that after a period
of 50 days feeding solely on _Artemia_ nauplii the reaction to
Balanus nauplii could no longer be shown. McBride, Idler, Jonas
and Tomlinson (1962) also found that fish (Pacific salmon smolts)
only showed responses to either zooplankton or _Artemia_ extracts
if they formed part of the fishes diet at the time of testing.
With regard to washings a similar situation was seen. An innate
reaction was shown to _Balanus_ nauplii washing in the earliest
stages. It was not possible to show a reaction to _Artemia_
washing at any stage, nor (due to lack of live nauplii) was it
possible to determine if the reaction to _Balanus_ nauplii washing
was lost with time. It is not known what specific substance or
substances cause the reaction to washings whether it is the same
substances present in the extract and leached to a small extent
from the intact organism or possibly some waste metabolites
excreted continually by the live organism, and of a different
nature to those causing the reaction to an extract. The fact that
even larvae which had conditioned themselves to react to _Artemia_
extract showed no reaction to _Artemia_ washing would tend to
support the second possibility. Obviously if there is to be a
difference between _Balanus_ and _Artemia_ nauplii washings in the
substance(s) responsible must not be of a very general nature
such as ammonia. Steven (1959) however was able to show feeding
behaviour in _Hepsitia_ in response to ammonia and lactic acid. In
view of their general occurrence it seems unlikely that they could
be alone responsible for the reaction to _Balanus_ nauplii in this

study. Little literature exists on the effectiveness of prey
washings as distinct from prey extracts in producing reactions
in fish. Steven (1959) was able to show feeding behaviour
induced by 'sea water which had contained zooplankton for a few
minutes' no clear quantification was given save the time an
unknown quantity of mainly copepods was in the water was 20-60 mins.
In contrast Winslade (1974) was unable to cause emergence of
sand eels to a feeding reaction by use of Artemia washing. From
his data the washings used were 16 mg nauplii/l/hr compared with
37 mg nauplii/l/hr in this study. Balanus washings were
effective stimuli at 37 mg nauplii/l/hr although the threshold
was not determined. Kleerkoper and Morgenson (1963) found the
lamprey Petromyzon to react to the odour of intact trout, the
reaction being specifically to an unknown amine (Amine F). McCleese
(1973) reported that as distinct from extracts, intact prey
organisms were poor attractants for the lobster Homarus americanus.
The exact nature of the stimulatory elements in extracts has been
the source of numerous investigations. In many cases both in
crustacea (Hodgson, 1958; Case and Gwilliam, 1961; Case, 1964; Laverack, 1964;
Levandowsky and Hodgson, 1965 and Ache, 1972) and fishes (Steven,
1959; Sutterlin, 1975; Carr, Gondeck and Delanoy, 1976) amino
acids have been shown to be effective in eliciting feeding
behaviour. Proteins of high molecular weight have been shown
as important simulants to feeding in marine invertebrates (Carr
and Gurin, 1975) but all behavioural studies on fish to date
have indicated that small molecules are the major feeding
stimulants. In this study an attempt to find the substance(s)
responsible was made by using dialysis of extracts. For both
Balanus and Artemia nauplii extracts the fraction passing through
the membrane was at least as effective as the whole extract.

This showed that small molecules of less than 12,000-14.000 mol wt were responsible for the response. Steven (1959) found that a dialysate of zooplankton extract was as effective as whole extract in eliciting feeding behaviour in Hepsitia. Carr, Gondeck and Delanoy (1975) in a study on pinfish found a fraction obtained by ultrafiltration containing substances with a molecular weight of less than ca 10,000 accounted for the response inducing characteristics of 5 tissue extracts.

The possibility of amino acids being the feeding stimulant was further supported by the examination in the present study of the effect of pure amino acids on activity. In the past workers have criticized conclusions drawn from amino acid stimulation of feeding response on the grounds of the relatively high concentrations necessary to trigger a response. In this study a standard concentration of 10^{-3} M was used giving a value of 10^{-4} M in the test chamber. In addition the experiment to find the threshold of response for glutamic acid fixed this at between $1-5 \times 10^{-6}$ M ($1-5\,\mu$M). We must now ask the question do the levels of amino acids at which a response is produced correspond with those found in extracts? The threshold of reaction to dialysed Artemia extracts (which might be expected more closely to correspond with pure substances) was found to be 0.02 g/l. An Artemia extract at threshold value would therefore have levels of the amino acids investigated as follows. Alanine 6.5×10^{-7} M, glutamic acid 1.2×10^{-7} M, proline, 3.5×10^{-7} M, aspartic acid, 1.7×10^{-7} M and glycine 3.2×10^{-7} M, these levels are lower than those used to produce a response; in particular glutamic acid, for which a threshold was determined, occurs at a lower level in extracts than its threshold. However three considerations are of importance. First except for glutamic acid no thresholds of response were

established. Secondly it has been found that synergistic effects
occur, that is mixtures of amino acids have a greater effect than
the sum of the effects of the component parts (Hashimoto et al.,
1968; McCleese, 1970; Carr and Gurin, 1975). Thirdly and
following from this, absence of a response does not imply absence
of detection. Electrophysiological studies have shown detection
of stimuli at levels of 10^{-7} M-10^{-8} M (Suzuki and Tucker, 1971;
Hara, Law and Hobden, 1973). From these considerations we can see
that a reaction to amino acids at lower levels may require the
presence of more than one amino acid, although it is possible
for the larva to detect the individual acids. Satou and Ueda (1975)
were able to show differences in nervous electrical discharge
patterns between different amino acids in the rainbow trout Salmo
gairdneri this would support the hypothesis that a range of amino
acids could be recognized as a group of individuals.

3. METHOD OF RESPONSE TO FEEDING-RELATED STIMULI

The results of analysis of movements of larvae of herring and
plaice in the fluvarium demonstrated that no simple, consistent
response to these stimuli was being shown. From the evidence
presented in this study therefore it has been shown that herring
and plaice larvae either do not detect changes in concentration
of the order produced or do not respond to such changes by simple
reflex-like changes in direction nor by changes in the relative
frequency of angle of turn to keep it in the same area of high
odour concentration (unless by a more subtle degree than that
detectable by the analysis method used. The only result of
detection of such a cue seems to be arousal into a state where the
larva is more ready or able to receive secondary, probably visual,
cues. Previous studies, as has been mentioned, present a range

of species varying in the degree to which feeding behaviour can be elicited by a purely olfactory stimulus. Using a standard bioassay technique Carr, Gondeck and Delanoy (1976) could induce biting behaviour (chemically) in pinfish (Lagodon rhomboides) and pigfish (Orthopristis chrysopterus) but not in the black sea bass (Centropristes striatus) or the pompano (Trachinotus carolinus). Grimm (1960) triggered a complete and stereotyped feeding behaviour in the goldfish (Carassius auratus) by electrical stimulation of the olfactory crura. This species will show feeding behaviour in response to an olfactory cue. In lower organisms such as crustacea (Carr and Gurin, 1975) a crab will seize and attempt to eat an imaginary prey as a result of chemical stimulation.

Two trends can be seen from those organisms which show complete feeding behaviour in response to olfactory cues. They usually have a simpler level of nervous organization, either inherently such as crustacea, or perhaps as a result of reduced visual powers. Secondly the mode of feeding and/or the degree of success encountered when undertaking a feeding act in response to a purely olfactory cue may, either genetically or by conditioning bring about this behaviour. One might expect bottom feeders or scavengers on non-motile prey to more readily show this association between cue and feeding. Adult herring (Blaxter and Holliday, 1958), plaice (Bateson, 1890; De Groot, 1972) and their respective larvae (Blaxter, 1965; 1968a) are predominantly visual feeders so it would be expected that these would be amongst those species requiring more than an olfactory cue to elicit a feeding response. Tiechmann and Tiechmann (1959) showed that in some cases at least; continuation of an olfactory stimulus may be enough to trigger a second stage in a feeding act. This was not found in this study. The ability of anchovy larvae to locate and remain in food patches in darkness (Hunter and

Thomas, 1974) does not require any degree of orientation by olfactory or tactile stimulation by prey but merely a continuation of the behaviour observed in light. Self-conditioning would ensure that if 'blind' feeding behaviour (perhaps in response to olfactory and tactile cues) was rewarded in the food patch by capture of prey sufficiently often, this behavioural pattern would become imprinted. Such results can be explained by increased swimming speed outside areas of food and decreased swimming speed in such a patch whether the patch be detected by vision, olfaction or other sense.

4. REACTIONS OF HERRING TO A SOCIAL IDENTIFIER.

It has been shown in this study that at two stages of larval life herring will show a response of increased activity to a substance or substances released by other larvae and detected by chemoreception. Additionally juvenile herring show response to water from a stock tank of herring. This was presumed to contain a similar substance(s) released by other herring. It is concluded from this that the response is present throughout the life of the fish. Since the fish examined were larval or juvenile it is assumed that this response is a social rather than a sexual phenomena. It seems likely that chemical recognition of conspecifics is an innate response. It is, however, possible that the response is a conditioned one; this would require the larvae to recognize their conspecifics in some other innate way and to associate them with the odour which would be continually present in the rearing tank. As the reaction would have to be conditioned within a few days of hatching the possibility is thought most unlikely. The presence of innate chemical responses has been mentioned previously in connection with feeding-related

stimuli. As well as the evidence from this study, Kleerkoper and Morgenson (1963) found the reaction of <u>Petromyzon</u> to 'trout water' to be innate and shown in animals reared in the laboratory away from contact with trout. Previously Schutz (1956) had found the shock reaction (Schreckreaktion) in a variety of cyprinids to be innate but only to appear at a certain age after onset of schooling behaviour. Pfeiffer (1966) by crossing 2 species of characinids possessing and not possessing the shock reaction, was able to show by back-crosses the genetic basis of the response. The olfactory recognition of conspecifics has been studied in fishes by several workers. Wrede (1932) showed that in a choice situation an individual minnow (<u>Phoxinus</u>), a shoaling species, showed a significant preference for a chamber containing the odour of another minnow. This work was followed by Göz (1941) using a conditioning technique. He showed this response was mediated by olfaction and that the minnow could distinguish between fishes of a number of genera as well as individual fishes of the same species, including both males and females. Olfactory recognition of conspecifics has subsequently been shown in a range of species (Keenleyside, 1955; Kuhme, 1964; Hemmings,1966 and Todd, Atema and Bardach, 1967).

In the present study no further attempts were made to discover the precise nature of the substance(s) involved but this has been the subject of much conjecture and experiment by previous workers on other species. Mucus (slime) has been shown to contain substance(s) responsible for the intraspecific behavioural effects in minnows (Wrede, 1932) and bullhead (Todd, 1971). More recently Doving, Nordeng and Oakley (1974) have shown mucus to be an effective stimulant of the olfactory system of char (<u>Salvelinus</u> <u>alpinus</u>) in an electrophysiological study. Racial and sexual

differences in response were also reported. Circumstantial evidence from the present study suggests mucus might be the 'social identifier' in herring as its characteristics fit in with the findings of this study as will be discussed. Although no direct evidence could be found it is probable mucus cells are present from time of hatching in herring larvae. Roberts, Bell and Young (1973) found mucus cells present from time of hatching in plaice and these increased in numbers with time. Mucus is produced continually and in large amounts. It is known that production is increased in response to stress such as crowding. Amongst fish in a group where the possibility of collision is increased production may be increased owing to the protective role of mucus (Jakowska, 1963). Additionally fish mucus has been shown to be effective in reducing drag (Rosen and Cornford, 1971) and so one can see a possible way in which the response may have evolved. Individuals in a shoal who could react positively by olfaction to mucus would require less energy for swimming and so gain a selective advantage.

In view of the difference in response between yolk sac and stage 3 larvae shown in the results of '% time active' and 'distance moved', it is interesting to note that Roberts, Bell and Young (1973) found histochemical differences between the mucus of newly-hatched and 30-day-old plaice larvae. In plaice at least, numbers of mucus cells also increase markedly with age, and so if mucus is indeed the source of the 'social identifier' then it is to be expected that production and also perhaps strength of reaction would be greater in older, larger larvae. It would be interesting to try and obtain a correlation between mucus cell abundance and threshold of reaction to a standard preparation of social identifier.

A comparison of the results of thresholds of reaction in herring larvae with Hemmings (1966) results for Rutilus is best done in terms of skin surface area. Hemmings experimental technique is closest to my method 3 of preparation. Using De Silva's (1973) formula for herring larvae surface area = $0.09 \times (length)^{2.27}$ the threshold for a washing prepared by method 3 is equivalent to 120 mm^2 skin/1/h. A value of 216-198 mm^2 skin/l/hr was calculated from Hemmings data using an empirically derived formula: Surface Area = $0.31 \times (length)^2$. Although this was not a threshold and the relative abundance and production of mucus cells are not known.

The advantages of interspecific attraction in the non-shoaling planktonic stage of a fish which shoals as an adult are several. In the case of larval herring which may take up to 3 months before shoaling begins, maintenance or setting up of aggregations (in the presence of disruptive water movements) is important if larvae are not to be dispersed such as to make creation of a shoal difficult to achieve. The existence of racial groupings of a genetic nature in herrings indicates that such a process must exist. Obviously other senses notably vision must play the major part in maintaining such aggregations, but at night, or when an individual strays more than its limited visual field from the periphery of such an aggregation, olfaction could play an important role in its return. Aggregations of pre-shoaling young fish have been reported by Shaw (1961) in Menidia, and from plankton studies (Longhurst, 1967). A further advantage of an aggregation of pre-shoaling fish is that in a situation where food supply is in patches (discussed by Hunter, 1972). An aggregation would be more effective in finding patches of food than individual larvae, especially if the aggregation could be more widely spaced than

allowed by the visual field. Increased activity in the region of the food patch might act to attract larvae in the aggregation by increased social identifier production. This would be additional to any chemical attraction of the patch itself. Finally an aggregation may have some of the defensive roles ascribed to a shoal by Breder (1967) and Morrow (1948).

In the case of a juvenile herring, where preference for the odour of other herrings was shown, it can be interpreted that a juvenile herring could localize and remain in the vicinity of other herring in the absence of cues other than olfaction. Keenleyside (1955) and Hemmings (1966a) found similar 'preferences' in rudd and roach. Keenleyside concluded that this was brought about by a simple orthokinesis. Hemmings (1966b) after a more intricate analysis of the fishes movements concluded a reverse klinokinesis was responsible. This 'preference' behaviour could act to maintain unpolarised aggregations at night when, as reported by many authors including Blaxter and Parrish (1965), shoals tend to break up. It has also been shown that olfaction plays some role in shoaling behaviour in light; although severing the olfactory tracts in bullfish will not affect shoaling behaviour, ablation of the fore-brain which affects the central connections of the olfactory systems will. Shoaling behaviour becomes more reflex-like and stereotyped (Aronson, 1963).

Although not a subject of investigation in this study, the role of the forebrain in olfactory-mediated behaviour is considered of such fundamental importance that the work of other authors in this field will be discussed. Although the forebrain is innervated mainly by fibres from the olfactory bulbs and was once thought to be mainly olfactory in function, it has recently been shown to play a major part in the learning process and

impinges on all sensory systems (Aronson and Kaplan, 1963;
1968, Aronson, 1968). In terms of electrophysiology, arousal
can be defined as fast activity, low voltage and de-synchronization,
reported in fish by Enger (1957) and Schade and Weiler (1959).
From this, an arousal of the forebrain by an olfactory stimulus
could affect reponse of learned or instinctive locomotor patterns
as well as other sensory systems. It is easy to see how a reaction
such as the 'searching behaviour' of herring larvae seen in this
study could be brought about by forebrain arousal to an olfactory
cue. Kleerkoper (1967) suggests that all olfactory behaviour in
fish can best be explained in terms of arousal rather than the
simple tropic reactions of invertebrates which have not been
satisfactorily proven in vertebrates. This is the conclusion of
this study.

SUMMARY

1.   In contrast to the literature existing on adult fish, no work has previously been undertaken on chemoreception in fish larvae. Because organ systems, especially sensory systems are undergoing development larval systems may be also functionally different from those of adults.

2.   The olfactory system was known to be the major centre of chemosense in teleosts, accordingly the morphological development of this system in herring larvae was studied; both to confirm the presence of a structurally complete system and to investigate the possibility of experimental ablation of the system.

3.   Changes in locomotor activity level due to detection of stimuli by herring and plaice larvae were examined using a thermistor activity monitoring system. Prey washings and extracts and amino acids were tested for detection by herring and plaice larvae. Additionally washings of herring larvae were tested to see if they were detectable by conspecifics.

4.   A fluvarium was used to produce a gradient of stimulus in order to examine the specific way in which a larva would respond to detection of a stimulus and if it could orientate in a concentration gradient so as to reach an area of high concentration.

5.   A structurally complete olfactory system was present in newly hatched herring larvae. This undergoes marked changes mainly associated with changes in head morphology. After metamorphosis the organ has yet to assume its adult form.

6.   Ablation of the olfactory system by severing the olfactory nerve in herring larvae produced a loss of a previous response to a prey extract which was retained in a group of sham-operated larvae. This showed the olfactory system to be responsible for the detection of the stimulus.

7.   Herring larvae showed an innate response of increased activity to washings and extracts of Balanus nauplii, an important natural prey, and to the amino acids glycine and proline. They did not react to extracts of Artemia nauplii prior to those forming the experimental diet. Following a diet change to Artemia nauplii as reaction was shown to Artemia extract (but not to washings) and to an increased range of amino acids.

8.   Plaice larvae showed reactions to a smaller range of amino acids than herring larvae on the same diet of Artemia nauplii. No reaction was shown to an extract of Artemia.

9.   A reaction by herring larvae to herring larva washing (termed social identifier) was present at all stages of development, including post-metamorphic juvenile fish. Threshold of reaction determinations using different methods of collection of washings showed production to be continuous and enhanced in groups of larvae and by crowding.

10.   Analysis of movements of larvae in a gradient of stimulus showed that, both to feeding-related stimuli and to the 'social identifier' larvae did not respond with a simple sterotyped reaction similar to those found in invertebrates. In herring larvae, responses to feeding-related stimuli of increased activity could be explained by an increased occurrence of a 'searching' behaviour pattern.

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APPENDIX I

DETAILS OF HOLE SIZES IN FLUVARIUM DISTRIBUTOR PLATE
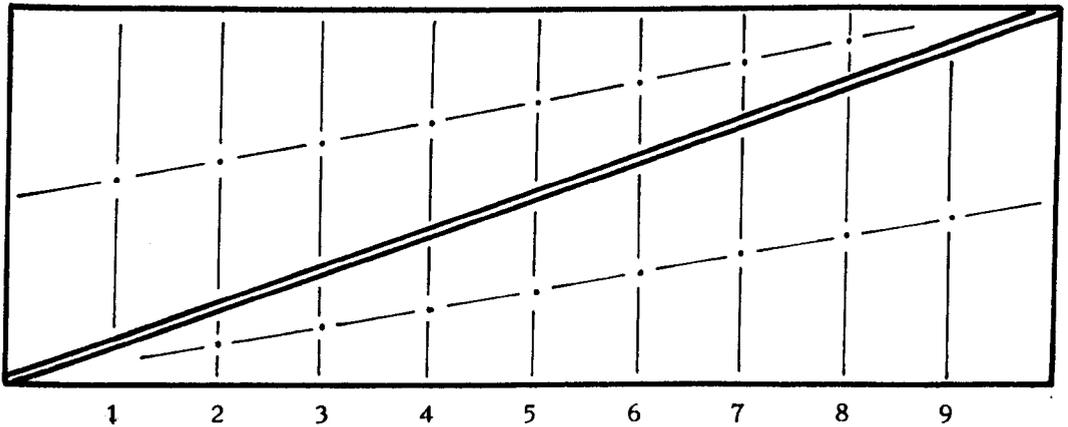
Position	Drill gauge number		Area (mm^2)		Area of holes as % of area of holes in position 1
	Upper	Lower	Upper	Lower	
1	35	–	6.16	–	100
2	38	61	5.31	0.79	99
3	41	54	4.72	1.54	101
4	43	50	3.98	2.54	105
5	48	48	2.99	2.99	97
6	50	43	2.54	3.98	105
7	54	41	1.54	4.72	101
8	61	38	0.79	5.31	99
9	–	35	–	6.16	100

APPENDIX II

FLOW CHARACTERISTICS OF THE FLUVARIUM.

The characteristics of flow and dilution factor of test substances in the fluvarium were tested by using a solution of Acid Fuchin as a test substance and taking samples from positions in the experimental chamber.

A solution of Acid Fuchin dye was pumped into the sea-water supply of the fluvarium in the same way as test substances during experiments. After 5 min a sample of approximately 5 ml of water was taken from each of the 10 sectors of the experimental chamber, by lowering an open glass tube into the chamber, sealing the upper end and transferring the contents to a test-tube.

Samples were scanned at 4250 A$^{\circ}$ using a Unicam SP600 spectrophotometer and the absorbance noted. Further samples were taken one hour after introduction of the dye to establish the longevity and stability of the gradient.

The above procedure was repeated using the other of the two inlets to the distribution chamber to introduce the dye. A calibration curve for deriving the relationship between absorbance and dilution of dye (Appendix Fig 1) was prepared scanning progressive dilutions of the stock solution.

A diagram (Appendix Fig 2) was prepared showing the dilution of the stock solution in the sectors of the experimental chamber.

Appendix Fig. 1.   Calibration curve showing the relationship

between dye dilution and absorbance of light

at 4250 $\overset{\circ}{A}$

Appendix Fig. 2.   Diagram showing the dilution of dye (= stock

solution of stimulus) in sectors of the

experimental chamber of the fluvarium.
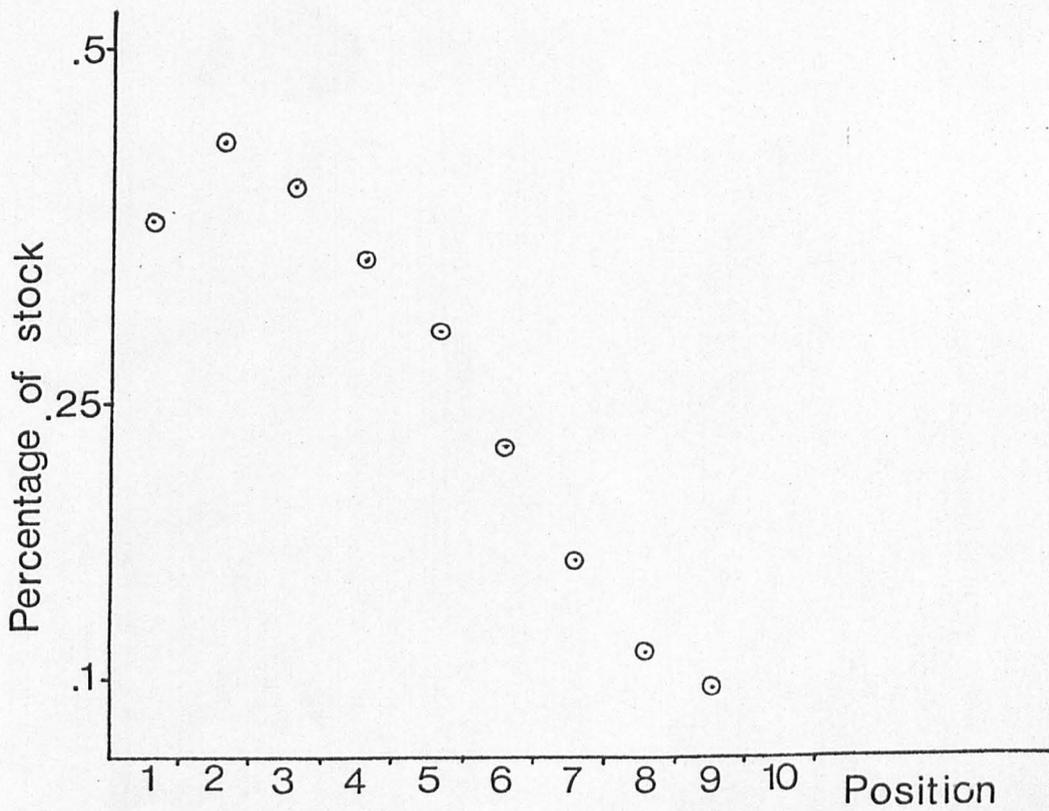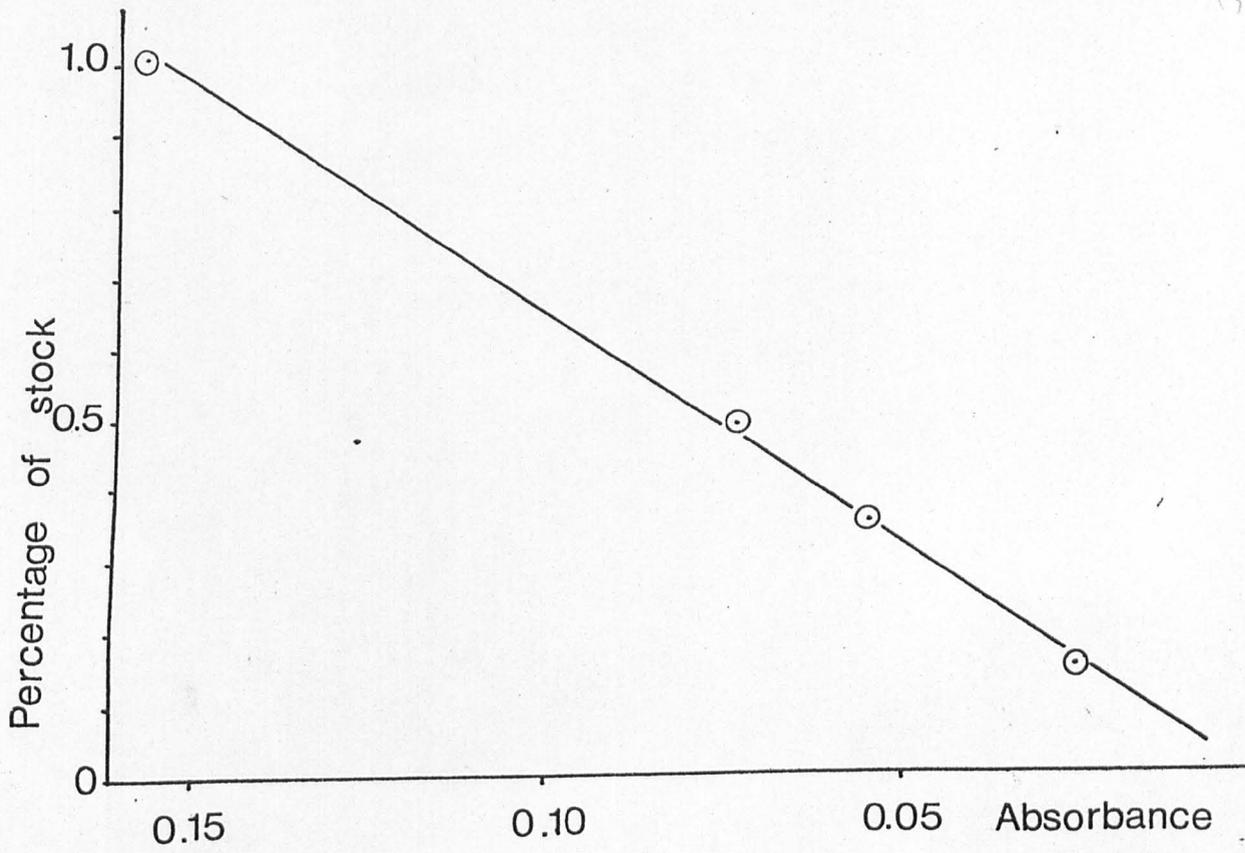
Appendix Plates 1-4.   These plates taken at 10 second intervals,

show the building up of a gradient in the

chamber after the initial arrival of dye

during a dye test.
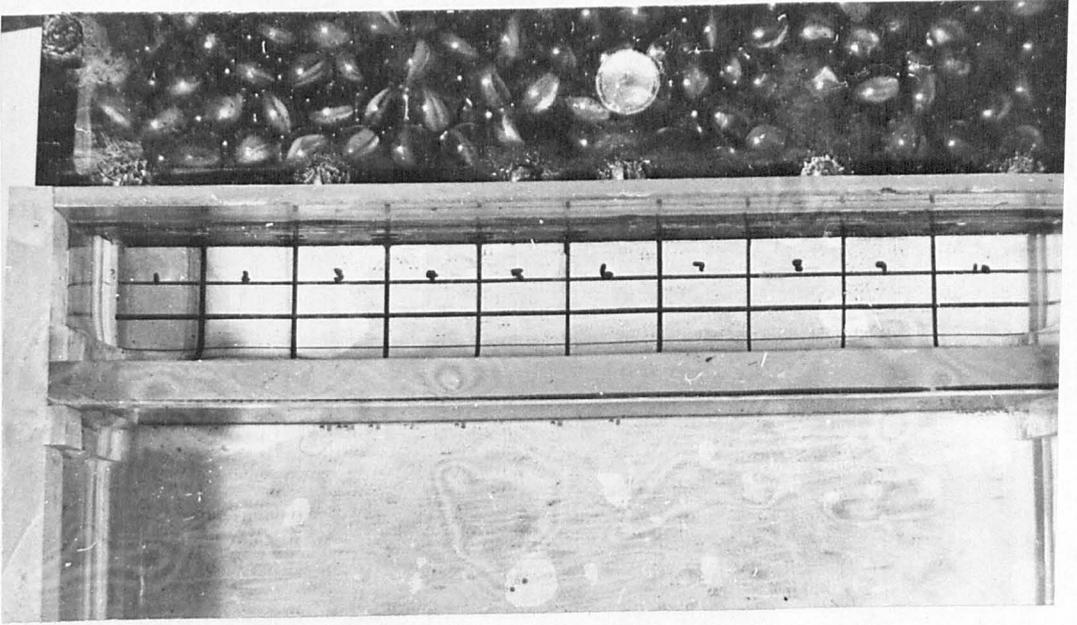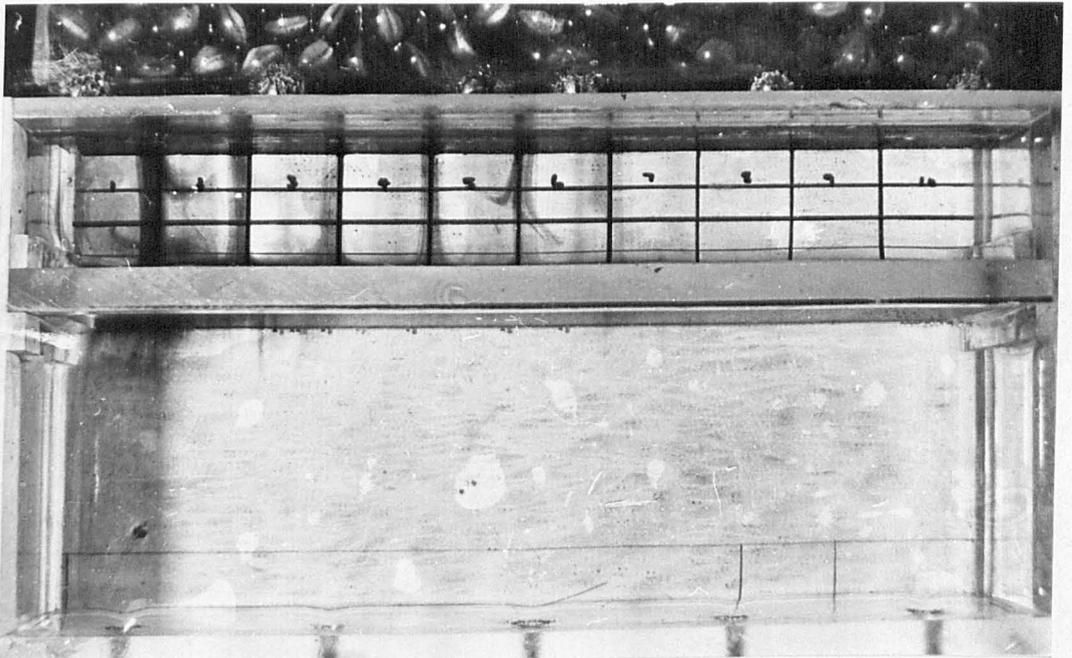
3
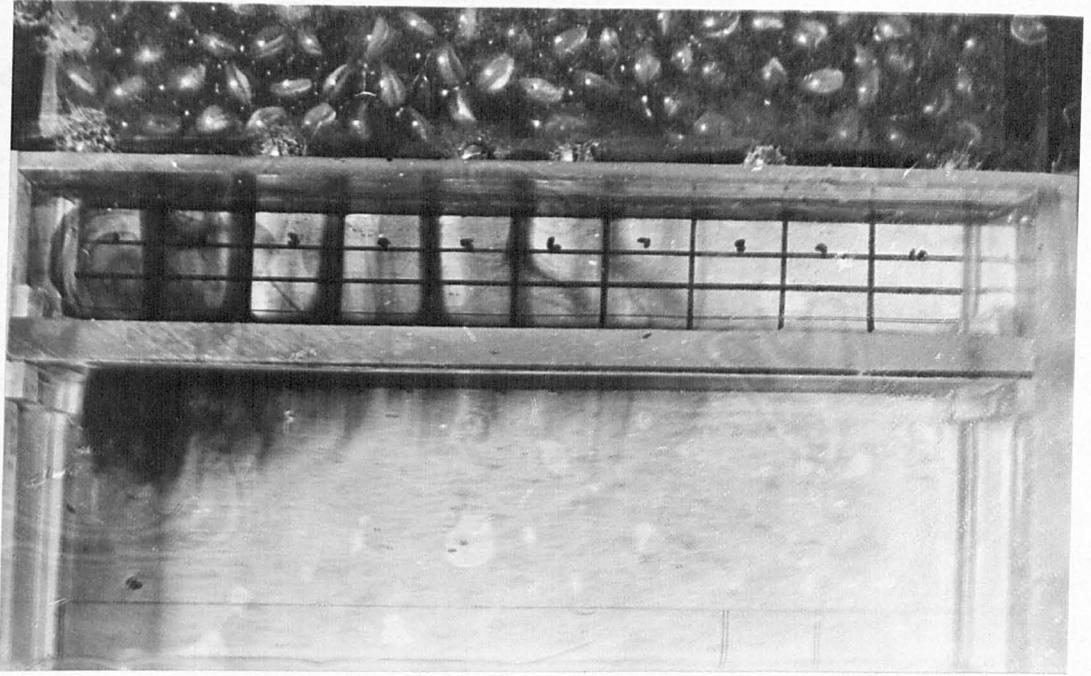
4
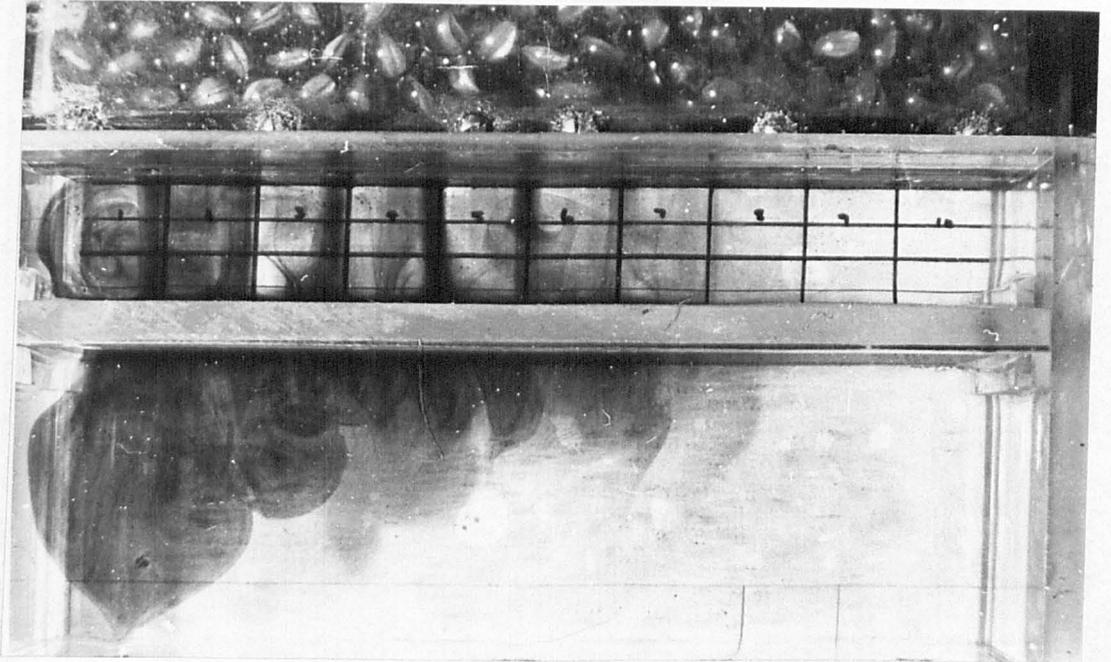
APPENDIX III

HAEMATOXALYN AND EOSIN STAINING METHOD FOR LARVAE AS USED IN THIS STUDY.

Solutions:

1. Mayers Haemalum.

2. Acid Alcohol. (0.5% HCl in 70% Ethanol).

3. Phenol Meths. (8% phenol in 90% methylated spirit).

4. 1% Aqueous Eosin.

5. Scotts' Tap Water Substitute.

> 3. 5 g Sodium Bicarbonate
>
> 20 g Magnesium Sulphate
>
> 100 ml Distilled Water

Method:

Sections were cut at 7 μm from formalin preserved material embedded in paraffin wax.

Sections were taken to water through two changes in xylene and graded alcohols including a 30 sec treatment in Phenol Meths.

Sections were stained in Mayers Haemalum for 20 min.

Sections were washed in running tap water for 2 min.

Sections were dipped twice in acid alcohol.

Sections were blued in Scotts' tap water substitute for 2-5 min.*

Sections were rinsed in distilled water for 1 min.

Sections were counterstained in 1% aqueous eosin for 45 min.

Sections were quickly dehydrated (10-20 sec in graded alcohols), cleared and mounted using D.P.X. medium.

* Local tap water was of a high pH (c.5.0).

APPENDIX IV

BODIAN'S (1936) SILVER IMPREGNATION TECHNIQUE AS USED IN THIS STUDY.

Solutions:

1.   1% Silver Protignate (Protargol).

2.   Bodians Reducing Solution.

>   1.0 g Hydroquinone
>
>   5.0 g Sodium Sulphate
>
>   100 ml Distilled Water

3.   1% Gold Chloride.

4.   2% Oxalic Acid.

5.   5% Sodium Thiosulphate (Hypo).

6.   Aniline Blue Solution.

>   0.1 g Aniline Blue
>
>   2.0 g Oxalic Acid
>
>   2.0 g Phosphomolybdic Acid
>
>   300 ml Distilled Water

Method:

Sections were cut at 7 μm from formalin preserved material embedded in

paraffin wax and mounted on slides using albumen adhesive.

Sections were taken to water through xylene and graded alcohols.

Sections were put in Coplin jars containing 1% 'Protargol' and 4-6 g

clean copper shot/100 ml was added, the jars were then incubated at

37°C for 48 h.

Sections received 3 changes of distilled water.

Sections were given 10 min in Bodians Reducing Solution.

Sections received 3 changes of distilled water.

Sections were toned in gold chloride for 10 min

Sections received 3 changes of distilled water.

APPENDIX IV cont.

Sections were left in 5% hypo for 5 min.

Sections were washed for 10-20 min.

Sections were counterstained with aniline blue by 3, 1 sec dips.

Sections were rinsed in distilled water.

Sections were dehydrated, cleaned and mounted using D.P.X. medium.

Results:

Nerve fibres and cell nuclei appear black on a grey-blue background.

APPENDIX V

COMPUTER PROGRAM FOR INFLUENCE OF A MOVE IN THE GRADIENT ON
SUBSEQUENT MOVES.

Fortran IV program used to analyse preferred direction of turn after

movment in the gradient of stimulus in the Fluvarium.


```
& JOB; 150027; FISH MOVMENTS; DEMPSEY;

& FORTRAN; L;

& TIME; 7,

      DIMENSION ARRAY (2,90)

      INTEGER O,P,RTUP,RTDN,RDN,Q,X,Y,A,B,RUP

      I=J=K=L=RTUP=RTND=RDN=RUP=M=N=O=P=ISTAT=IVERT=0

      READ (7,10) IEXPNO

      WRITE (2,9) IEXPNO

   9 FORMAT ('EXPERIMENT NUMBER F', I4)

      READ (7,10)((IRRAY (ID,JD), ID=1,2) JD=1,90)

  10 FORMAT (20I4)

      WRITE (2,10)(IRRAY(ID,JD), ID=1,2), JD=1,90)

      X1=IRRAY (1,1)

      Y1=IRRAY (2,1)

  11 DO 50 IN=2,29

      X2=IRRAY (1,IN)

      Y2=IRRAY (2,IN)

      A=X2-X1

      B=Y2-Y1

C   THIS GIVES THE MM VALUE OF THE FISHES CURRENT COORDINATES

      IF (A.EQ.0)GOTO 27

      GOTO 29

  27 IF (B.EQ.0)GOTO 28

      GOTO 29
```

APPENDIX V cont

```
   37 RTUP=1

   38 CONTINUE

C     WE NOW HAVE DIRECTION OF TRAVEL FOR MOVE IN HELD IN A TEMP STORE
      IF (IN.LT.3)GOTO 49
C     THE ABOVE CARD PREVENTS ASSIGNATION OF FIRST MOVE TO PERM STORE
C     MOVE ONE IS MADE WITHOUT A PREVIOUS DIRECTION OF TRAVEL (FROM REST)
      IF (Q.GT.0)GOTO 39
      M=M+I
      N=N+J
      O=O+K
      P=P+L
      RUP=RUP+RTUP
      RDN=RDN+RTDN
      GOTO 48
      IF (IVERT.LE.0)GOTO 39
      M2=M2+I
      N2=N2+J
      O2=O2+K
      P2=P2+L
      RUP2=RUP2+RPTUP
      RDN2=RDN2+RTDN
      GOTO 48
   39 M1=M1+I
      N1=N1+J
      O1=O1+K
      P1=P1+L
      RUP1=RUP1+RTUP
      RDN1=RDN1+RTDN
```

APPENDIX V cont.

```
 28 ISTAT=ISTAT+1

    GOTO 49

 29 CONTINUE

C   THIS PART OF THE PROGRAM HAS DEALT WITH NON MOVES STORED IN ISTAT

    Q=I+K

    I=0

    J=0

    K=0

    L=0

    RTUP=0

    RTDN=0

C   WE NOW HAVE A MEASURE OF IF LAST MOVE WAS TO LEFT OR RIGHT

    IF (A.GT.0)GOTO 30

    IF (B.LT.0)GOTO 31

    I=1

    GOTO 35

 30 IF (B.GE.0)GOTO 33

    IF (B.LT.0)GOTO 34

    GOTO 35

 31 K=1

    GOTO 35

 33 J=1

    GOTO 35

 34 L=1

 35 CONTINUE

    IF (A.EQ.0)GOTO 36

    GOTO 38

 36 IF (B.LT.0)GOTO 37

    RTDN=1

    GOTO 38
```

APPENDIX V cont.

```
 48 CONTINUE

    Q=0

    IVERT=0

    IF (A.EQ.0)IVERT=1

 49 CONTINUE

    X1=X2

    Y1=Y2

 50 CONTINUE

    WRITE (2,12) M,N,O,P,RUP,RDN

 12 FORMAT ('CONTROL, AFTER MOVE TO LEFT 1-90',I2,'91-179', I2,
    1'181-270',I2,'271-359',I2,'UP',I2,'DOWN',I2)

    WRITE (2,13) M1,N1,O1,P1,RUP1,RDN1

 13 FORMAT ('CONTROL, AFTER MOVE TO RIGHT 1-90',I2,'91-179',I2,
    1'181-270',I2,'271-359',I2,'UP',I2,'DOWN',I2)

    WRITE (2,14)M2,N2,O2,P2,RUP2,RDN2

 14 FORMAT ('CONTROL AFTER NO LATERAL MOVEMENT 1-90',I2,'91-179'
    1,I2,'181-270',I2,'271-359',I2,'UP',I2,'DOWN',I2)

    WRITE (2,15)ISTAT

 15 FORMAT ('FISH STATIONARY FOR',I2,'MOVES')
```

This is the program for the first 30 observations (control period) the above Do-loop from statement 11 to format statement 15 is repeated twice for the two halves of the test period with changes only to statement numbers and format detail to identify the results.

APPENDIX VI

COMPUTER PROGRAM FOR ANGLE OF TURN AND STEP LENGTH

Fortran IV program used to calculate the angle of turn and step length between observations of the larvas position.

```
& JOB; 150027;  ANGLE OF TURN DEMPSEY;

& ASSIGN;   3;DV;40;PAD;

& FORTRAN; L;

& TIME; 7;
      DIMENSION IRRAY (2,90)
      DO 51 JK = 1,28
      INUM = 90
      ONGLE = 0
      READ (3,8) IEXPNO
    8 FORMAT (13)
      READ (3,10) ((IRRAY (ID,JD), ID = 1,2), JD = 1, INUM)
   10 FORMAT (10I4)
      WRITE (2,9) IEXPNO
    9 FORMAT ('EXPERIMENT NUMBER F',I4)
      X1 = IRRAY (1,1)
      Y1 = IRRAY (2,1)
      DO 50 IN = 2, INUM
      X2 = IRRAY (1, IN)
      Y2 = IRRAY (2, IN)
      A  = X2 - X1
      B  = Y2 - Y1
      INO = IN - 1
      IF (A.EQ.0) GOTO 27
      GOTO 29
   27 IF (B.EQ.0) GOTO 28
      GOTO 29
```

APPENDIX VI cont.

```
   28 WRITE (2,12) INO

   12 FORMAT ('MOVE NUMBER', I3, 'FISH STATIONARY')

      GOTO 49

   29 CONTINUE

      IF (C.LT.0) GOTO 25

      IF (S.GT.0) GOTO 26

      XO = XO + (44/7)

      GOTO 26

   25 XO = XO + (22/7)

   26 CONTINUE

      WRITE (2,14)

   14 FORMAT ('CONTROL PERIOD')

      WRITE (2,13) IN, R, XO

   13 FORMAT ('NO OF TURNS=', I4, 'RESULTANT =', F10.5, 'MEAN DIR =', F8.5)

      C = 0

      S = 0
```

The above is the program for the control period, the two test periods
are analysed by a similar method.

APPENDIX VII
COMPUTER PROGRAM FOR ANGULAR STATISTICS.

Fortran IV program used to calculate the resultant and mean angle from angle of turn results.

```
& JOB; 150027/R44/2;  DEMPSEY, PFD6;

& ASSIGN; 1,DC;50; ANGSTAT, CZ1;

& OPTIONS;

& FORTRAN; L;

& TIME; 7;

      DIMENSION IRRAY 1 (1,1000)

      DIMENSION IRRAY 2 (1,1000)

      DIMENSION IRRAY 3 (1,1000)

      K = 3

      DO 300 JK = 1,21

      READ (K,10) IDEXP, IN1, IN1, IN3

   10 FORMAT (I3, 3I4)

      WRITE (2,11) IDEXP

   11 FORMAT ('EXPERIMENT IDENTIFICATION NUMBER', I3)

      READ (K,12) (IRRAY 1 (I,JD), JD=1, IN1)

      READ (K,12) (IRRAY 2 (I,JD), JD=1, IN2)

      READ (K,12) (IRRAY 3 (I,JD), JD=1, IN3)

   12 FORMAT (10I4)

      C = 0

      S = 0

      DO 30 I = 1, IN1

      INGLE = IRRAY 1 (1,I)

      ANGLE = INGLE * 0.01746

      C = C + COS (ANGLE)

      S = S + SIN (ANGLE)
```

APPENDIX VII cont.

```
   30 CONTINUE

      RB = SQRT (((C/IN1)**2) + ((S/IN1)**2))

      R  = RB/IN1

      XO = ATAN ((S/IN1)/(C/IN1))

      DIST = SQRT ((A*A) + (B*B))

      ANGLE = SO9AAF (ABS (B)/DIST, 0)

      ANGLE = ANGLE/0.01745

      IF (A.LT.0) GOTO 32

      IF (B.LT.0) GOTO 30

      ANGLE = 90 - ANGLE

      GOTO 34

   30 ANGLE = 90 + ANGLE

      GOTO 34

   32 IF (B.LT.0) GOTO 33

      ANGLE = 270 + ANGLE

      GOTO 34

   33 ANGLE = 270 - ANGLE

   34 CONTINUE

      IF (ONGLE.LT.ANGLE) ONGLE = ONGLE + 360

      AOT = ONGLE - ANGLE

      WRITE (2,13) INO, DIST, AOT

   13 FORMAT ('MOVE NO', I3, 'DIST MVD', F4.0, 'TURN ANGLE', F4.0)

      ONGLE = ANGLE

      X1 = X2

      Y1 = Y2

   49 CONTINE

   50 CONTINUE

   51 CONTINUE

      STOP

      END
```

APPENDIX VIII

ANGLE OF TURN DISTRIBUTIONS AND ANGULAR STATISTICS
DESCRIBING MOVEMENTS OF HERRING AND PLAICE LARVAE
IN FLUVARIUM GRADIENTS OF FEEDING-RELATED STIMULI.

This appendix contains histograms depicting the relative
proportions of the angles of turn made by larvae in control
and test periods of trials using feeding-related stimuli.
Tables 1 and 2 present the angular statistics 'mean direction'
and 'degree of dispersion' of angles of turn about this value.
The derivation of these statistics is given in Appendix IX.

Appendix Fig 3.   Distribution of angles of turn of herring larvae in
                   a fluvarium gradient in control and test situations.

                   Test stimuli and stage of larvae.
                       1. Control experiment, Stage 1c larvae

                       2. Control experiment, Stage 3 larvae

                       3. Artemia nauplii washing, Yolk sac larvae

                       4. Balanus nauplii washing, Yolk sac larvae

                       5. Artemia nauplii washing, Balanus feeding larvae

                       6. Balanus nauplii washing, Balanus feeding larvae

                       7. Artemia nauplii washing, Artemia feeding larvae

Control   Test 1   Test 2

Relative Frequency
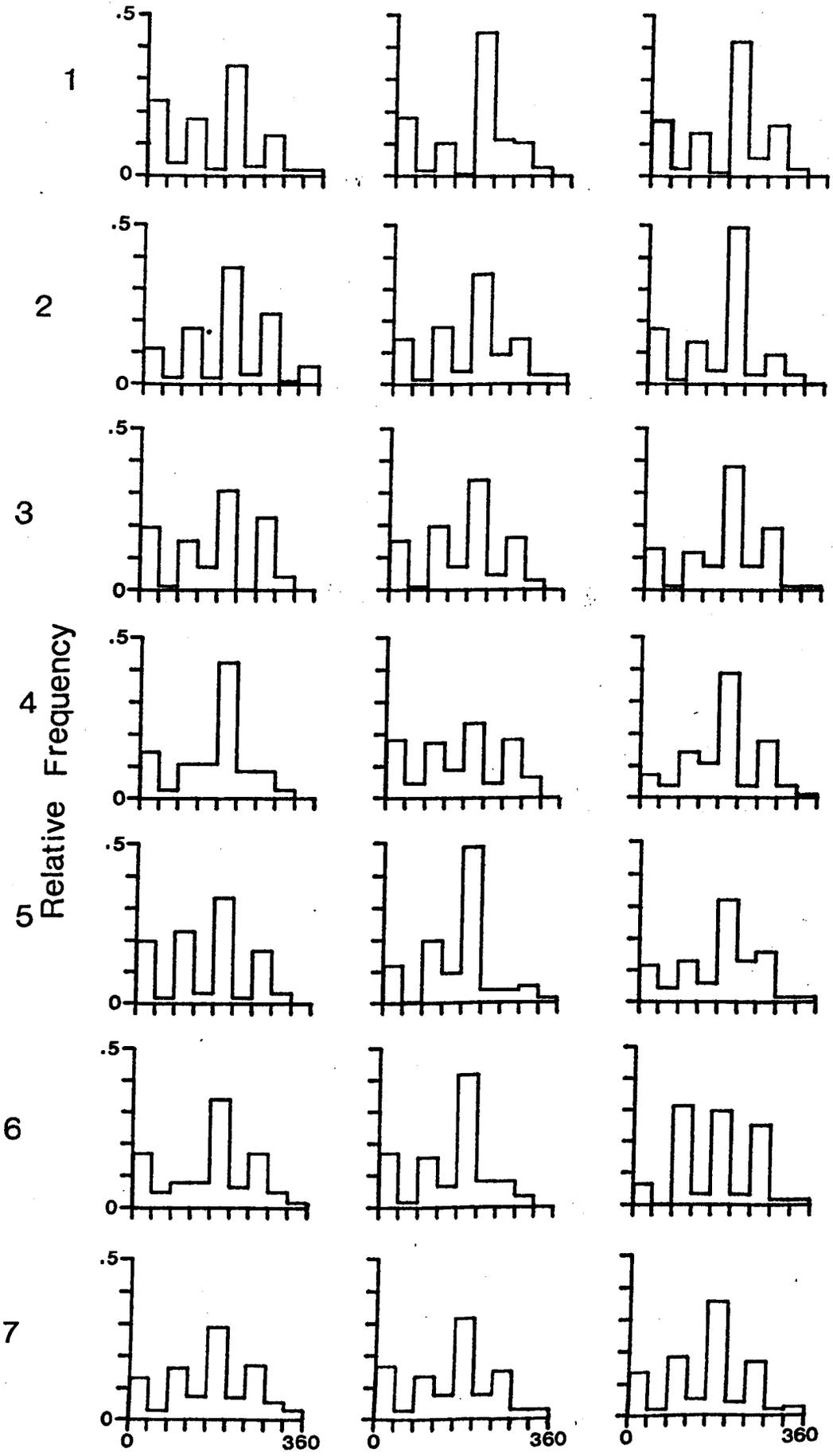
Angle of Turn

Appendix Fig 4.   Distribution of angles of turn of herring larvae in

a fluvarium gradient in control and test situations.

Test stimuli and stage of larvae

1.   Artemia nauplii extract. Yolk sac larvae

2.   Balanus nauplii extract, Yolk sac larvae

3.   Artemia nauplii extract, Balanus feeding larvae

4.   Balanus nauplii extract, Balanus feeding larvae

5.   Dialysed Balanus nauplii extract, Balanus

feeding larvae

6.   Artemia nauplii extract, Artemia feeding larvae

Control   Test 1   Test 2

Relative Frequency
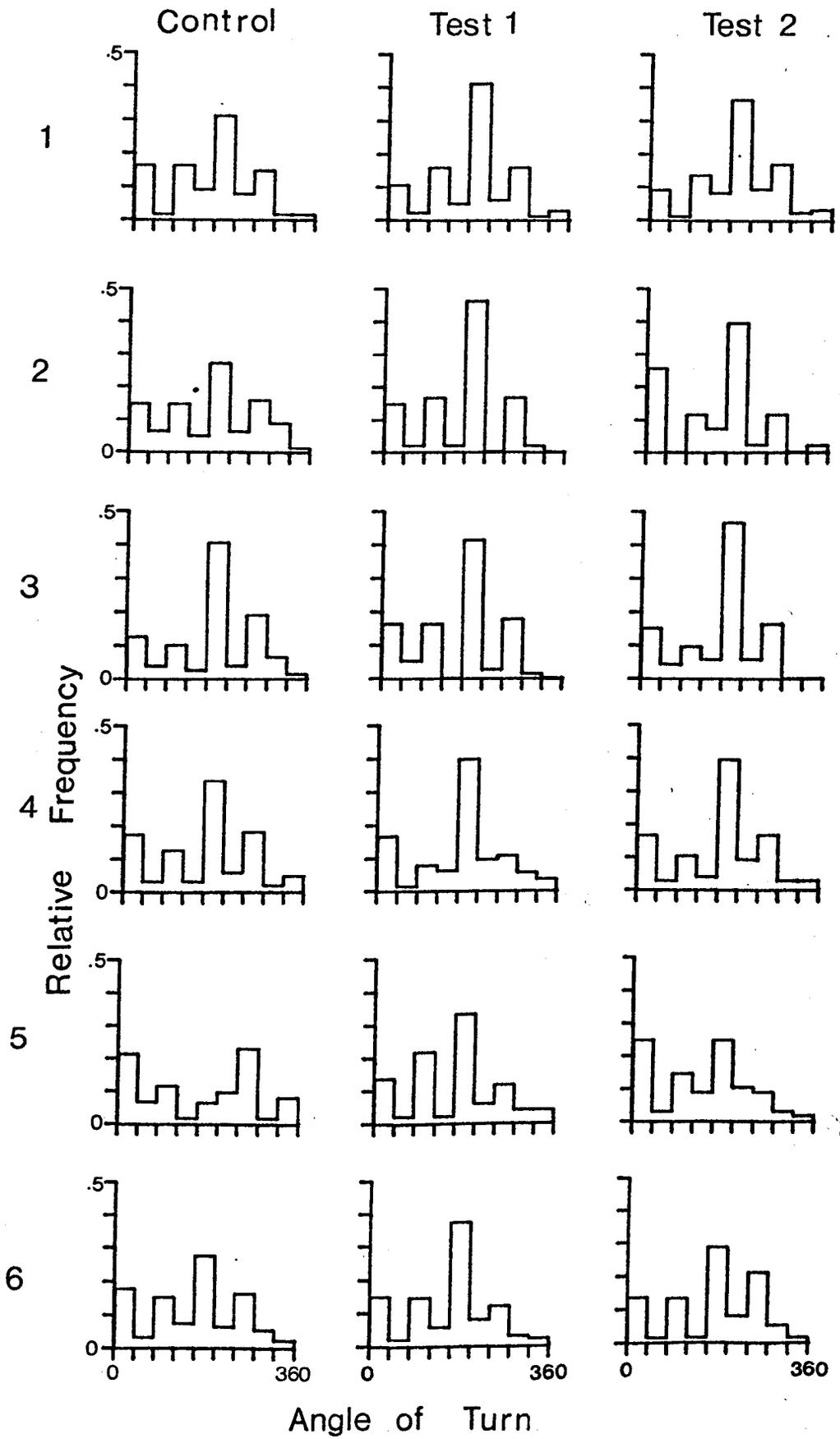
Angle of Turn

Appendix Fig 5. Distribution of angles of turn of herring larvae in

a fluvarium gradient in control and test situations.

Test stimuli and stage of larvae

1. Glutamic acid, Yolk sac larvae

2. Aspartic acid, Yolk sac larvae

3. Glycine, Yolk sac larvae

4. Methionine, Yolk sac larvae

5. Alanine, Yolk sac larvae

6. Proline, Yolk sac larvae

7. Cysteine, Yolk sac larvae
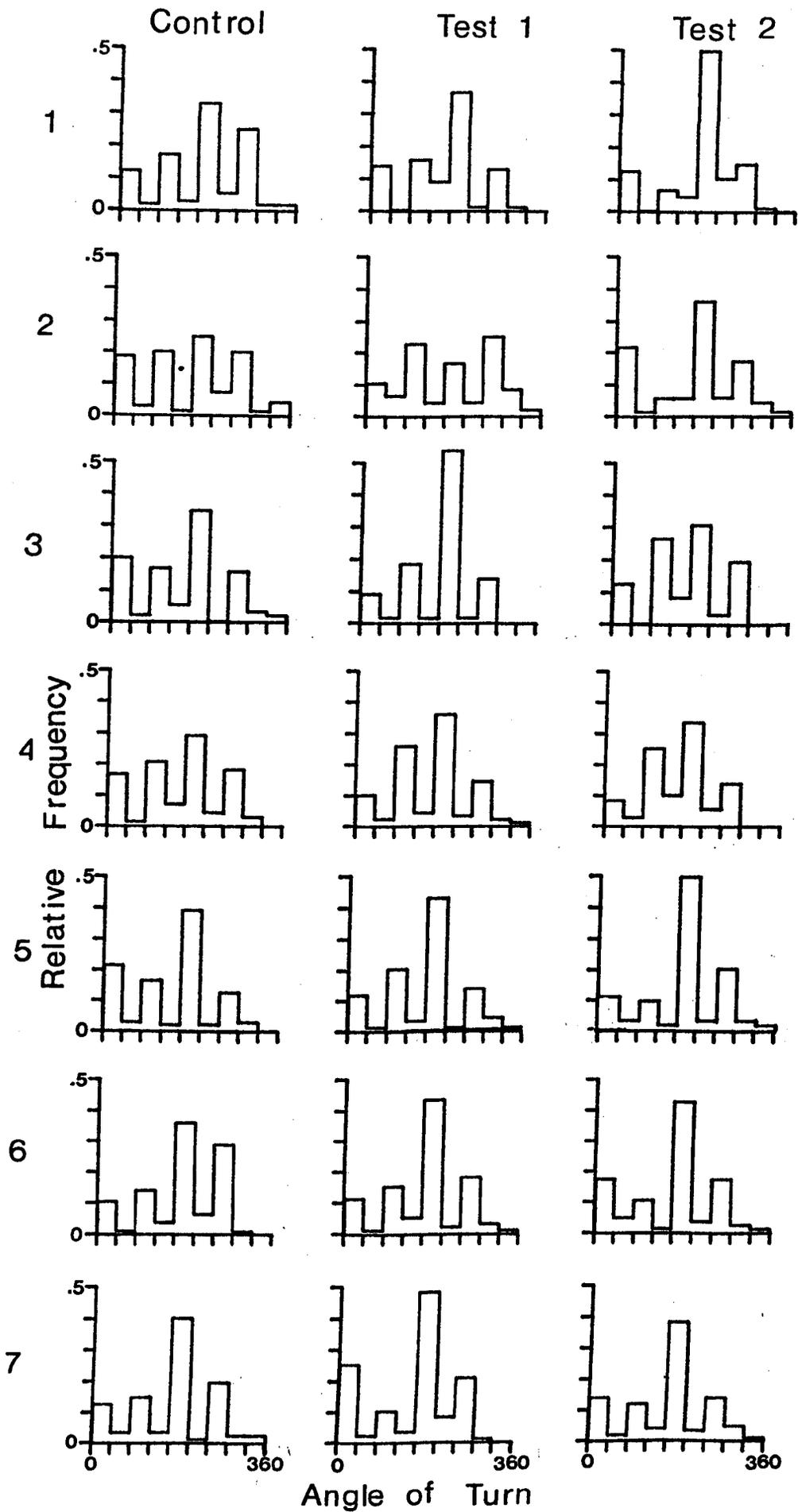
Angle of Turn

Appendix Fig 6. Distribution of angles of turn of herring larvae in
the fluvarium in control and test situations.

Test stimuli and stage of larvae

1. Glutamic acid, <u>Artemia</u> feeding larvae

2. Aspartic acid, <u>Artemia</u> feeding larvae

3. Glycine, <u>Artemia</u> feeding larvae

4. Methionine, <u>Artemia</u> feeding larvae

5. Alanine, <u>Artemia</u> feeding larvae

6. Proline, <u>Artemia</u> feeding larvae

7. Cysteine, <u>Artemia</u> feeding larvae

Control   Test 1   Test 2

Relative Frequency
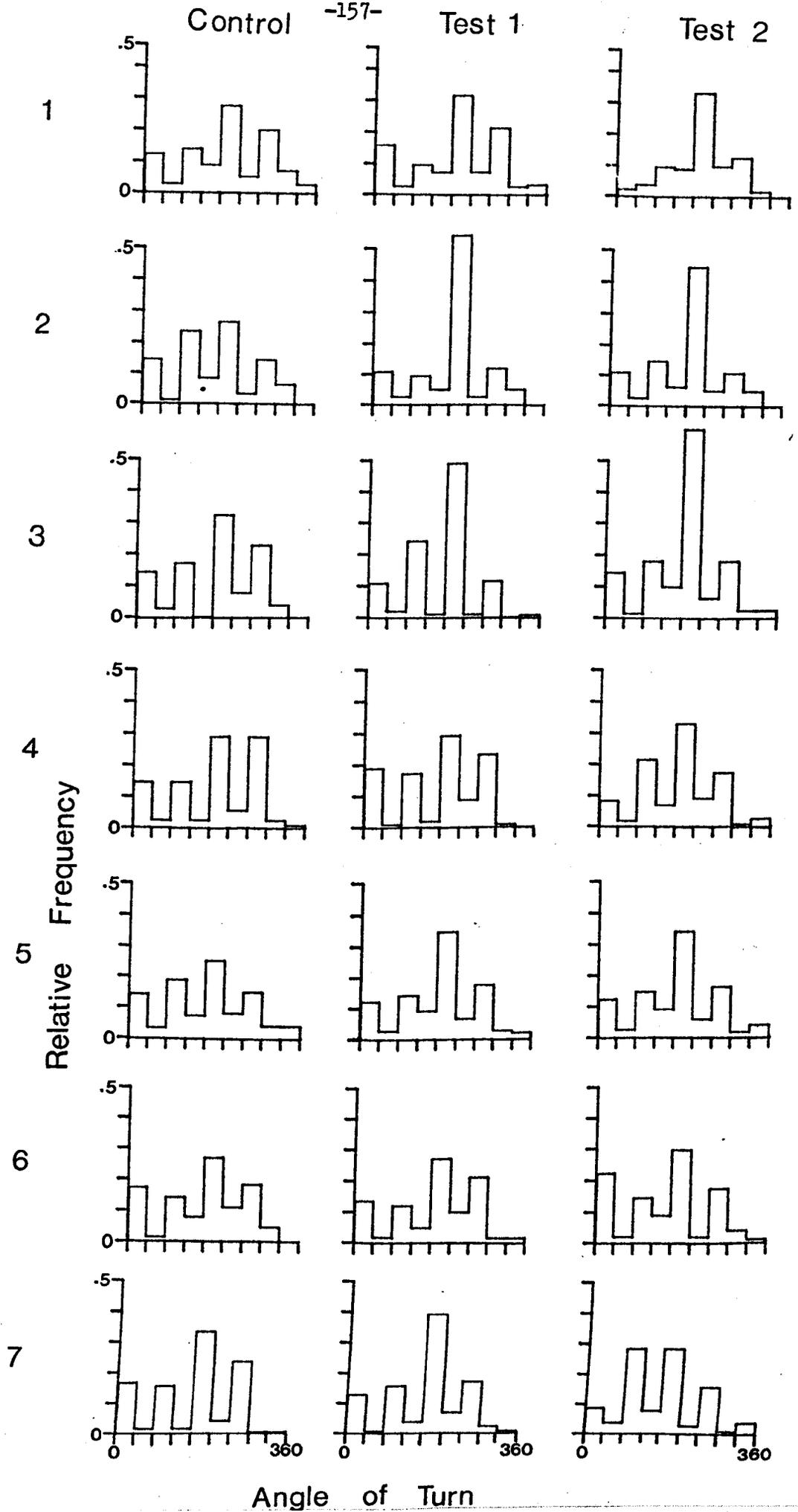
Angle of Turn

Appendix Fig 7.   Distribution of angles of turn of plaice larvae in

a fluvarium gradiènt in control and test situations.

Test stimuli

1.   <u>Balanus</u> nauplii extract

2.   <u>Artemia</u> nauplii extract

3.   <u>Artemia</u> nauplii washing
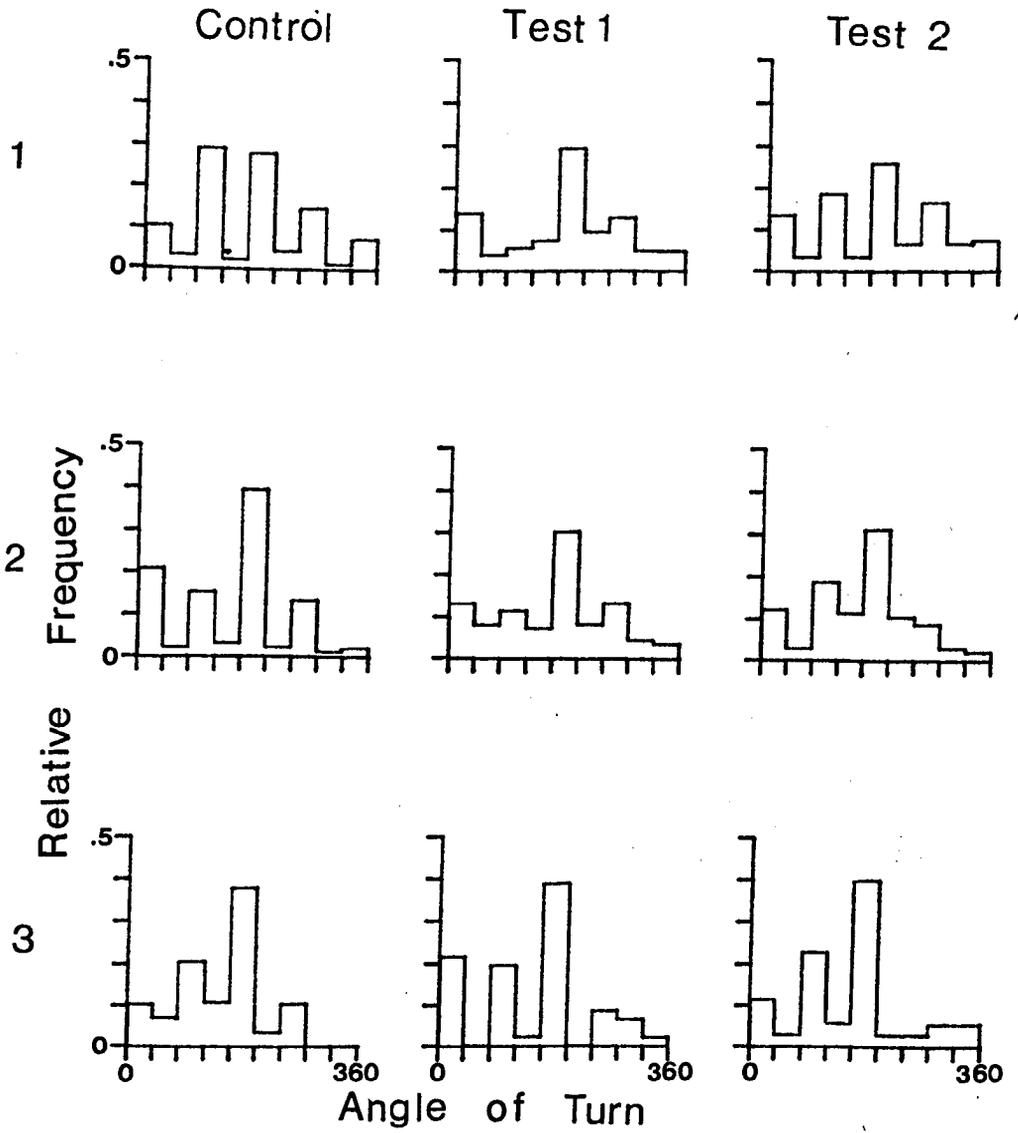
Angle of Turn

Appendix Fig 8.   Distribution of angles of turn of plaice larvae in
                   a fluvarium gradient in control and test situations.

   Test stimuli
                        1.   Glutamic acid

                        2.   Aspartic acid

                        3.   Glycine

                        4.   Methionine

                        5.   Alanine

                        6.   Proline

                        7.   Cysteine

Control   Test 1   Test 2

Frequency

Relative
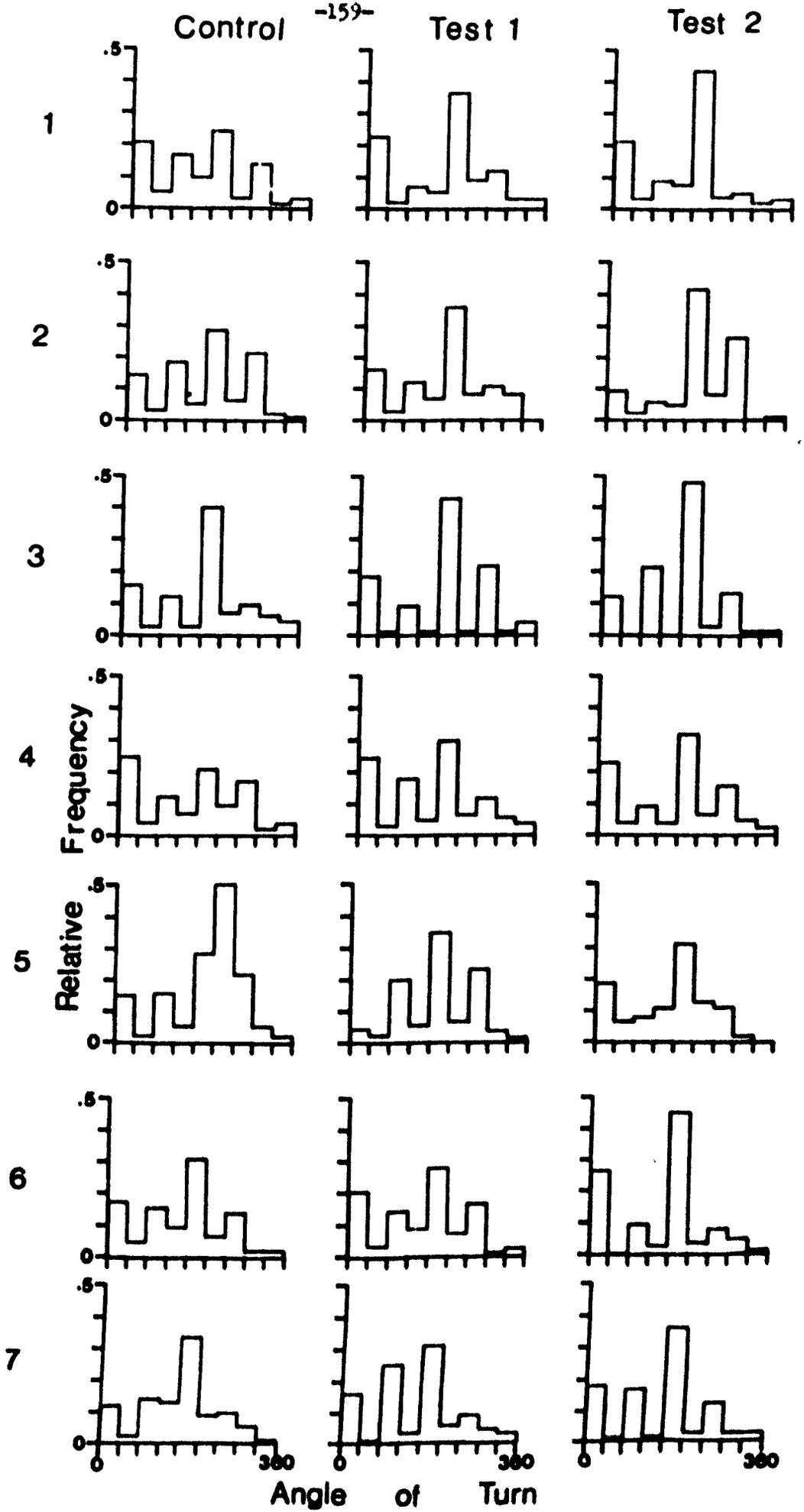
Angle   of   Turn

APPENDIX TABLE I

ANGULAR STATISTICS QUANTIFYING THE DISTRIBUTION OF ANGLES OF TURN

BETWEEN POSITIONS OF HERRING LARVAE IN A FLUVARIUM GRADIENT OF

FEEDING-RELATED STIMULUS

Shown are the mean direction $(\bar{X}o)$ and the degree of dispersion (So)

of angles of turn about this value in the control and test periods.

STAGE OF LARVAE AND TEST STIMULUS	CONTROL		TEST 1		TEST 2	
	$\bar{X}o$	So	$\bar{X}o$	So	$\bar{X}o$	So
YOLK SAC STAGE						
Balanus nauplii washing	-4.5	.99519	6.1	.99867	-3.4	.99609
Balanus nauplii extract	15.9	.99793	-4.3	.99438	-3.6	.99488
Artemia nauplii washing	11.6	.99777	-8.5	.99725	11.4	.99588
Artemia nauplii extract	4.9	.99661	-4.3	.99557	4.8	.99541
Glutamic acid 10^{-3}M	14.8	.99736	-12.8	.99592	13.6	.99423
Aspartic acid 10^{-3}M	12.3	.99796	16.9	.99776	34.6	.99604
Glycine 10^{-3}M	-6.6	.99796	-9.3	.99217	-16.7	.99512
Methionine 10^{-3}M	-14.8	.99656	-21.8	.99658	-18.9	.99323
Alanine 10^{-3}M	-16.4	.99814	-5.9	.99629	12.6	.99417
Proline 10^{-3}M	12.4	.99597	4.7	.99605	6.9	.99628
Cysteine 10^{-3}M	6.4	.99664	16.3	.99663	7.1	.99724
CONTROL EXPERIMENT 1	-5.2	.99858	4.8	.99701	9.6	.99605
BALANUS FEEDING STAGE						
Balanus nauplii washing	20.8	.99536	-12.7	.99436	-7.9	.99518
Balanus nauplii extract	10.2	.99725	20.5	.99738	13.1	.99678
Dialysed Balanus nauplii extract	-50.1	.99624	-0.3	.99587	-32.5	.99673
Artemia nauplii washing	-21.7	.99695	-14.6	.99402	8.2	.99464
Artemia nauplii extract	21.8	.99575	-4.6	.99648	7.2	.99378
Glycine 10^{-3}M	-20.5	.99763	-21.8	.99641	-1.3	.99511

APPENDIX TABLE I cont.

STAGE OF LARVAE	CONTROL		TEST 1		TEST 2	
AND TEST STIMULUS	$\overline{X}o$	So	$\overline{X}o$	So	$\overline{X}o$	So
ARTEMIA FEEDING STAGE						
Artemia nauplii washing	9.0	.99882	-4.1	.99891	-0.1	.99874
Artemia nauplii extract	-1.0	.99925	0.2	.99902	20.5	.99908
Glutamic acid $10^{-3}M$	18.0	.99789	24.7	.99773	2.8	.99783
Aspartic acid $10^{-3}M$	-25.4	.99766	2.8	.99415	-3.7	.99491
Glycine $10^{-3}M$	28.2	.99777	-17.3	.99556	1.3	.99616
Methionine $10^{-3}M$	29.5	.99774	20.0	.99732	-1.9	.99662
Alanine $10^{-3}M$	22.4	.99759	5.5	.99560	-3.9	.99533
Proline $10^{-3}M$	9.1	.99694	22.9	.99744	-7.0	.99824
Cysteine $10^{-3}M$	17.7	.99799	6.6	.99706	-18.0	.99674
CONTROL EXPERIMENT 2	18.9	.99724	-2.8	.99630	-4.8	.99521

APPENDIX TABLE 2

ANGULAR STATISTICS QUANTIFYING THE DISTRIBUTION OF ANGLES OF TURN

BETWEEN POSITIONS OF PLAICE LARVAE IN A FLUVARIUM GRADIENT OF

FEEDING-RELATED STIMULUS.

Shown are the mean direction ($\bar{X}o$) and the degree of dispersion (So)

of angles of turn about this value in the control and test periods.

TEST STIMULUS	CONTROL		TEST 1		TEST 2	
	$\bar{X}o$	So	$\bar{X}o$	So	$\bar{X}o$	So
Balanus nauplii extract	-5.3	.99784	-2.8	.99791	-21.2	.99665
Artemia nauplii washing	-35.4	.98974	-19.0	.99363	-28.8	.989711
Artemia nauplii extract	-17.2	.99729	14.4	.99624	21.7	.99869
Glutamic acid $10^{-3}M$	-43.4	.99909	19.3	.99859	-14.6	.99820
Aspartic acid $10^{-3}M$	25.4	.99726	- 4.7	.99572	10.4	.99343
Glycine $10^{-3}M$	18.8	.99757	31.8	.99708	-10.8	.99506
Methionine $10^{-3}M$	35.5	.99953	25.4	.99904	33.5	.99834
Alanine $10^{-3}M$	22.4	.99759	5.5	.99560	- 3.9	.99533
Proline $10^{-3}M$	-4.6	.99747	-8.8	.99782	10.5	.99860
Cysteine $10^{-3}M$	-6.5	.99624	-31.9	.99724	-17.0	.99708

APPENDIX IX

DERIVATION OF ANGULAR STATISTICS

The angles of turn of the fish in the fluvarium were subjected to a vector addition in order to obtain two values, the mean direction $(\bar{X}o)$ and the circular variance (So). These were used to quantify any difference in turning behaviour between control and test periods. The circular variance gives a quantification of the degree of dispersion of the angles of turn about the mean angle; where So is nearly zero the angles are clustered around the mean angle, and where So is nearly one the angles are widely dispersed. The way in which these values are derived are detailed below.

Mean direction, resultant and circular variance.

Let Pi be the point of the unit circle, centre at O corresponding to the angle θi. Then the mean direction $\bar{X}o$ of θ_1, θ_2,........θ_n is defined to be the direction of the resultant of the unit vectors OP_1, OP_2,.......OP_n. The co-ordinates of Pi are (Cos θi, Sin θi) so that the centre of gravity of these points is $(\bar{c},\bar{s})$ where:

$$\bar{c} = \frac{1}{n} \sum_{i=1}^{n} \text{Cos } \theta i \quad , \quad \bar{s} = \frac{1}{n} \sum_{i=1}^{n} \text{Sin } \theta i$$

The resultant, $R = n\bar{R}$

Where: $\bar{R} = (\bar{c}^2 + \bar{s}^2)^{\frac{1}{2}}$

The mean direction is given by

$$\bar{X} = \begin{cases} \bar{X}_o^i & \text{if } \bar{s} > 0 \text{ and } \bar{c} > 0 \\ \bar{X}_o^i + \pi & \text{if } \bar{c} < 0 \\ \bar{X}_o^i + 2\pi & \text{if } \bar{s} < 0 \text{ and } \bar{c} > 0 \end{cases}$$

Where

$$\bar{X}_o^i = \text{arctan } (\bar{s}/\bar{c}) \quad , \quad -\frac{\pi}{2} < \bar{X}_o^{-1} < \frac{\pi}{2}$$

The circular variance (So) is given by

$$So = 1 - \frac{1}{n} \sum_{i=1}^{n} \text{Cos } (\theta i - \bar{X}o)$$

Which can be shown to be equal to

$$So = 1 - \bar{R} \quad , \quad 0 \leq So \leq 1$$

APPENDIX X.

LEVELS OF FREE AMINO ACIDS IN THE NAUPLII OF BALANUS BALANOIDES

AND ARTEMIA SALINA.

Values given compare levels in Artemia salina found by Emerson (1967)

with those for the same amino acids in Balanus balanoides found by

Barnes and Blackstock (1976). Levels are given in uM/g. Dashes

indicate no value given by the author for an amino acid.

APPENDIX TABLE 3

Amino acid	Value Balanus	Value Artemia
Alanine	35.2	32.6
Glutamic acid	29.3	6.0
Proline	165.1	17.7
Aspartic acid	5.1	8.6
Serine	8.1	12.2
Glycine	92.7	16.0
Threonine	7.1	10.9
Tyrosine	6.4	5.5
Arginine	4.7	4.0
Histidine	5.1	2.8
Ph. Alanine	2.0	1.9
Lysine	11.2	2.9
Cystine	-	3.7
Valine	6.7	3.3
(Leucine) (Isoleucine)	5.8	2.4
Taurine	77.3	-
Methionine	1.9	-