SKIN DEFENCE MECHANISMS IN FISH LARVAE

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by

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ABSTRACT

Wound healing and recovery from injury were investigated in eggs and larvae of herring (Clupea harengus L.), plaice (Pleuronectes platessa L.) and salmon (Salmo salar L.). The resistance of herring eggs to mechanical damage was first examined. The chorion of eggs before and just after fertilisation could be burst by loads of 4-30 g but eggs 5 h post-fertilisation could withstand over 1000 g without bursting. Resistance remained high until just before hatching when it decreased to 20-680 g. The vitelline membrane, however, showed a lower resistance at all stages.

Early herring and plaice larvae were caught and eaten by medusae (Aurelia aurita, Tiaropsis multiserrata, Bougainvillea sp.), hydroids (Sarsia sp.), megalopa larvae of the prawn Nephrops norvegicus and adult mysids. Early herring larvae survived minor stings from an Aurelia ephyra, and also experimentally inflicted lesions such as superficial scratches, suction wounds and amputation of up to 2 mm of the tail in sea water. The caudal region of the primordial fin regenerated within a month when less than 1 mm was cut off. Yolk sac and first feeding herring also survived an incision of 0.3 mm long through the body ventral to the notochord and dorsal to the gut; in starving larvae survival was poorer in the later stages of starvation.

When skin was removed in larvae of all 3 species the mortality depended on the area of the lesion, the maximum area tolerated increasing with larval size. In sea water the threshold area was 0.1-0.2 mm^2 for 6-8 mm long plaice, < 0.3 mm^2 for 10-13 mm long

herring and 0.3–0.4 mm^2 for 14–17 mm long herring. In river water the threshold was 1–2 mm^2 for 19–21 mm long salmon and 6.5–8 mm^2 for 26–28 mm long salmon. The thresholds were about 1–3% of the total body surface area. Tolerance was increased in isosmotic salinities, the threshold area being as high as 10–14% of the body surface in 24–28 mm long salmon in 8‰.

Healing of skin lesions was observed *in vivo* and by histology, the main response being a mass migration of epidermal cells from the periphery of the lesion. Wound areas of 0.1–7 mm^2 closed in 4–12 h, the mean rates of cell migration being 40–110 µm/h at 10–11°C. The rate of migration was temperature dependent. The normal skin structure was restored with regeneration of a new basement membrane and dermis within 3 weeks. Older stage larvae showed an inflammatory response similar to adult fish.

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I INTRODUCTION

Fish larvae tend to be susceptible to injury because of their delicate nature, especially their thin integument, and it is not surprising that there are several reports of their sensitivity to injuries inflicted by predators and parasites. The survival of fish eggs and larvae appears to be important in determining year-class strength and recruitment to fish stocks and so the need for studies on the recovery of larvae from predatory strikes was indicated at a colloquium on larval fish mortality at La Jolla, California (see Hunter, 1976). The present study concerns wound healing and recovery from injury in larvae of herring (Clupea harengus L.), plaice (Pleuronectes platessa L.), and salmon (Salmo salar L.) and, to a lesser extent, the mechanical resistance of herring eggs.

It seems that many organisms can fatally injure fish larvae. In an early attempt to rear marine fish larvae, Garstang (1900) found that mortalities of larvae of the blenny (Blennius ocellaris) coincided with large numbers of harpacticoid copepods in the rearing tanks. In an experiment to see if the copepods would attack living larvae, he observed harpacticoids (Idya furcata) attacking healthy young gobies (Gobius minutus) and browsing on the fin membrane, and one of the gobies was dead the next day. Davis (1959), on noticing that dead fish fry taken in plankton samples had several cyclopoid copepods clinging to them found that in the laboratory the cyclopoid Mesocyclops edax bit and damaged fry of the rockbass (Ambloplites rupestris). After about an hour of harassment, the fry showed signs of exhaustion. Lakshmanan (1969) also showed that Mesocyclops sp.

caused high mortality of early carp fry. In one experiment about 200 of these copepods were offered as food to 10 fry of 5 - 7 mm in length. Within 3 min one fry had been fatally wounded by a copepod which had become attached near the head, and within 4 h all 10 fry had been devoured.

Rosenthal (1967) also described the harmful effects of copepods on fish larvae. Reared herring larvae fed on wild plankton were attacked by two copepod species which occurred in the plankton: the copepodite stage of the parasitic copepod Lernaeocera sp. and adult Caligus rapax. The larval lernaeocerids found their host within 10 min. They moved around over the whole body surface of the herring and eventually attached themselves and began to feed, usually in the region of the anus. Within 2 h of the attachment of a copepod, the swimming of the herring was adversely affected and it ceased to feed. A few hours later the infested larva died and the copepod moved off in search of a new host. Attacks by Caligus rapax were not as serious and did not seem to be fatal. Kabata (1970), as well as dealing with the damage caused by parasitic crustaceans to adult fish, reviewed the harmful effects of free-living copepods on fish larvae, stressing the abundance of crustaceans in the upper layers of water where fish larvae are found in greatest numbers. In a more experimental study of predation by pontellid marine copepods (Labidocera trispinosa, L. jollae and Pontellopsis occidentalis) on anchovy (Engraulis mordax) larvae, Lillelund and Lasker (1971) observed that the copepods did not always ingest larvae which they captured, but often dropped them after inflicting a wound which was always fatal.

Novotny and Mahnken (1971) reported that juvenile pink salmon (Oncorhynchus gorbuscha) were attacked by a marine isopod (Rocinella belliceps pugettensis) which was offered as food. The isopods inflicted small but deep wounds which penetrated the body wall and the young salmon fell to the bottom where they died within minutes. Similar fatal attacks were observed in the wild at night. Westernhagen and Rosenthal (1976) found that in the laboratory yolk sac larvae of the Pacific herring (Clupea pallasi) were eaten by the hyperiid amphipod Hyperoche medusarum. Frequently the amphipods grasped the larvae for a few seconds only and then released them; larvae never survived such attacks.

It seems then that wounds inflicted by predatory organisms may be an important cause of larval fish mortality, especially in the sea. The mechanical resistance of fish eggs has been studied for several species (see Pommeranz, 1974), but no experimental study has been made previously to assess the resistance of larvae to traumatic damage.

In addition to predation, starvation of larvae at sea due to a scarcity of suitable food organisms after yolk resorption is widely considered to be a major cause of larval mortality, and it was suggested at La Jolla that starvation and predation may interact (see Hunter, 1976). Starvation lowers the resistance of young fish to various deleterious environmental factors including toxic substances such as phenol, pH, low O_2 tension, parasitic and fungal infections and predation (Ivlev, 1961). It seems likely that starvation may also increase the susceptibility of delicate larvae to damage.

Fish skin, as well as providing some degree of mechanical protection, assists in maintaining the osmotic concentration of the body fluids at a relatively stable level by means of its low permeability

to salts and water. Damage to the skin may allow an uncontrolled flow of salts and water across the skin, which can often result in an osmotic imbalance (Parry, 1966). Mortalities associated with the handling of fish have often been attributed to osmotic disturbance caused by skin damage (Parrish, Blaxter and Holliday, 1958; Holliday and Blaxter, 1961; Potts and Parry, 1964; Lewis, 1971) and similar changes in the osmotic concentration of the body fluids of fish with bacterial or fungal infections are also attributed to surface damage associated with the infections (Gardner, 1974; Mulcahy, 1975). It has been shown that mortality of damaged fish can be reduced if the fish are held in water of a salinity which reduces the osmotic difference between the internal body fluids and the external medium (Parrish et al., 1958; Collins and Hulsey, 1963; Lewis, 1971; Wedemeyer, 1972). Holliday and Blaxter (1961) noted large increases in the osmotic concentration, judged by freezing point depression, of the blood of badly descaled herring before the fish died and they suggested that fish damaged in tagging experiments should be held for at least a few hours in water of less than 15.8‰ (the salinity isosmotic with the blood) before being returned to the sea. It seems that nothing is known about the effect of salinity on the survival of damaged larvae. For people concerned with fish rearing, such knowledge seems desirable since larvae may be subject to various harmful mechanical stimuli under rearing conditions.

It has been shown that in adult teleosts skin lesions are covered rapidly by epidermis (see Mittal and Munshi, 1974; Anderson and Roberts, 1975; Laird, Roberts, Shearer and McArdle, 1975; Phromsuthirak, 1977), and that mature fish have a highly evolved system of defence involving the infiltration of lesions by blood-derived phagocytic

macrophages and polymorphonuclear leucocytes and by lymphocytes (see also Finn, 1970; Finn and Nielsen, 1971a, b; Roberts, Mac Queen, Shearer and Young, 1973; Ellis, Munro and Roberts, 1976; Phromsuthirak, 1977). Very little is known, however, about the healing mechanisms of fish larvae.

Nusbaum and Sidoriak (1900) studied the regeneration of the posterior portion of the body in newly-hatched trout (Salmo fario) alevins, and found that within 48 h at 1 - 2°C the notochord which protruded by 2 - 3 mm was covered by epidermis 5 - 12 cells thick. Closure of the wound was brought about by the migration of cells from the adjacent epithelium rather than by cell division. The posterior part of the body could regenerate in 10 weeks even when cuts were made anterior to the anus. Prazdnikov and Mikhailova (1966) showed that in humpback salmon (Oncorhynchus gorbuscha) embryos the first response to the introduction of threads soaked with carmine and bacteria was phagocytosis by cells of the periosteum, epithelium and endomysium. Blood cells were not important in the early stages of the response but phagocytosis by polymorphonuclear leucocytes was observed after the third day. No work at all has been published on wound healing or regeneration in herring or plaice larvae.

The present study was made to investigate the resistance of herring embryos, and herring, plaice, and salmon larvae to damage, especially damage to the skin, and to examine some of the cellular defensive mechanisms of larvae which may be important for their survival. The term "larva" is used here to refer to all stages from hatching up to and including metamorphosis in herring and plaice, and for the period from hatching to yolk resorption in salmon, i.e.,

the alevin stage (see Blaxter, 1969). Herring and plaice larvae, two phylogenetically distinct marine species, are especially interesting because the early larvae are particularly fragile and vulnerable to predator attack. They hatch at a very embryonic stage of development with a thin delicate epithelium and a poorly developed circulatory system, the blood being colourless until metamorphosis. Salmon, on the other hand, hatch at a more advanced stage of development, in freshwater. The newly-hatched alevins are considerably larger than newly-hatched herring and plaice and they have a relatively tough, thicker skin. The blood pigments are present in salmon for some time before hatching and at hatching most of the cellular elements of blood are already present, including monocytes and polymorphonuclear leucocytes.

The mechanical resistance of herring eggs to pressure and the recovery of the embryo after deformation was examined first. Observations were also made on the attacks of some zooplanktonic organisms on herring and plaice larvae but most of the study concerns the responses of larvae to experimentally inflicted lesions. The survival of both feeding and starving early herring larvae after various types of injury was examined. An attempt was also made to assess how much skin damage herring, plaice, and salmon larvae could withstand, and how important it was for their survival for damaged larvae to be maintained in a salinity isosmotic with their body fluids. The restoration of epithelial continuity after damage would seem to be one of the most important aspects of wound healing for larvae in a hypo- or hyper-osmotic environment and so the rate and mechanism of wound closure was investigated in some detail. In addition, the ability of herring and plaice larvae to mount a cellular inflammatory response

to injury was investigated in relation to the immaturity of their blood system. The powers of regeneration in early herring larvae were also examined by making some observations on the regeneration of the caudal fin.

II REARING OF LARVAE

A Source and incubation of eggs

1. Herring.

Eggs were obtained from spring-spawning herring captured by trammel net fishermen on the Ballantrae Bank, in the Firth of Clyde in February 1974 - 1977 and from autumn-spawning herring captured off Douglas, Isle of Man in September 1975. Gonads, dissected from male and female fish immediately after capture, were placed individually in dry glass jars and stored on ice in vacuum flasks for transport to the Dunstaffnage Laboratory.

The eggs were fertilised at the laboratory about 14 - 16 h later and were hatched and the larvae reared using the methods of Blaxter (1968). To fertilise the eggs, ground glass or glass plates of 20 x 20 cm, 10 x 20 cm and 27 x 27 cm were placed on the bottom of shallow, plastic containers and covered with sea water to a depth of about 15 cm. Groups of eggs were removed from an ovary on the tip of a dry scalpel and dispersed onto the plates by shaking the scalpel in sea water. When the eggs were evenly distributed but not overcrowded the plates were transferred to a suspension of milt from several males for 15 - 30 min, then rinsed in clean sea water and arranged vertically around the sides of 200 l or 500 l round, black plastic tanks with a slow circulation of sea water. Plates were transferred to clean sea water every second day. The eggs were incubated in darkness by covering the tanks with black plastic sheeting. During incubation the water temperature was 7 - 9^{o}C in the spring and 13.5 - 14.5^{o}C in the autumn in the main aquarium (see Fig. 1), and 9 - 11^{o}C in the autumn in a constant temperature room. The time from

Fig. 1

Mean weekly water temperature during rearing of herring and plaice larvae.

In aquarium:

O, 1974 herring and plaice

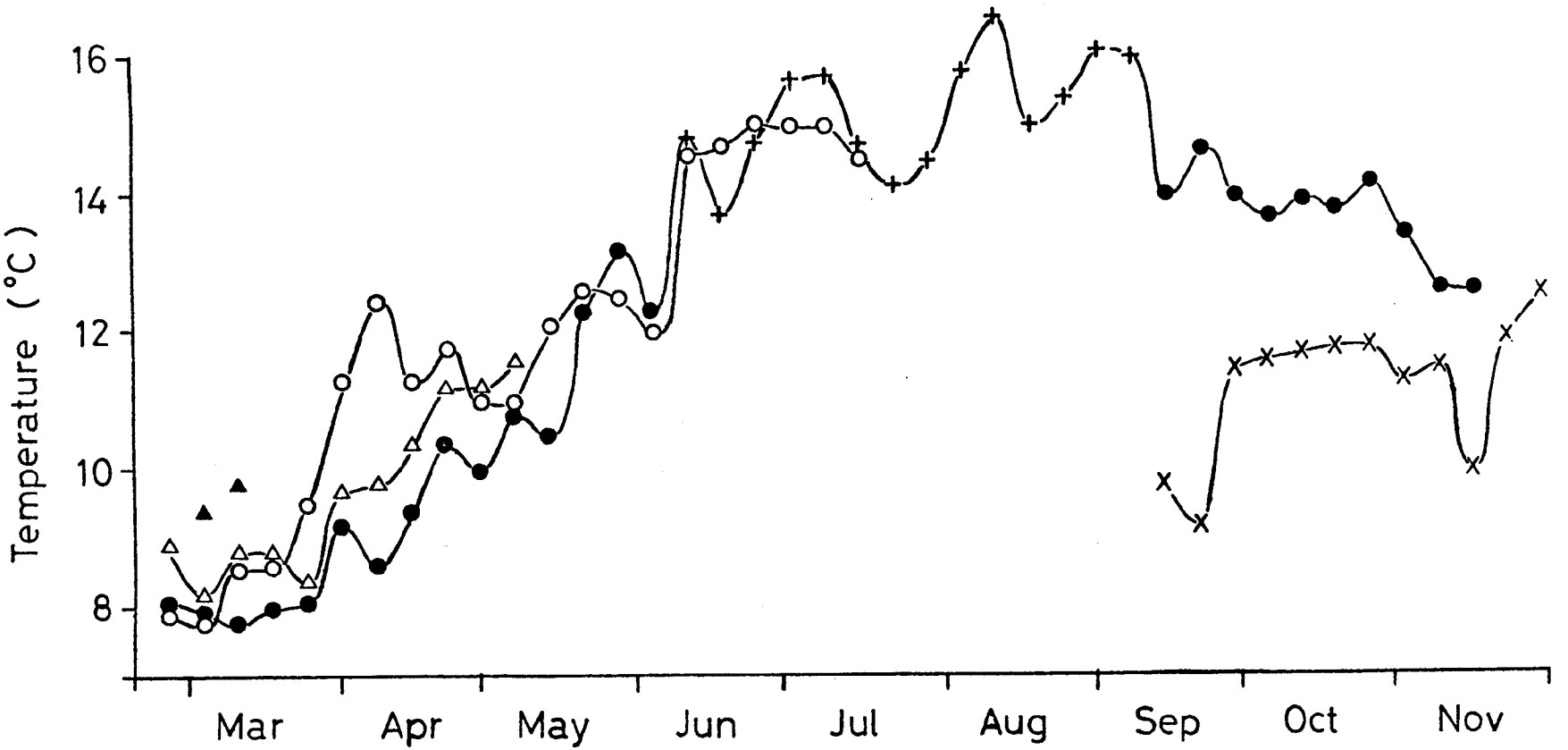
●, 1975 spring herring and early plaice, and autumn herring and late plaice

+, 1975 late plaice only

Δ, 1976 herring and plaice

▲, 1977 herring eggs

X, 1975 autumn herring


Temperature (°C)
16
14
12
10
8
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov

fertilisation to first hatching was 12 - 13 days in the spring and 8 days in the autumn in the aquarium, and 12 days in the autumn in the constant temperature room.

2. Plaice.

Plaice larvae were hatched and reared using methods described by Shelbourne (1964). Pond-spawned, naturally fertilised eggs were obtained from the Department of Marine Biology, University of Liverpool, Port Erin, Isle of Man, and artificially fertilised eggs from the White Fish Authority at Artoe, Ardnamurchan in April 1974 - 1976. The eggs, floating in sea water, were transported to Dunstaffnage in vacuum flasks some days after fertilisation.

At the laboratory, the eggs were transferred to standing sea water in 20 l black plastic tanks. Where necessary the salinity of the laboratory sea water was raised to 34‰ by the addition of sodium chloride, thereby ensuring that the eggs would float at the surface. Eggs were maintained until hatching in standing sea water containing 50 IU/ml of crystalline sodium penicillin (Glaxo) with 0.5 mg/ml streptomycin sulphate (Glaxo). The water temperature was 9 - 12°C. The time to hatching depended on the stage of development of the eggs on arrival at Dunstaffnage, but was around 15 days from fertilisation.

3. Salmon.

Salmon eggs were not incubated at the Dunstaffnage laboratory because of the danger of high copper levels in the freshwater supply at that laboratory. In 1975 the salmon were reared at a small hatchery south of Oban belonging to Mr. David Kilpatrick and in 1976 they

were reared by Mr. L.B. Servant in the Awe Fishery Board hatchery at Inverawe, 12 miles from the laboratory. The rearing methods used by Mr. Servant are typical and will be described here.

Eggs were fertilised in November by stripping eggs and sperm from ripe salmon into a plastic basin. The eggs were washed and hardened in water and then placed in meshed plastic trays held within rigid trays 56 cm in diameter. The outer trays were stacked eleven high. River water was fed into the top tray and then flowed through each tray to the bottom at a rate of about 45 - 70 l/min. During incubation eggs were treated with malachite green and dead eggs were removed frequently to prevent fungal infection. The hatchery was kept in darkness throughout development of the eggs and alevins. The water temperature during incubation of the eggs was 4 - 9^{o}C. Hatching began in March, some four months after fertilisation.

When required eggs and alevins were brought to Dunstaffnage in 2 l plastic containers of river water. River water was brought from the hatchery, or from streams near the laboratory, in 20 l plastic carboys. At the laboratory the eggs were maintained in a constant temperature room at 6.5^{o}C in 2 l or 5 l containers of river water with continuous aeration. They were transferred to clean water every second day.

B Rearing of larvae

1. Herring and plaice.

After hatching herring larvae were maintained in black plastic tanks of 200 l or 500 l capacity with a sea water flow rate of 0.2 - 0.4 l/min. Plaice larvae were held in similar tanks of 20 l

or 200 l with flow rates of about 0.1 l/min and 0.2 l/min respectively. Before sea water was distributed to the rearing tanks it was pumped into a 500 l header tank where it was slightly heated and aerated vigorously to minimise supersaturation. This aeration greatly reduced mortality of herring larvae associated with bubbles of gas in their gut.

In all tanks the outflow pipe was protected by a screen of plankton netting or coarser gauze to prevent loss of larvae. Dead larvae and debris were removed by siphoning regularly, using glass tubing with a narrow bore to avoid siphoning living larvae. The bacterial film on the surface of the tanks was skimmed off with a beaker each day and care was taken to keep tanks free of potential predators such as hydroids, small medusae, and mysids. The temperature in each herring and plaice tank was recorded daily (see Fig. 1). Illumination to the tanks was provided by two 80 W fluorescent tubes with a diffuser, situated 1.5 m above the tanks. A time switch turned the lights on and off at dawn and dusk. Some 2 months after hatching, when the herring were approaching metamorphosis, they were transferred to a translucent fibreglass tank, the sides of which were covered with black plastic sheeting. This tank, of 3000 l capacity, was situated in the main aquarium where it received natural daylight.

Spring herring larvae were first provided with food about 6 days after hatching. They were offered barnacle (Balanus balanoides) nauplii, obtained from ripe barnacles on the shore, and natural plankton which was sieved through a 400 μm screen. About a week later they were provided with nauplii of the brine-shrimp (Artemia salina). Barnacle nauplii were provided until the adults had finished spawning in mid-April. Autumn herring larvae were first offered rotifers

(Brachionus plicatilis) about 2 days after hatching. Later they were fed on Artemia nauplii as well as rotifers. In 1976 the nutritional value of the Artemia nauplii was improved by feeding the nauplii for 24 h after harvesting, on the commercially available dried green alga, "Spirulina", and on reconstituted dried yeast. These ongrown nauplii were fed to the herring larvae about three weeks after first feeding. As the herring approached metamorphosis they were also offered the copepod Tigriopus sp., which was collected from rock pools.

Plaice larvae were first offered Artemia nauplii about 2 days after hatching and were fed up to metamorphosis on Artemia nauplii only.

2. Salmon.

After hatching salmon alevins were kept at the hatchery for 4 - 6 weeks in the same trays and under similar conditions as the eggs had been. The water temperature was 6 - 10°C.

Alevins brought to the laboratory were held in river water in plastic tubs of 2 l, 5 l or 20 l with continuous aeration, in a constant temperature room at around 6.5°C or 10 - 11°C. They were transferred to clean water every second day to avoid accumulation of toxic wastes.

Some six weeks after hatching, before the yolk was fully resorbed, alevins were first provided with food. They were offered Artemia nauplii, dried food pellets and mysids. After first feeding some salmon were held in tanks with a circulation of laboratory tap water and they survived well.

III THE EFFECT OF DAMAGE ON SURVIVAL OF EMBRYOS AND LARVAE

This section deals with the resistance of herring eggs to mechanical pressure, predator attacks on herring and plaice larvae, recovery of feeding and starving herring larvae after various experimental lesions, and finally the effect of lesion area and isosmotic salinity on survival of herring, plaice, and salmon larvae of different ages.

A Methods

1. Resistance of herring eggs to mechanical pressure.

Mechanical resistance was measured by the force required to burst the chorion and vitelline membrane of herring eggs at intervals from before fertilisation to hatching. In 1974 only resistance of the chorion was measured and so further experiments were carried out in 1977 to measure resistance of the vitelline membrane and to make more frequent observations on chorion resistance in the early stages of development. Eggs for experiments were incubated on glass microscope slides. They were gently prised from the slide using tissue paper and placed individually between two glass slides hinged at one end by adhesive tape. The hinged slides were put on the pan of a Mettler E1000 balance and the ball point of a "biro" pen attached to a Palmer stand was wound down on the glass to apply increasing pressure to the egg. During this process the egg was observed under a dissecting microscope to record at what load the vitelline membrane burst. The pointer of the balance jerked back when the chorion burst. In 1977 experiments, the diameter of the egg at bursting of the chorion and of the vitelline membrane was measured so that the area of the egg in

contact with the glass, and from that the applied pressure, could be calculated. Resistance was measured for 20 eggs at each stage of development. The maximum load recordable on the balance was 1 kg.

To investigate the effect of deformation on survival of the embryo, eggs were deformed but not burst. The initial diameter of the egg was measured using an eyepiece graticule and pressure was applied until the diameter of the egg as seen from above was either 33% or 50% greater than the initial diameter. The load necessary to produce these deformations was recorded from the balance. In 1974 eggs were not deformed by 50% until 3 days after fertilisation and so additional experiments were made in 1977 to investigate survival after 50% deformation and also after the vitelline membrane was burst at 21 h, 54 h and 3 days after fertilisation. Survival after the chorion was pierced with a needle at 54 h after fertilisation was also examined. Eggs were deformed in groups of 10 and then incubated in sea water in 250 ml glass beakers. The number of viable eggs was counted 24 h after deformation and daily thereafter until hatching. Non-viable eggs were white and opaque, whereas viable eggs were translucent.

2. Recovery of larvae from injury.

For experiments, herring, salmon, and early stage plaice larvae were caught from the rearing tanks using a wide-mouthed pipette with a tip rounded by heating in a flame. To take out plaice after they had settled on the bottom of the tank, they were driven towards the surface of the water by means of a net and then removed from the tank in a glass beaker. All experiments were carried out in a constant temperature room at 10 - 11^{o}C. Herring and early stage plaice larvae

were anaesthetised in a 1 : 20,000 solution of methane tricaine sulphonate (MS-222; Sandoz Ltd.) and salmon and later plaice stages in a 1 : 15,000 solution of MS-222. All observations and measurements were made with the aid of a Wild M5 dissecting microscope. Small larvae were measured using an eyepiece graticule, and larvae greater than about 10 mm in length were measured against a graduated scale (graph paper) placed beneath the glass dish containing the larvae. The notochord length of early herring and plaice larvae was measured rather than the total length, because the tip of the caudal fin was difficult to see. The total length of salmon alevins and of later herring and plaice was measured. The stage of development of herring and plaice larvae (based on external morphology) was also noted in some cases. The staging system of Ryland (1966) was used for plaice (see Appendix 1) and that of Doyle (1977) for herring (see Appendix 2). Length or stage of development, rather than age, was always used to group the larvae for experiments, because size and developmental hierarchies occur in reared plaice and herring populations due to variations in the growth rate of individuals (see Doyle, 1977).

Operations on larvae were performed in a groove cut out of 3% agar in a petri dish, the groove serving to immobilise the larvae during an operation. Salmon were also immobilised by removing the MS-222 solution from the petri dish and "stranding" the alevin. Herring and plaice, however, especially the early stage larvae, were kept submerged at all times because if they came out of water for even a short time the fins became frayed and such larvae usually died. Larvae were manipulated in the operating dish and held in position using loops of baby's hair inserted into the tip of a fine glass tube and held in position with wax (Rugh, 1962). The instruments used to produce wounds were tungsten needles, a borrodaile knife, watchmaker's

forceps and a pair of scissors all with finely ground points. A scalpel was also used for the oldest larvae. Tungsten needles were made by inserting a length of tungsten wire of about 10 - 15 mm into a glass rod and dipping the tungsten into boiling sodium nitrite until a fine point was obtained.

The larvae were transferred after wounding to a container of clean medium without MS-222. Older larvae were resuscitated by agitating the water to cause a flow past the gills. Early stage herring and plaice larvae were maintained post-operatively in 1 - 4 l glass beakers or crystallising dishes, depending on the size and number of the larvae in the group. The walls of the containers were surrounded by black plastic to provide contrast for feeding. Plaice at metamorphosis and salmon alevins were kept in 2 l or 5 l white plastic tubs, and the salmon tubs were aerated continuously. Salmon alevins maintained post-operatively for more than 24 h were transferred to clean water each day. A darkening in colour of the alevins indicated that they became less healthy if not changed regularly. Herring, plaice, and salmon larvae were fed on Artemia nauplii.

In each experiment control larvae were anaesthetised, handled and measured in the same way as wounded larvae, but were not wounded. They were then maintained under the same conditions as the wounded larvae. Survival of wounded and control larvae was recorded by counting the number of dead and moribund larvae after 24 or 48 h in early experiments and each day for up to 10 days in later experiments. Herring and plaice larvae were considered moribund as soon as the brain became white as larvae never survived once this condition was manifest. It was difficult to decide when salmon alevins were moribund, so they were considered to have survived until the heart stopped beating.

(i) Predator attacks on herring and plaice larvae.

The observations made on predatory attacks were of a very preliminary nature. Daily plankton samples taken outside Dunstaffnage Bay, and some samples from Loch Sween, were examined for potential predators of larvae. These organisms were then isolated in beakers of sea water with early herring or plaice larvae to see if they would attack the larvae. The potential predators tested were medusae of several species including Aurelia aurita, Tiaropsis multiserrata, Sarsia tubulosa, Rathkea octopunctata and Bougainvillea sp. The megalopa larva of the Dublin Bay prawn Nephrops norvegicus, the copepod Euchaeta norvegica, mysids which entered the larval rearing tanks with the sea water, and hydroids (Sarsia sp.) which tended to become established on the walls of the rearing tanks were also tested. In some cases larvae were removed from the beaker after they had been attacked and their survival in sea water was recorded.

In one experiment 10 first feeding herring larvae of 10 - 14 mm long were anaesthetised and individually presented under microscopic observation to an Aurelia ephyra of 10.4 mm in diameter. The ephyra immediately grasped the larva in its manubrium. After 30 sec each larva was rescued and the number of larvae surviving in sea water after 48 h was recorded.

(ii) Recovery of herring larvae from various lesions.

In all experiments described in this section, larvae were wounded and maintained in sea water. The experiments were confined to yolk sac stage and first feeding herring larvae. Lillelund and Lasker (1971) observed that anchovy larvae always died if their delicate skin was punctured by a copepod. To test if herring larvae were

equally susceptible, feeding larvae of 8.8 - 10.4 mm in length were wounded by piercing the skin with a tungsten needle with a tip of 20 µm. The wound was made on the left side of the body dorsal to the anus, taking care to just penetrate to the muscle and not to puncture the notochord. Ten larvae were wounded and 10 larvae used as controls. Survival of both groups was recorded 24 h after wounding.

The effect of a wound produced by suction was also investigated. The apparatus used to produce negative pressure consisted of a micropipette connected to a reservoir of water. The micropipette was made by drawing out catheter tubing over a flame to produce a tip 0.26 mm in diameter. Using a microscope and a micromanipulator the pipette was brought up to the skin of the larva, and negative pressure of 40 cm of water applied to the skin for 30 sec. The skin was sucked into the pipette and a standard lesion of 0.26 mm in diameter was inflicted. Ten feeding larvae of 10.8 - 12.8 mm in length were wounded and survival was recorded after 24 h.

Lillelund and Lasker (1971) found that copepods were attracted by the tail beat of anchovy larvae and Westernhagen and Rosenthal (1976) observed that the amphipod Hyperoche medusarum grasped the tail of herring larvae more often than any other part of the body. My observations also showed that the megalopa larva of Nephrops norvegicus frequently grasped and chewed the tail of herring larvae, so it seemed desirable to investigate experimentally the chances of larvae surviving after damage to the tail. Yolk sac herring larvae of 8.2 - 9.6 mm in length were wounded in sea water by cutting off a 1 mm, 2 mm or 3 mm length from the posterior end of the notochord.

Groups of 10 larvae were wounded and survival of the larvae was recorded each day for 6 days.

(iii) The effect of starvation on recovery of damaged herring larvae.

Blaxter and Hempel (1963) introduced and defined the term "the point-of-no-return" (PNR) as the point at which 50% of the starving larvae in a population become too weak to feed even if suitable food becomes available, the larvae having then reached a point of irreversible starvation. In a preliminary experiment to examine the effect of damage on survival of starving larvae, the caudal fin was amputated from 20 non-feeding larvae of 10.5 - 12 mm in length, after yolk resorption. The starving larvae were selected from the stock tanks judged by the lack of food in their gut, the concavity of their heads and a tendency to hang head down in the water, the latter two criteria indicating that they were starved to, or nearly to, the point-of-no-return. Survival of the wounded larvae was recorded each day for 7 days.

In a second experiment, recovery from injury of herring larvae starved for different numbers of days after yolk resorption was compared with that of yolk sac and first feeding larvae. About 200 larvae were hatched in a 20 l tank in a constant temperature room at 10 - 11^{o}C. Six days after hatching, half of the larvae were transferred to a second 20 l tub. Larvae in one tank were fed and larvae in the second tank were not. Twenty yolk sac larvae were wounded at 6 and 9 days from hatching by making an incision of about 0.3 mm long through the body just posterior to the yolk sac and ventral to the notochord. After yolk resorption 20 feeding and 20 non-feeding larvae were wounded in a similar way at 3-day intervals until the starving larvae had reached PNR. Survival was recorded after 24, 48 and 72 h.

(iv) The effect of lesion area and isosmotic salinity on survival of herring, plaice, and salmon larvae.

Tolerance to skin damage was assessed by measuring the area of lesion which larvae could withstand. Yolk sac herring larvae of 10.5 - 12.5 mm and stage 2 larvae of 14 - 16.5 mm in length were used in experiments. Mortality of plaice larvae was high in the stock tanks at the time of experiments and so only yolk sac larvae of 6 - 8 mm and plaice at metamorphosis of 10.5 - 20 mm in length were used. Newly-hatched salmon alevins of 19 - 21 mm and alevins of 24 - 27.5 mm, some 6 weeks after hatching, were also studied.

Examples of the site of skin lesions in herring, plaice, and salmon larvae are shown in Fig. 2. A wound of a given area usually caused more severe damage in early herring and plaice larvae because it was difficult to cut out an area of the delicate integument without damaging a good deal of the underlying muscle, and sometimes even the notochord. On the other hand, the thicker skin of salmon and older plaice could be removed causing only superficial damage to the muscle. Different areas of skin were excised from larvae in groups of 10 usually, but in groups of 5 for the larger wounds which were difficult to produce. The length and breadth of each wound was measured with an ocular micrometer. The median wound area was calculated for each group of larvae.

The osmotic concentration of the body fluids of herring and plaice larvae is equivalent to 12.5‰ NaCl (Holliday and Blaxter, 1960) and that of salmonids equivalent to 7.5 - 9.5‰ NaCl (Parry, 1961; Rao, 1969). To test the effect of isosmotic salinity on survival after skin damage, herring and plaice larvae were wounded and maintained in full-strength sea water of 28 - 32‰, or in a salinity of 12.5‰ made by

Fig. 2

Yolk sac herring and plaice larvae and a newly-hatched salmon alevin showing the maximum area from which skin was removed in isosmotic salinity. Smaller wounds were the same width but shorter in length. Similar sites were wounded in older larvae.

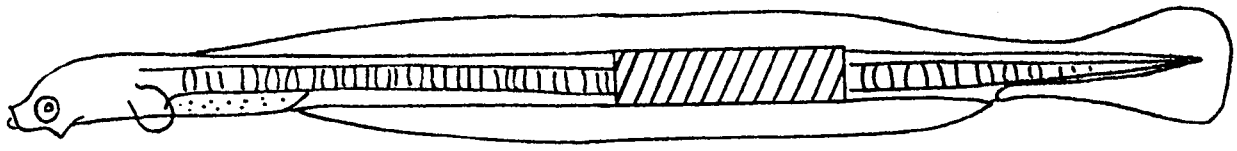
 , area from which skin was removed

A. Herring of 10.5 mm, showing a wound of 1 mm^2 (0.5 x 2 mm)

B. Plaice of 7 mm, showing a wound of 0.75 mm^2 (0.3 x 24 mm)

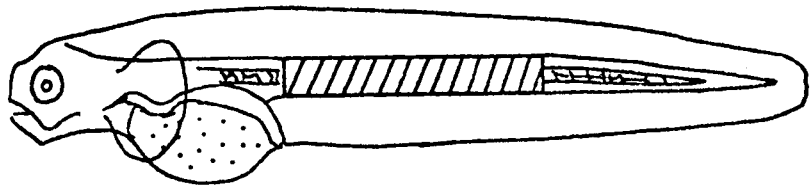
C. Salmon of 19 mm, showing a wound of 12.5 mm^2 (10 x 1.25 mm)

A. Herring



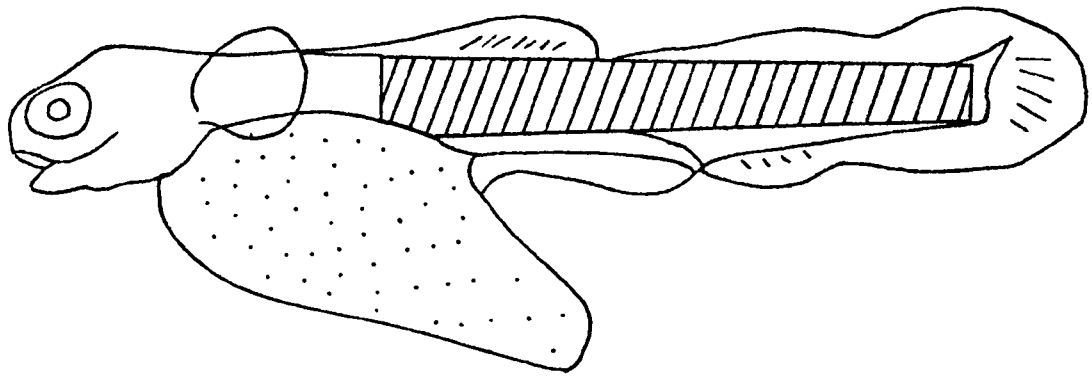
1mm

B. Plaice



1mm

C. Salmon



2mm

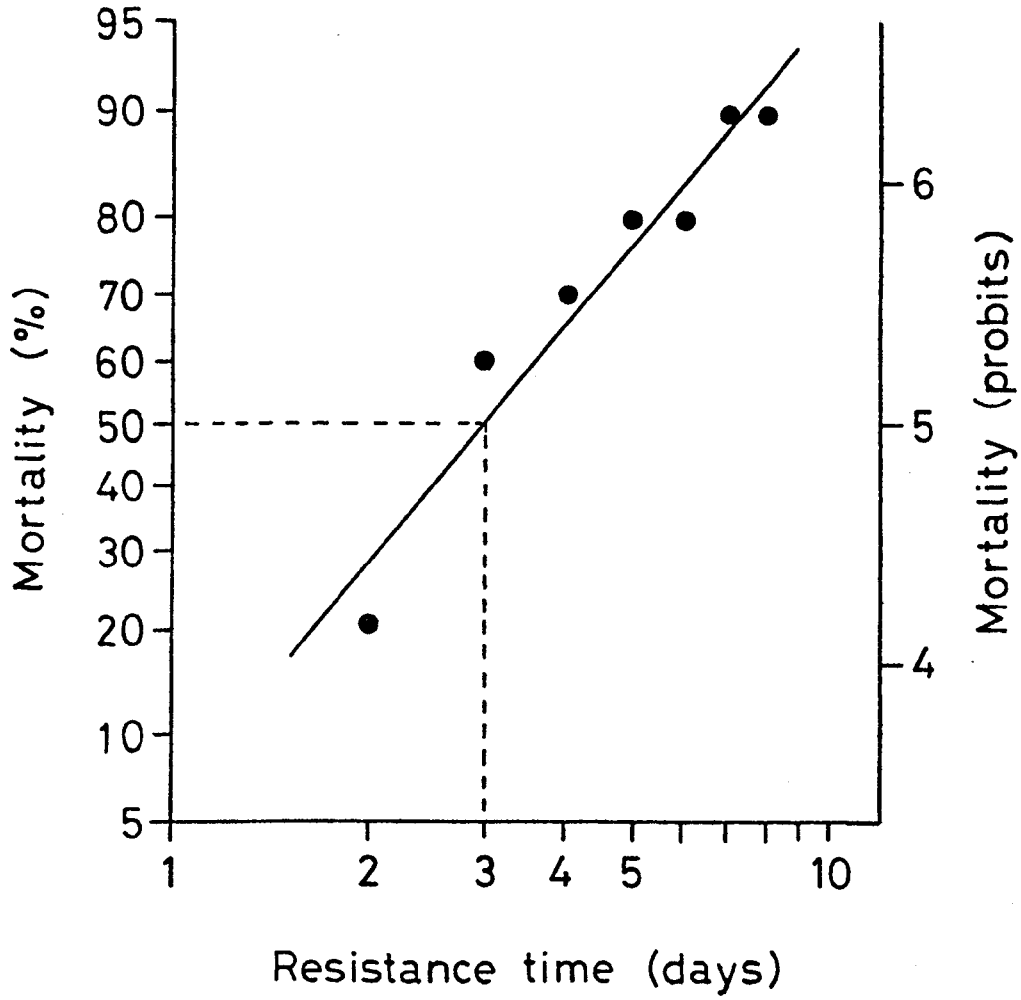
diluting sea water with distilled water. Salmon alevins were wounded and held in river water, or in a salinity of 8‰ made by adding sea water to river water. Generally, wounded and control larvae were held in the experimental salinity for at least 10 days, and the number of dead and moribund larvae was counted each day. In one experiment, 5 salmon alevins of 27 - 27.5 mm long were wounded in a salinity of 8‰ and held in that salinity for 24 h. They were then transferred to river water and survival was recorded daily for 5 days.

(a) Treatment of data: The time to 50% mortality (median resistance time) after each wound area was estimated using the method of Litchfield (1949). In many time-mortality experiments the distribution of tolerances is approximately normal if time is transformed into logarithms, so that when cumulative percentage mortality is plotted against the logarithm of time the resulting curve will take on a sigmoid shape typical of a cumulative normal distribution (see Shepard, 1955; Finney, 1971). This curve can be converted to a linear form by converting percentage mortalities to their probits (units of standard deviation above and below the mean with a value of 5 added to avoid using negative numbers). Cumulative percentage mortality of larvae after a wound of a given area was plotted against time on logarithmic-probability paper and a regression line fitted to the points by eye. The estimated time to 50% mortality was obtained by interpolation. An example is shown in Fig. 3.

To show the general pattern of survival after different lesion areas median resistance time was plotted against wound area for larvae of different species and ages and in different salinities. The area of wound which causes 50% mortality (the median lethal wound area) would be a suitable measure of the tolerance of larvae to damage (see

Fig. 3

Log-probit plot to estimate the median resistance time or time to 50% mortality (ET_{50}) for yolk sac herring larvae in 12.5‰ salinity following a lesion of 1.1 mm^2 (see Fig. 11). Cumulative percentage mortality was plotted on a probit scale against survival time on a logarithmic scale and a regression line was fitted to the points by eye. The ET_{50} obtained by interpolation was 3 days.


95
90
80
70
60
50
40
30
20
10
5
Mortality (%)
6
5
4
Mortality (probits)
1
2
3
4
5
10
Resistance time (days)

Finney, 1971) but the data were not suitable for estimation of this. Therefore for each group of larvae a range of areas is given between which the median lethal wound area lies. The lower limit of this range is the maximum area tested which caused less than 50% mortality in 10 days, and the upper limit is the minimum area tested which caused greater than 50% mortality in 10 days. In my experiments survival for 10 days is considered to indicate indefinite survival.

Wound areas which define the lethal threshold are also expressed as a percentage of the total body surface area. Total surface areas (including fins) of herring and yolk sac plaice larvae were estimated using the equations of de Silva (1974); $\log \hat{y} = 0.16 + 2.14 \log x$ for herring, and $\log \hat{y} = 0.12 + 2.58 \log x$ for plaice, where $\hat{y}$ is the total surface area and x is the length. The surface area of plaice longer than 15 mm was estimated by drawing the outline of the plaice on graph paper with the aid of a camera lucida attachment on a Wild M5 binocular microscope. The area of one side was doubled to give the total surface area. The body surface area of salmon alevins (excluding the fins) was calculated as being approximately equal to that of a cylinder, π d l, where d is the mean diameter and l the notochord length. The mean diameter of the body was calculated from measurements of depth and width at seven positions along the length of the body, with the part of the yolk sac attached to the body being included in these measurements. The surface area of the yolk sac not attached to the body was calculated separately, also as being approximately equal to a cylinder. The area of fins, measured on camera lucida drawings of the alevins, was added to the body and yolk sac surface area to give the total surface area of an alevin.

B Results

1. Resistance of herring eggs to mechanical pressure.

(i) Resistance of the chorion.

The chorion of unfertilised eggs was relatively delicate and could be burst by loads of 4 - 15 g (Fig. 4A). It had not hardened at all by 2 h after fertilisation before cell division had started, but by 5 h after fertilisation, at the 4 - 8 cell stage, resistance had increased dramatically and the toughest eggs could resist a force of over 1 kg before bursting (Fig. 4B). Eggs were remarkably flattened by the loads required to burst the chorion in its resistant stages, and the area in contact with the slide under a load of 1 kg was about 12 mm^2. The pressure applied to the egg by 1 kg was therefore 8.33 kg/cm^2. At 2 h after fertilisation the chorion was burst by pressures of about 0.29 kg/cm^2, with an area of about 4 mm^2 in contact with the slide. After the rapid hardening of the chorion in the first few hours after fertilisation, it remained highly resistant until just before hatching when it began to soften (Fig. 4A and B). Although some eggs just before hatching resisted less than 50 g, many of the eggs could still withstand forces of over 500 g. Even though eggs deformed between two slides showed high resistance throughout most of their development, the chorion of eggs at 54 h after fertilisation could be punctured quite easily with a sharp tungsten needle, the force required being only 1 - 2 g.

Apart from the anchovy egg, the chorion of which can resist forces of only up to 200 g (de Ciechomski, 1967), the eggs of other species previously studied can withstand forces as great as or greater than the herring eggs studied here. Galkina (1957) showed

Fig. 4 A

Load required to burst the chorion of 1974 herring eggs during development. Each point represents the median value of 20 measurements; the vertical lines give the range of values. The first point is for unfertilised eggs. One thousand g was the maximum load applied.

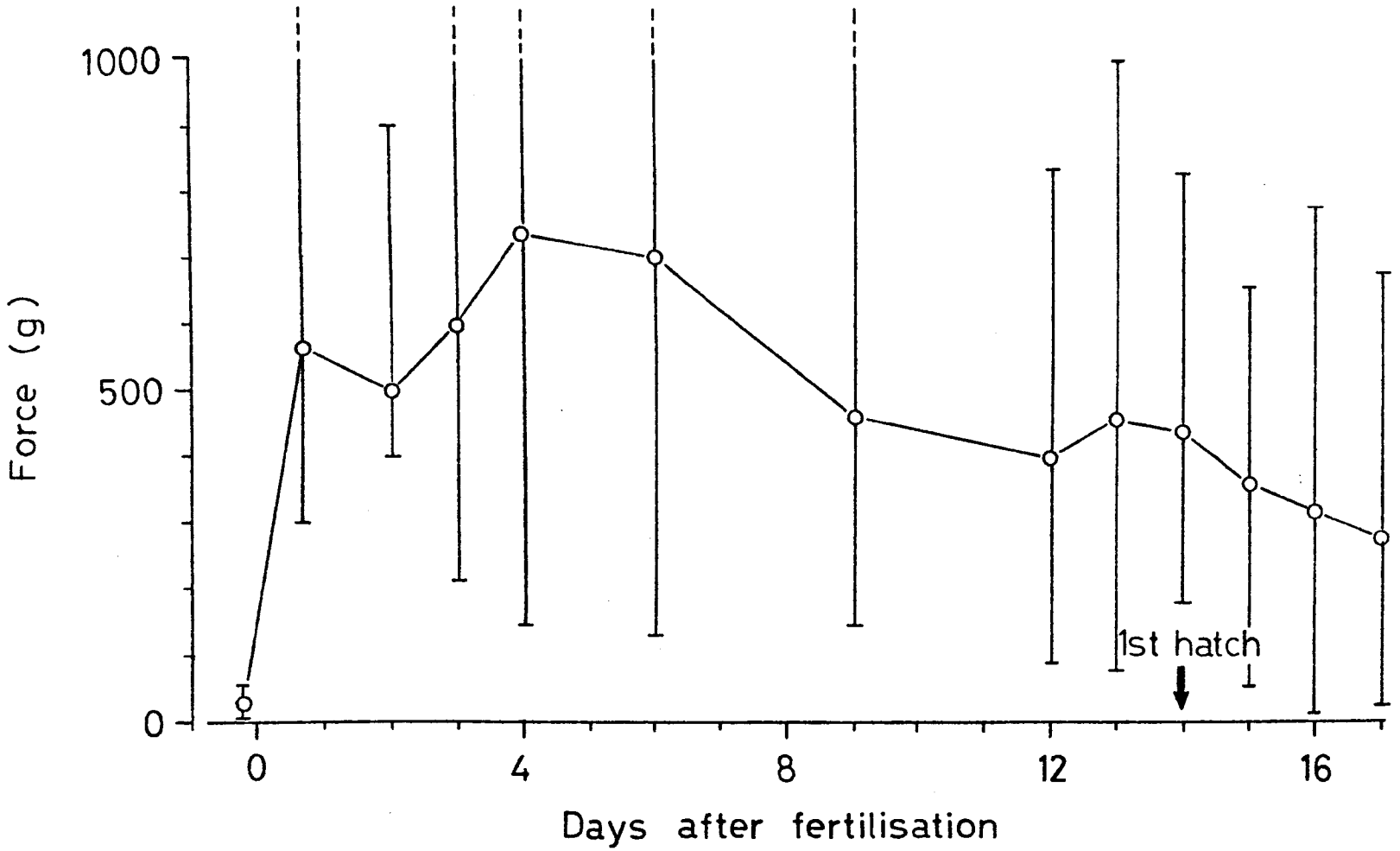
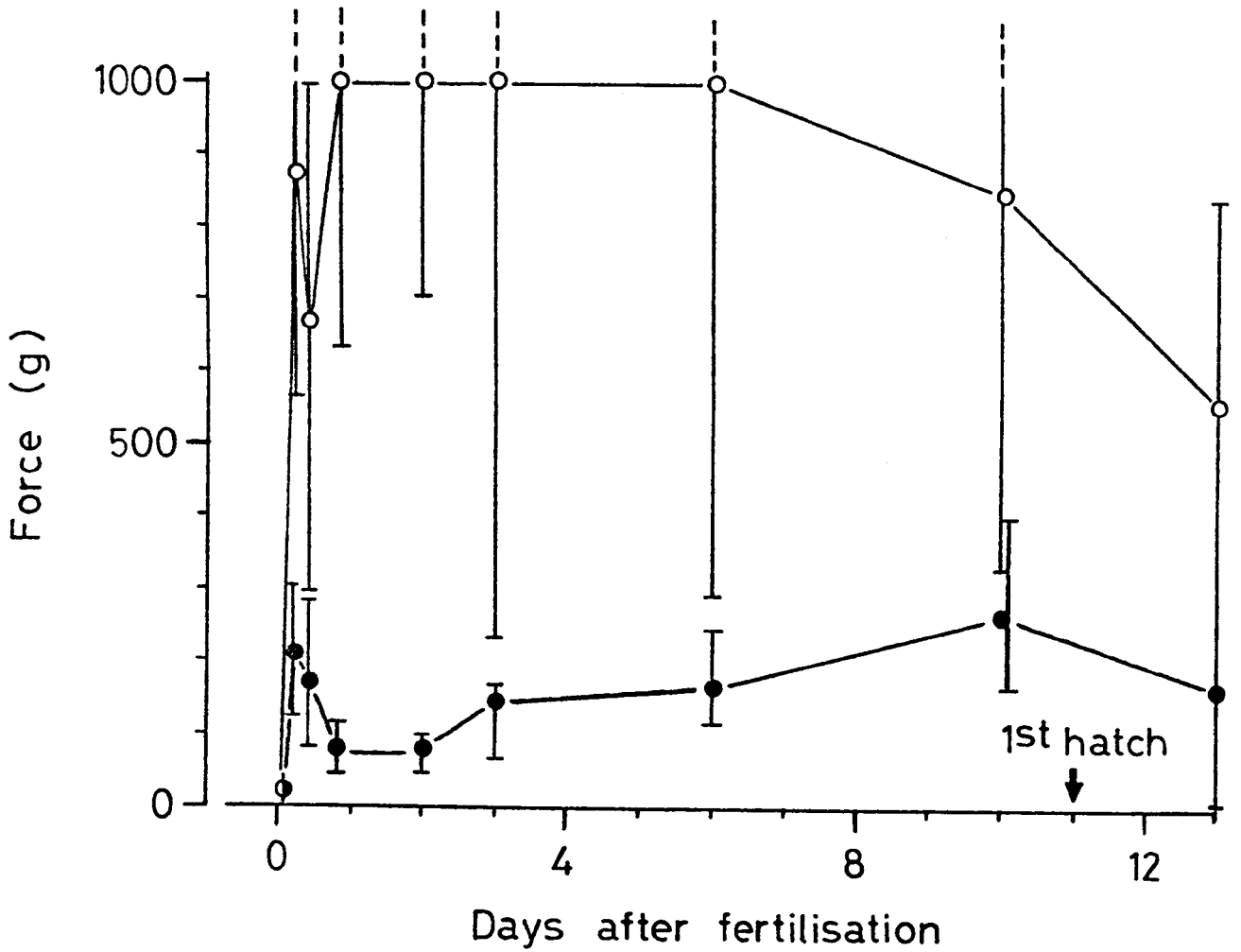

1000
500
0
Force (g)
0
4
8
12
16
Days after fertilisation
1st hatch

Fig. 4 B

Load required to burst the chorion and vitelline membrane of 1977 herring eggs during development. Each point represents the median value of 20 measurements; the vertical lines give the range of values.

O —— O, chorion

● —— ●, vitelline membrane


1000
500
0
Force (g)
0
4
8
12
Days after fertilisation
1st hatch

that the chorion of the Okhotsk herring could resist loads of up to 4 kg before bursting. Pommeranz (1974) found that plaice eggs at the most resistant stages could withstand forces greater than 3.5 kg, but the mean loads to burst the chorion throughout development, about 400 - 700 g, were less than the median loads required to burst the chorion of the herring eggs in my experiments. It seems that a load of about 400 g was equivalent to a breaking-pressure of 2.7 kg/cm^2 for plaice eggs. It may be that demersal herring eggs are more liable to mechanical shock than are pelagic plaice or anchovy eggs, and therefore herring eggs have a more resistant chorion. Salmonid eggs which are often buried in stony ground also have a very tough chorion. Gray (1932) found that trout eggs usually burst at loads of 1.5 - 2.0 kg, and he calculated that a load of 2.3 kg exerted a pressure of 7.7 kg/cm^2, which is similar to the breaking pressure for herring eggs. Zotin (1958), on the other hand, found that pressures of 19 - 21 kg/cm^2 were required to rupture the chorion of salmon and trout when it was removed from the embryo, but the resistance of the chorion of intact eggs was 2.5 - 3.5 kg. Zotin (1958) also showed that eggs of the whitefish (Coregonus lavaretus baeri) could resist forces of 1.8 - 2.2 kg.

(ii) Resistance of the vitelline membrane.

Fig. 4B shows that the forces necessary to burst the vitelline membrane or the embryo were much lower than those to burst the chorion, except at 2 h after fertilisation and in some eggs just before hatching when the chorion burst before the vitelline membrane did. The resistance of the vitelline membrane was relatively high at the 4 - 8 cell stage, 5 h after fertilisation; it decreased to a minimum during gastrulation and then began to increase again after the blastopore had

closed. Resistance continued to increase with development until just before hatching when the vitelline membrane decreased in resistance at the same time as the chorion did.

(iii) Survival of the embryo after deformation of the egg.

The results of 1974 experiments on survival after deformation are summarised in Fig. 5 and complete results from 1977 are shown in Fig. 6. Embryos at all stages of development could survive deformation of the egg by 33% (Fig. 5), a degree of deformation produced by relatively small forces (see Fig. 7). At 21 and 54 h after fertilisation in 1977, before the blastopore had closed, deformation by 50% ruptured the vitelline membrane of most eggs and subsequent survival was low (Fig. 6A and B). At 3 days, however, after the blastopore had closed, the vitelline membrane was burst in only 1 of 10 eggs deformed by 50% and survival was good, with 7 healthy larvae hatching from the deformed eggs (Fig. 6C). In 1974 the blastopore was not closed by 3 days after fertilisation, and deformation by 50% at this stage caused high mortality (Fig. 5). Later embryos in 1974 did not survive 50% deformation of the egg until 9 days after fertilisation when the tail was completely free from the yolk and the embryo quite motile (Fig. 5). The higher mortality in 1974 than in 1977 of embryos deformed by 50% after the blastopore had closed was probably because greater forces were applied in 1974 to produce this degree of deformation (Fig. 7). Deformation of eggs just sufficiently to burst the vitelline membrane caused high mortality of embryos at 21 h, 54 h and 3 days after fertilisation (Fig. 6). Later stages were not tested.

The chorion was punctured with a tungsten needle in eggs at only one stage of development, 54 h after fertilisation. The embryo was not physically damaged by the needle, but some of the perivitelline

Fig. 5

Survival of 1974 herring eggs deformed at intervals after fertilisation. Each point represents the percentage survival or percentage hatch of 10 embryos.

A. Percentage survival 48 h after deformation.

B. Percentage hatch after deformation.

X, control

O, 33% deformation

●, 50% deformation

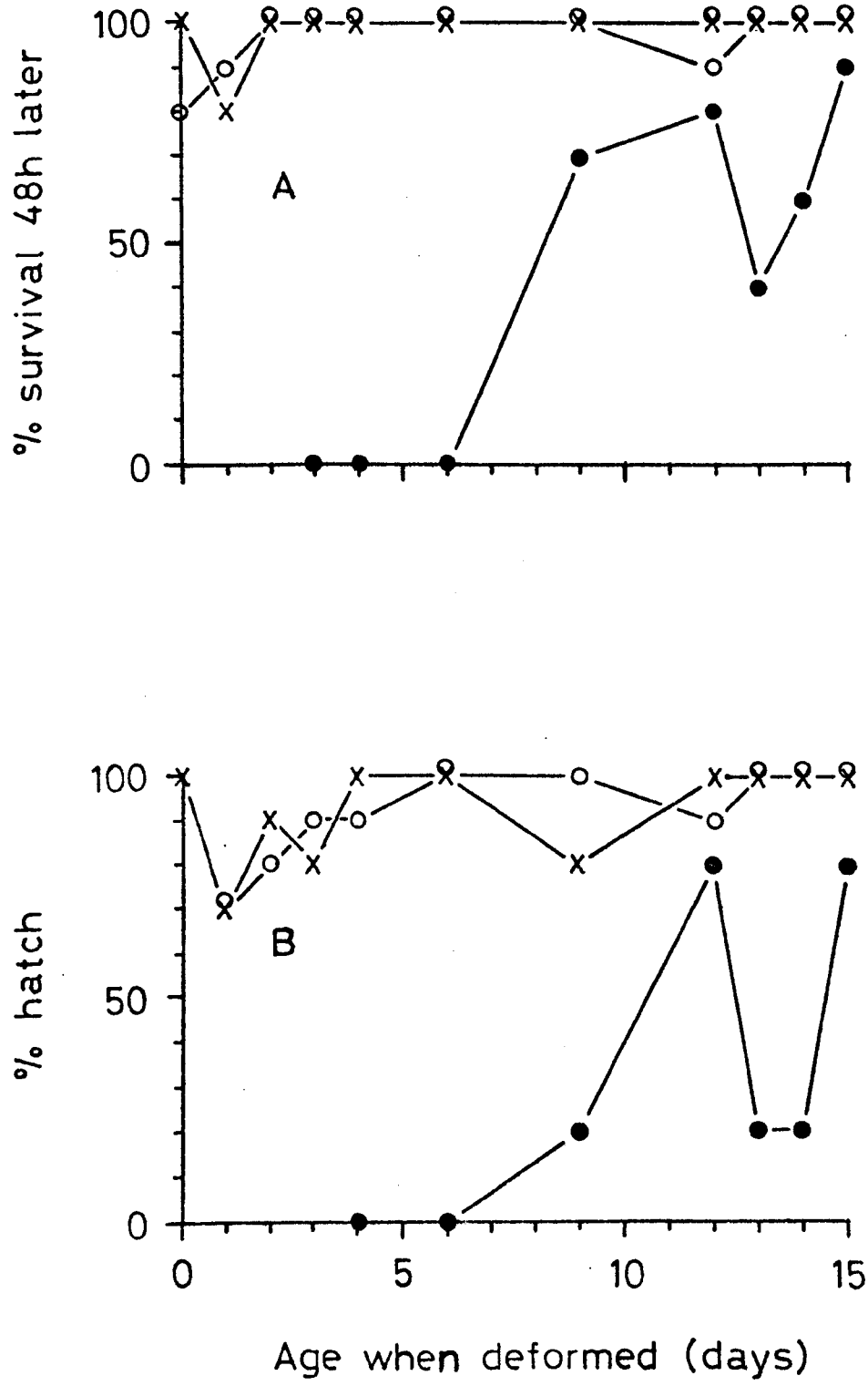

100
50
0
% survival 48h later
A
100
50
0
% hatch
B
0
5
10
15
Age when deformed (days)

Fig. 6

Survival of 1977 herring eggs after deformation or damage at (A) 21 h, (B) 54 h, (C) 3 days after fertilisation. Cumulative percentage survival is plotted at least up to hatching. Each point represents the percentage survival of 10 embryos.

X, control

●, 50% deformation

Δ, vitelline membrane burst

+, chorion pierced

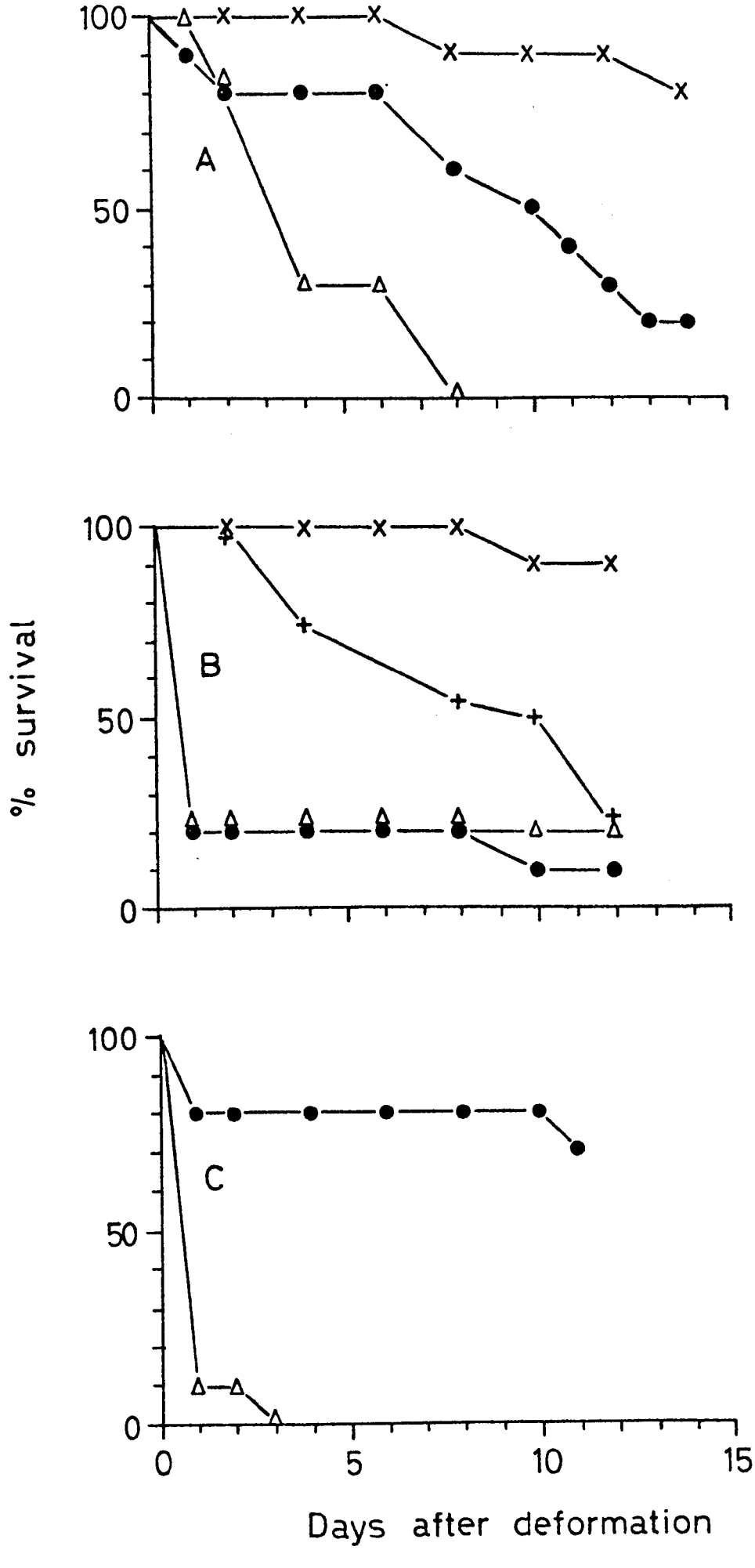

100
50
0
A
B
C
% survival
0
5
10
15
Days after deformation

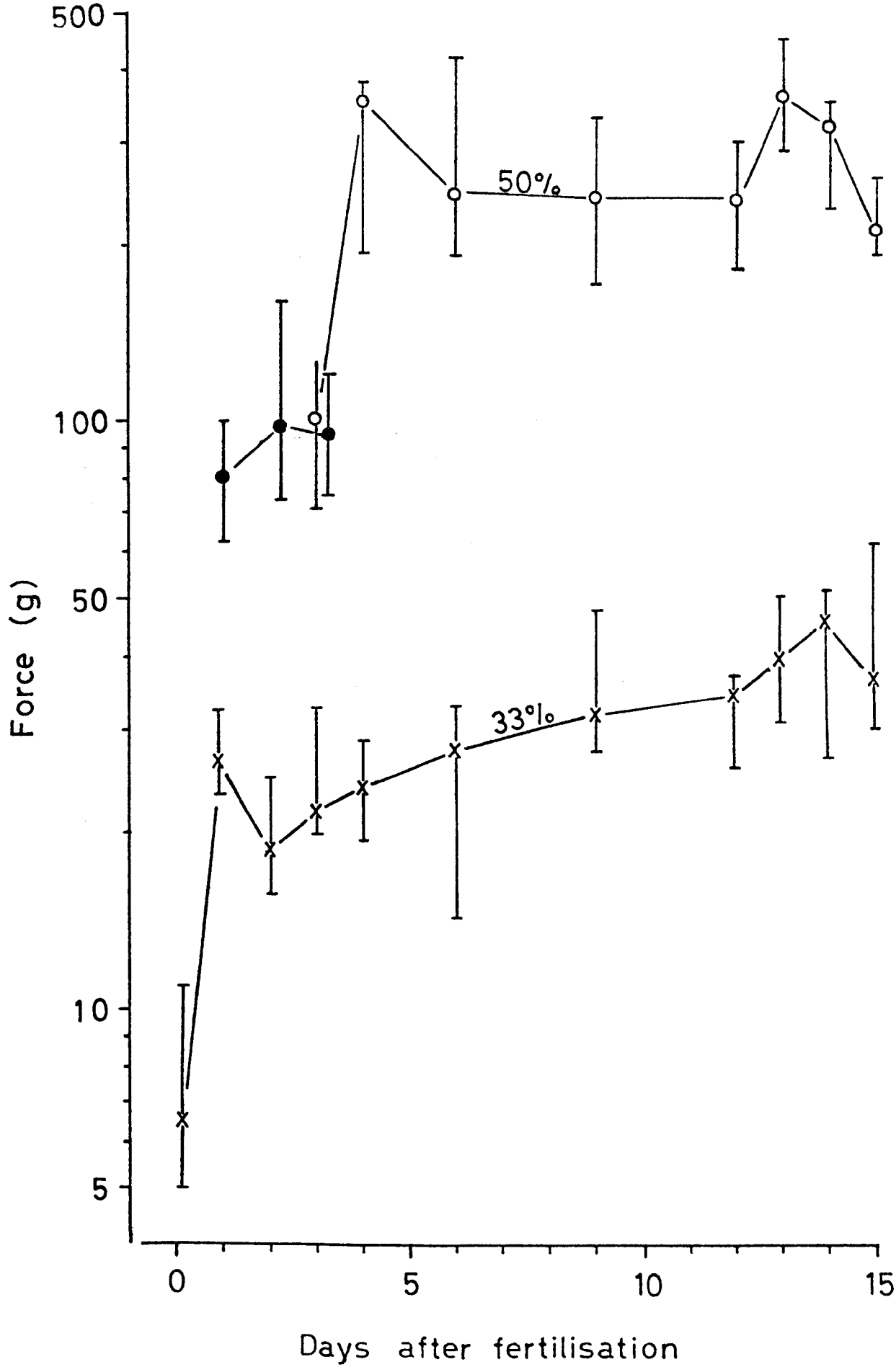
Fig. 7

Load required to deform herring eggs by 30% and 50% during development.

X, 33% deformation in 1974 eggs

0, 50% deformation in 1974 eggs

●, 50% deformation in 1977 eggs


500
100
50
10
5
Force (g)
50%
33%
0
5
10
15
Days after fertilisation

fluid escaped and the egg was deflated slightly. Although the hole was sealed and the eggs seemed to have regained their normal turgidity a day later, survival of the embryos was poor. Almost half of the embryos were dead before the time of hatching, and most of the larvae which hatched could not swim properly and died within 1 or 2 days after hatching (Fig. 6B).

The present findings on sensitivity of the embryo to damage at different stages are in agreement with those of Galkina (1957), who showed that the vitelline membrane of the Okhotsk herring was more resistant in the blastula stage than at the beginning of gastrulation. It is interesting that in the present study at the 4 - 8 cell stage, 5 h after fertilisation, the median resistance of the vitelline membrane to applied pressure was 213 g almost as great as the maximum median resistance (266 g) found 10 days after fertilisation when the yolk was protected by several layers of embryonic tissue. The vitelline membrane seems to weaken during the overgrowth of the blastoderm, leaving a very sensitive period until after closure of the blastopore. The increased resistance from then on is probably brought about by the increase in complexity and thickness of the cell layers covering the yolk. De Ciechomski (1967) and Pommeranz (1974) also showed that the mechanical resistance of anchovy and plaice eggs respectively increased after the closure of the blastopore.

2. Recovery of larvae from injury.

(i) Predator attacks on herring and plaice larvae.

Aurelia aurita medusae were common in the plankton throughout the larval rearing season, ephyrae being abundant at the time of first feeding of herring larvae. All stages, from ephyrae of 8 mm to medusae

of 30 mm in diameter, captured and ingested young herring and plaice larvae readily. Most larvae, especially in the yolk sac stage, were eaten when stung and trapped by the tentacles, but one herring larva of 20 mm was stung badly without being caught; within 10 min the whole body had become white and the larva lay on the bottom of the container. Examination showed that the skin was torn in many places. Another herring larva taken from the manubrium of an Aurelia medusa recovered in sea water and was feeding within 24 h. Table 1 shows that there was 90% survival of larvae anaesthetised and stung by an Aurelia ephyra. The damage to the skin was superficial and was evident as white patches towards the posterior end of the larva. Of the other medusae tested, Tiaropsis multiserrata from Loch Sween was even more efficient at capturing herring and plaice larva than was Aurelia, and Bougainvillea sp. also ate herring larvae. Colonies of Sarsia hydroids captured herring larvae of 12 - 18 mm and plaice larvae of about 7 mm as soon as the larvae touched the polyps. The larvae were usually caught by about 10 polyps at once and they never escaped.

Mysids, and Nephrops norvegicus megalopa larvae also attacked and ate herring larvae and often scratched the larvae while attempting to catch them. One larva was rescued after its tail had been eaten and body scratched by a Nephrops larva; it died within 24 h.

(ii) Recovery of herring larvae from various lesions.

Because of previous reports of the fragility of larvae and of their great susceptibility to damage, my exploratory experiments involved relatively slight injuries. Table 1 shows that first feeding herring larvae survived well after the skin was pierced with a tungsten needle and after a superficial wound of 0.05 mm^2 was produced by suction.

Table 1. Preliminary observations on recovery of first feeding herring larvae from various superficial injuries in sea water.

Type of lesion	Number of larvae used	% survival 48 h after wounding	
		Control	Wounded
Puncture of skin with a tungsten needle	10	100	100
Wound of 0.05mm^2 caused by suction	10	90	80
Stings of an Aurelia ephyra	10		90

The swimming of larvae was not affected by these small wounds, and most of the larvae were feeding within one or two days after wounding. Other experiments, on the healing of skin wounds, showed that young herring larvae also recovered well after the skin was scratched with a tungsten needle along the body dorsal to the anus, simulating damage which could be inflicted by the spines of a predator. The incision of 0.6 mm long removed a strip of epidermis and caused some damage to the dermis, making the body of the larva bend at the area damaged. The information on survival obtained in these experiments on healing was incidental and is not included in Table 1 because the number of wounded larvae was continually being reduced by sampling.

The effect on survival of cutting off different lengths of the tail from yolk sac herring larvae (8.2 - 9.6 mm) is shown in Fig. 8. Usually the amputations resulted in the loss of a large amount of blood relative to the total volume of blood in the larva. The colourless blood seemed to flow out from the cut end of the dorsal aorta for some time without clotting. Survival was good after 1 mm and 2 mm lengths of the tail were cut off in sea water so it seems that the loss of a relatively large volume of blood was not lethal for larvae at this early stage of development. After 1 mm of the tail was cut off larvae could swim quite well, but swimming was severely affected after a 2 mm length was cut off and 13 days later the larvae could still only swim slowly and not very well. All of the larvae which had lost 3 mm of the tail did not die until day 6 (Fig. 8B), but they were effectively moribund long before that even though the brain was not white. About half of the body was white and although they struggled to swim they spent most of the time lying on the bottom of the container. There were no gross signs of regeneration of the tail in any of the

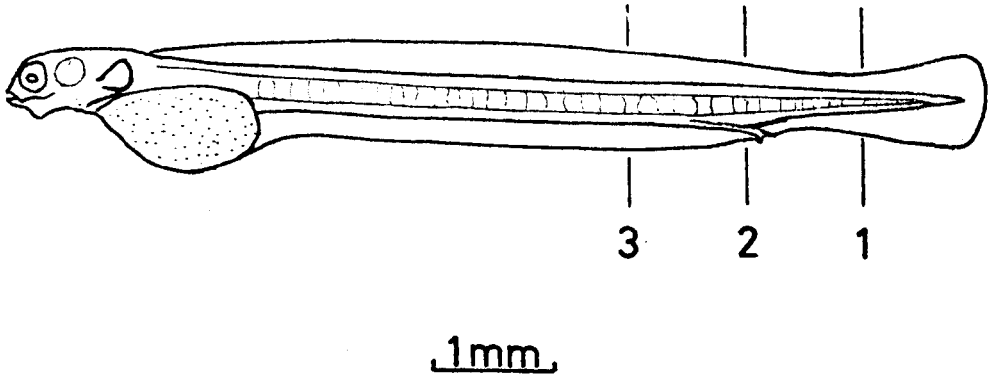
Fig. 8

Recovery of yolk sac herring larvae of 8.2 - 9.6 mm after amputation of different lengths of the tail in sea water.

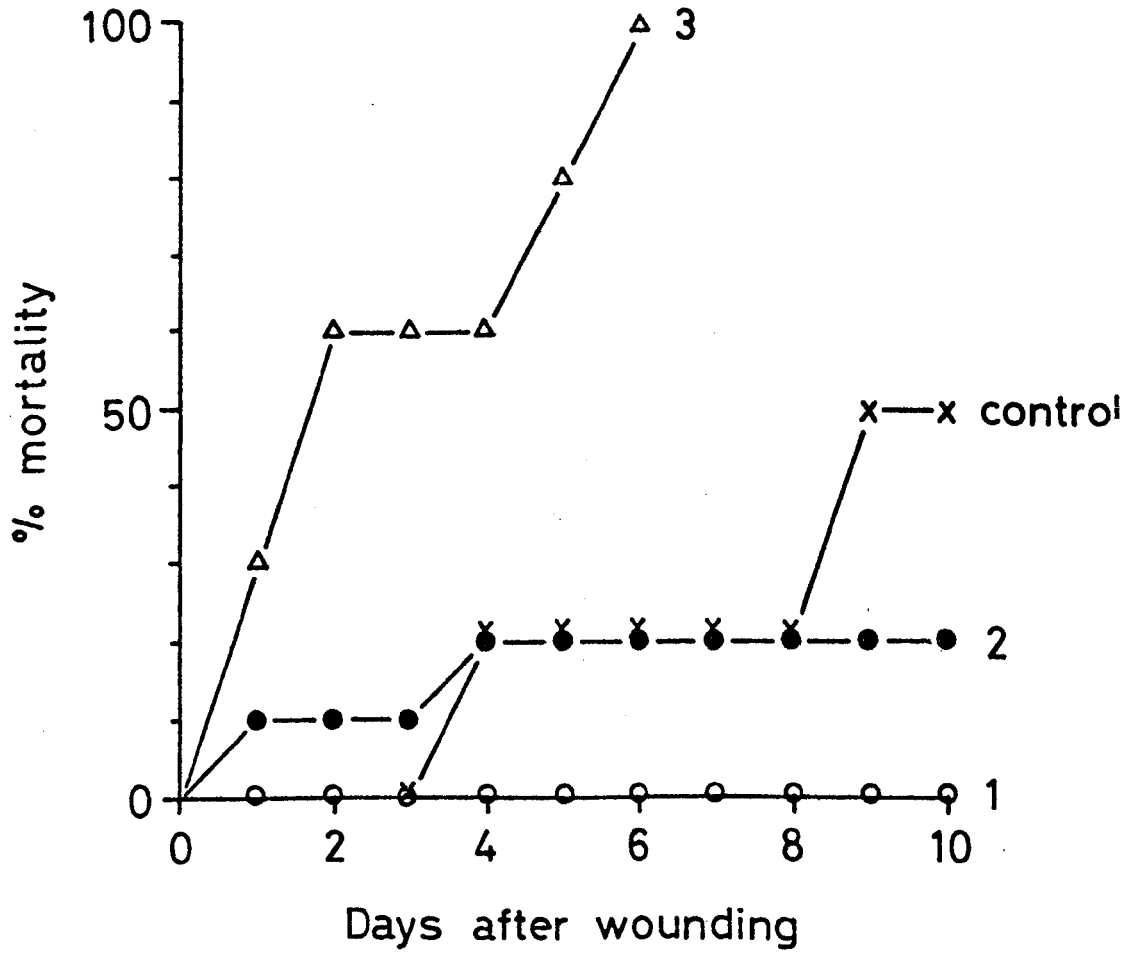
A. Two day old larva showing where cuts were made to remove a 1 mm, 2 mm or 3 mm length of tail.

B. Cumulative percentage mortality for each day up to 10 days after wounding. The figure with each line shows what length of the tail was cut off in mm. Each point represents the percentage mortality of 10 larvae. The high mortality of control larvae on day 9 coincided with the addition of barnacle nauplii to the beakers, but it is not known why the larvae died.

A.


3
2
1
1mm

B.


100
50
0
% mortality
3
x—x control
2
1
0
2
4
6
8
10
Days after wounding

larvae including 2 which survived for 27 days after 1 mm was cut off and 8 which were kept for 17 days after a length of 2 mm was cut off.

(iii) The effect of starvation on recovery of damaged herring larvae.

Fig. 9 shows the effect of cutting off a 1 mm length of tail from herring larvae of 10.5 - 12 mm starved to, or nearly to, PNR after yolk resorption. There was, not surprisingly, a high mortality even of the undamaged starving larvae but the wounded larvae tended to show a lower resistance time. Further experiments showed that up to 6 days after yolk resorption non-feeding larvae tolerated an incision through the body as well as yolk sac or feeding larvae did, but after starvation to PNR tolerance was reduced (Fig. 10A and B). Mortality of undamaged larvae starved to PNR was also high, however, and so again the main effect of injury was to decrease the resistance time (Fig. 10C).

(iv) The effect of lesion area and isosmotic salinity on survival of herring, plaice, and salmon larvae.

Larvae began to swim immediately after recovery from anaesthesia when only a relatively small area of skin was removed, such as an area of 0.2 mm^2 from stage 2 herring or of 3 mm^2 from 24 - 26 mm salmon. Following extensive lethal lesions, however, the larvae could not swim, e.g., 24 - 27.5 mm salmon with lesions of 35 mm^2 lay still on the bottom of the container for 4 - 5 days until they died. Larvae with non-lethal lesions generally fed as well as did the control. There were no obvious signs of fungal infection in any of the wounds but some herring larvae developed fluid-filled vesicles which may have been associated with bacterial infection.

Fig. 9

Effect of starvation after yolk resorption on recovery of 10.5 - 12 mm herring larvae after amputation of the caudal fin in sea water. Cumulative percentage mortality of larvae wounded at or near PNR is shown for up to 7 days after wounding. Each point represents the percentage mortality of 20 larvae.

O, control starving

●, wounded starving

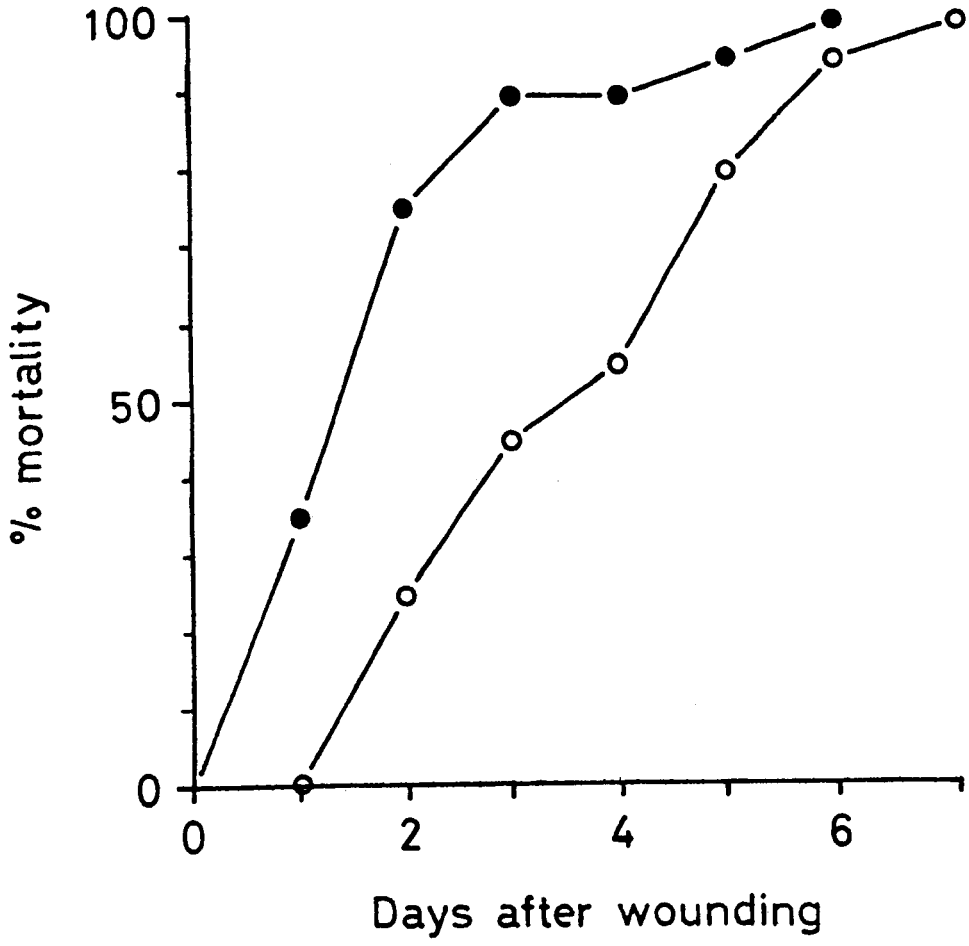

100
50
0
% mortality
0
2
4
6
Days after wounding

Fig. 10

Effect of starvation on recovery of herring larvae in sea water after an incision of 0.3 mm long through the body. Percentage mortality is shown for yolk sac and first feeding larvae and for larvae starved for different numbers of days after yolk resorption to PNR. Each point represents the percentage mortality of 20 larvae.

O —— O, wounded yolk sac or feeding larvae

Δ —— Δ, control yolk sac or feeding larvae

● ---- ●, wounded starving larvae

▲ ---- ▲, control starving larvae

EYS, end yolk sac

PNR, point-of-no-return

A. Percentage mortality of larvae of different ages 24 h after wounding.

B. Percentage mortality of larvae of different ages 48 h after wounding.

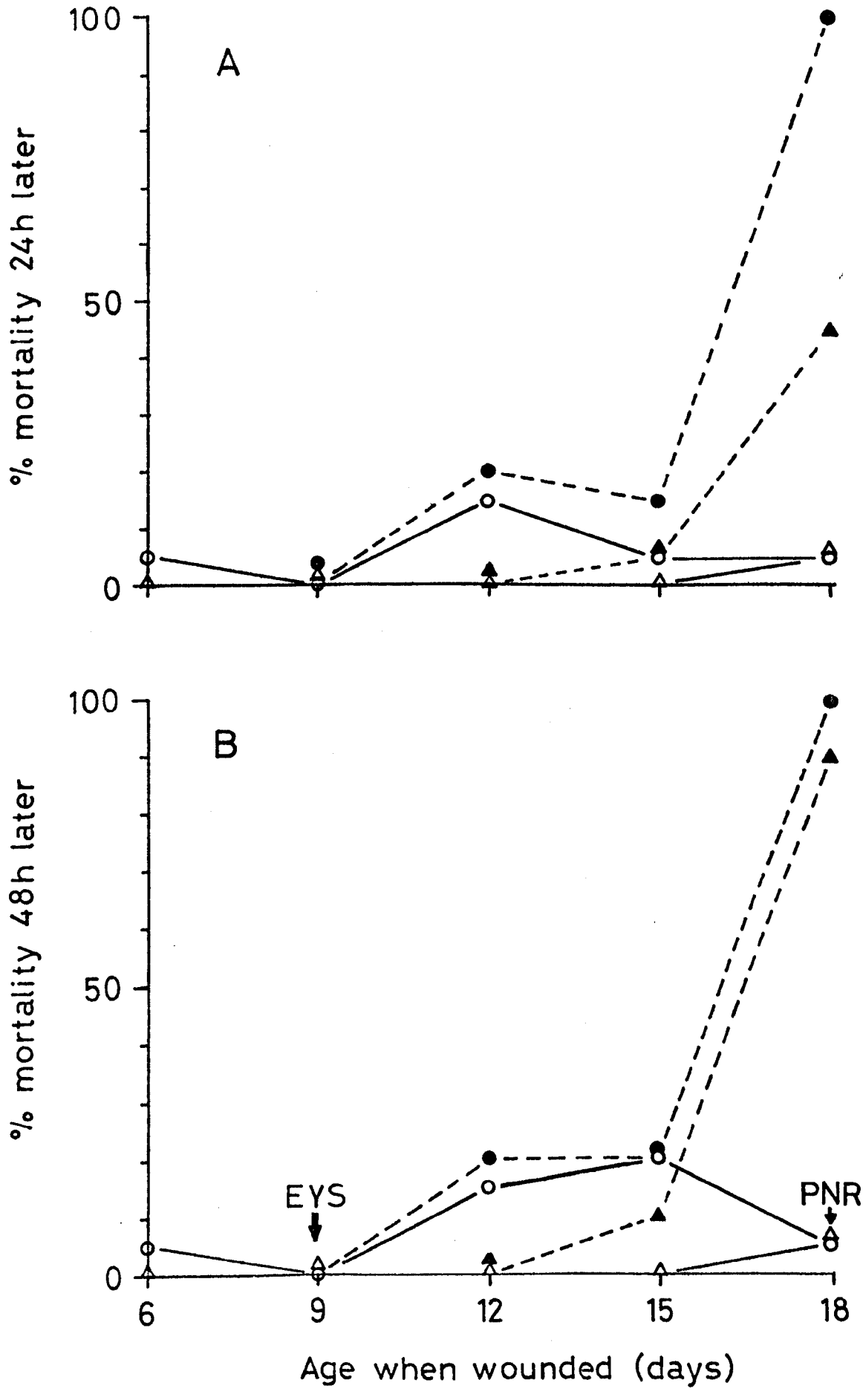

A
100
50
0
% mortality 24h later
B
100
50
0
% mortality 48h later
EYS
PNR
6
9
12
15
18
Age when wounded (days)

Fig. 10 contd.

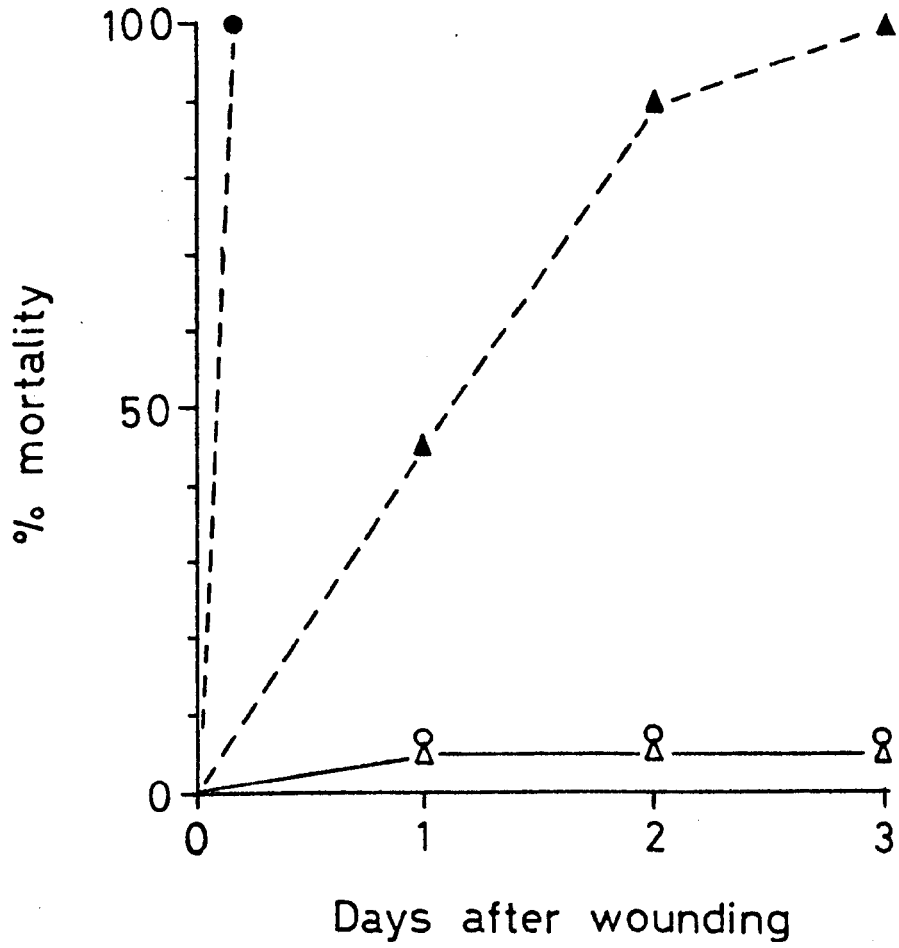
C. Cumulative percentage mortality for 3 days after wounding of larvae wounded 18 days after hatching, when starving larvae had reached PNR.

O —— O, wounded feeding

Δ —— Δ, control feeding

● ---- ●, wounded starving

▲ ---- ▲, control starving


100
50
0
% mortality
0
1
2
3
Days after wounding

(a) Herring: Figs. 11 and 12 show cumulative mortality of herring larvae after lesions of different area. These results are summarised in Fig. 13, and in Table 2 with results on plaice and salmon. In both sea water and in a salinity of 12.5‰, yolk sac larvae of 10.5 - 12.5 mm were more sensitive to damage than were stage 2 larvae of 14 - 16.5 mm (Fig. 13 and Table 2). Tolerance to damage of both size groups was increased when they were held in a salinity of 12.5‰ isosmotic with their body fluids (Fig. 13 and Table 2). Although acute mortality was certainly reduced in 12.5‰, damaged stage 2 larvae tended to show signs of distress after several days in that salinity. Many of them seemed to lose their sense of equilibrium swimming in tight circles or very erratically, and their heads were bent downwards giving them a curved appearance. They also tended to swim very close to or lie on the bottom of the container. There was delayed mortality of the damaged stage 2 larvae in 12.5‰ even after wounds which would not have been lethal in sea water (Figs.12 and 13). The control larvae, however, showed no mortality over a period of 10 days in 12.5‰ (Fig. 12).

(b) Plaice: Figs. 14 and 15 show cumulative mortality of plaice larvae following lesions of different area. These results are summarised for yolk sac larvae in Fig. 16, and for older larvae and yolk sac larvae in Table 2. Yolk sac plaice larvae, at least in 12.5‰ salinity, could withstand less damage than could yolk sac herring, but the maximum area of lesion tolerated represented about the same proportion (around 2%) of the total body surface (Table 2).

In both sea water and in 12.5‰ salinity, plaice larvae of 10.5 - 20 mm survived larger wounds than did yolk sac larvae of 6 - 8 mm. The older larvae also seemed to tolerate the removal of skin from a

Fig. 11

Effect of skin wounds of different area on survival of yolk sac herring (10.5 - 12.5 mm) in (A) sea water and (B) 12.5‰ salinity. Cumulative percentage mortality is plotted for 10 days after wounding. The wound area in mm^2 is shown after each line. Each point represents the percentage mortality of 10 larvae.

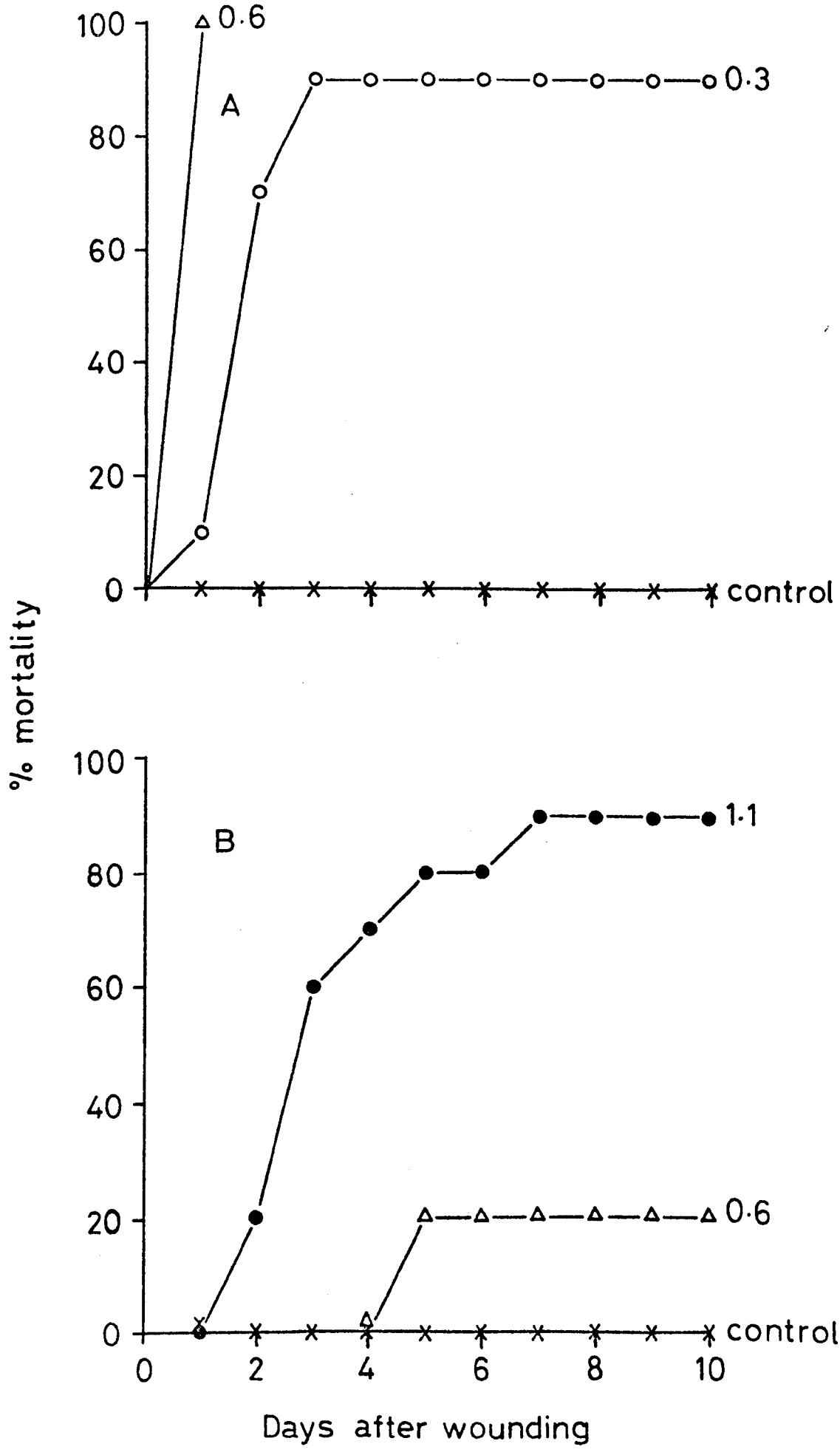

100
80
60
40
20
0
% mortality
A
0·6
0·3
control
B
1·1
0·6
control
0
2
4
6
8
10
Days after wounding

Fig. 12

Effect of skin wounds of different area on survival of stage 2 herring (14 - 16.5 mm) in (A) sea water and (B) 12.5‰ salinity. Cumulative percentage mortality is plotted for 10 days after wounding. The wound area in mm^2 is shown after each line, but where necessary a symbol after the wound area indicates to which line the area refers. Each point represents the percentage mortality of 10 larvae.

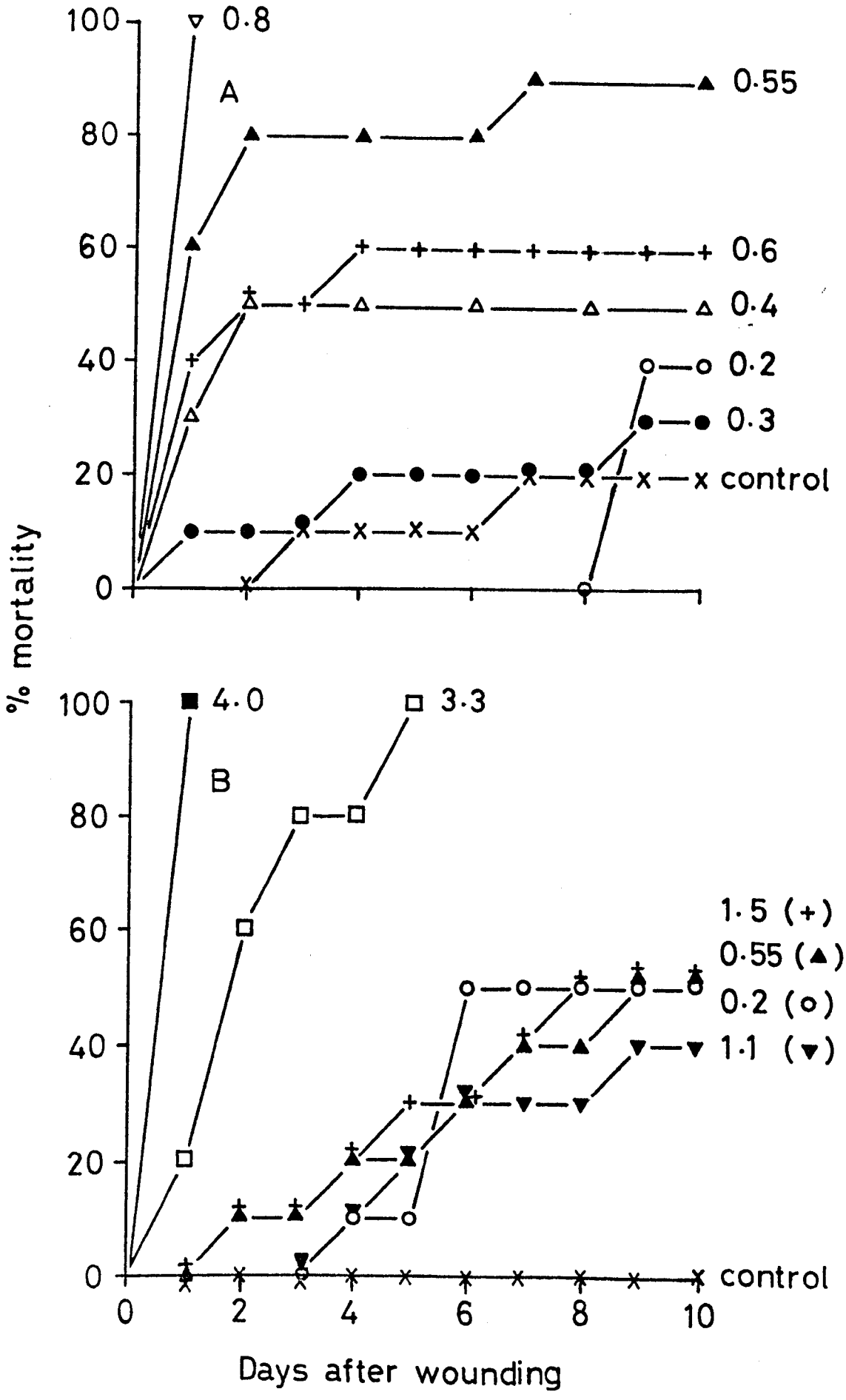

100
80
60
40
20
0
% mortality
0·8
A
0·55
0·6
0·4
0·2
0·3
control
4·0
3·3
B
1·5 (+)
0·55 (▲)
0·2 (o)
1·1 (▼)
control
0
2
4
6
8
10
Days after wounding

Fig. 13

Estimated time to 50% mortality (ET_{50}) plotted against wound area for yolk sac and stage 2 herring in sea water and 12.5‰ salinity. The points plotted at greater than 10 days indicate less than 50% mortality by 10 days after a given wound area.

● —— ●, yolk sac in seawater

O --- O, yolk sac in 12.5‰

▲ —— ▲, stage 2 in sea water

Δ --- Δ, stage 2 in 12.5‰

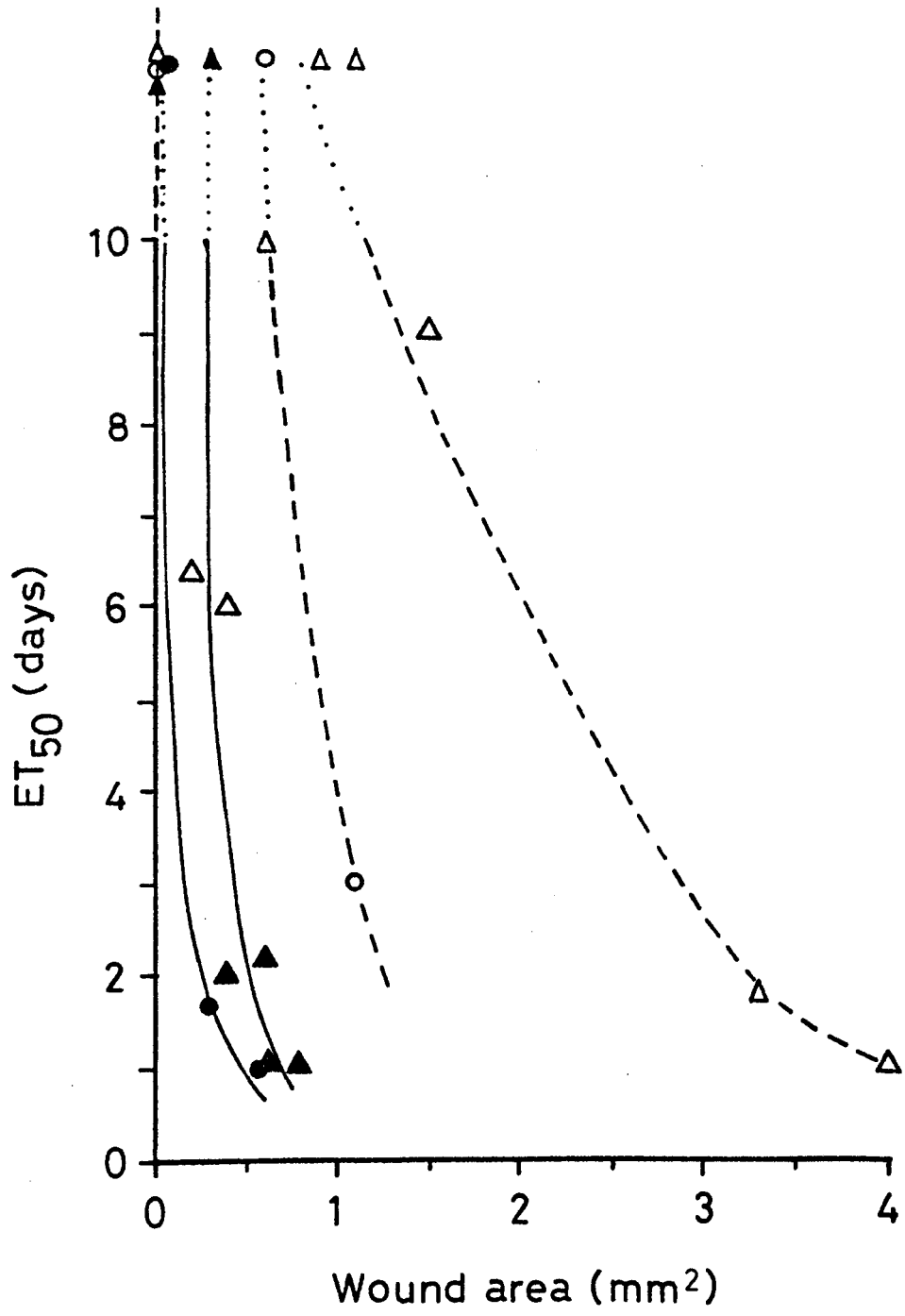

ET50 (days)
10
8
6
4
2
0
0
1
2
3
4
Wound area (mm2)

Table 2. Tolerance to skin damage of herring, plaice, and salmon larvae of different ages and in different salinities. For each group of larvae a range of lesion areas is given, the lower limit of which caused less than 50% mortality and the upper limit of which caused greater than 50% mortality.

		Limits between which there will be 50% mortality			
		In natural medium		In isosmotic medium	
	Length (mm)	Wound area (mm^2)	Wound area as a % of total body surface area*	Wound area (mm^2)	Wound area as a % of total body surface area*
Herring					
Yolk sac	10.5 - 12.5	< 0.3	< 0.9%	0.6 - 1.1	1.8 - 3.3%
Stage 2	14 - 16.5	0.3 - 0.4	0.5 - 0.7%	1.5 - 3.3	2.6 - 5.8%
Plaice					
Yolk sac	6 - 8	0.1 - 0.2	0.6 - 1.1%	0.3 - 0.4	1.7 - 2.3%
At metamorphosis	14 - 20	1.8 - 6.3	< 3.8%	> 6.2	> 3.8%
Salmon					
	19 - 21	1.0 - 1.9	0.6 - 1.1%	>12.5	> 6.5%
	24 - 26	3.1 - 4.0	1.4 - 1.8%	-	-
	26 - 27.5	6.5 - 8.2	2.7 - 3.4%	-	-
	24 - 27.5			25.4 - 35.1	10.5 - 14.5%

* The total body surface area was estimated for a larva of median length in each group.

Fig. 14

Effect of skin wounds of different area on survival of yolk sac plaice (6 - 8 mm) in (A) sea water and (B) 12.5‰ salinity. Cumulative percentage mortality is plotted for 10 days after wounding. The wound area in mm^2 is shown after each line, but where necessary a symbol after the wound area indicates to which line the area refers. Each point represents the percentage mortality of 10 larvae.

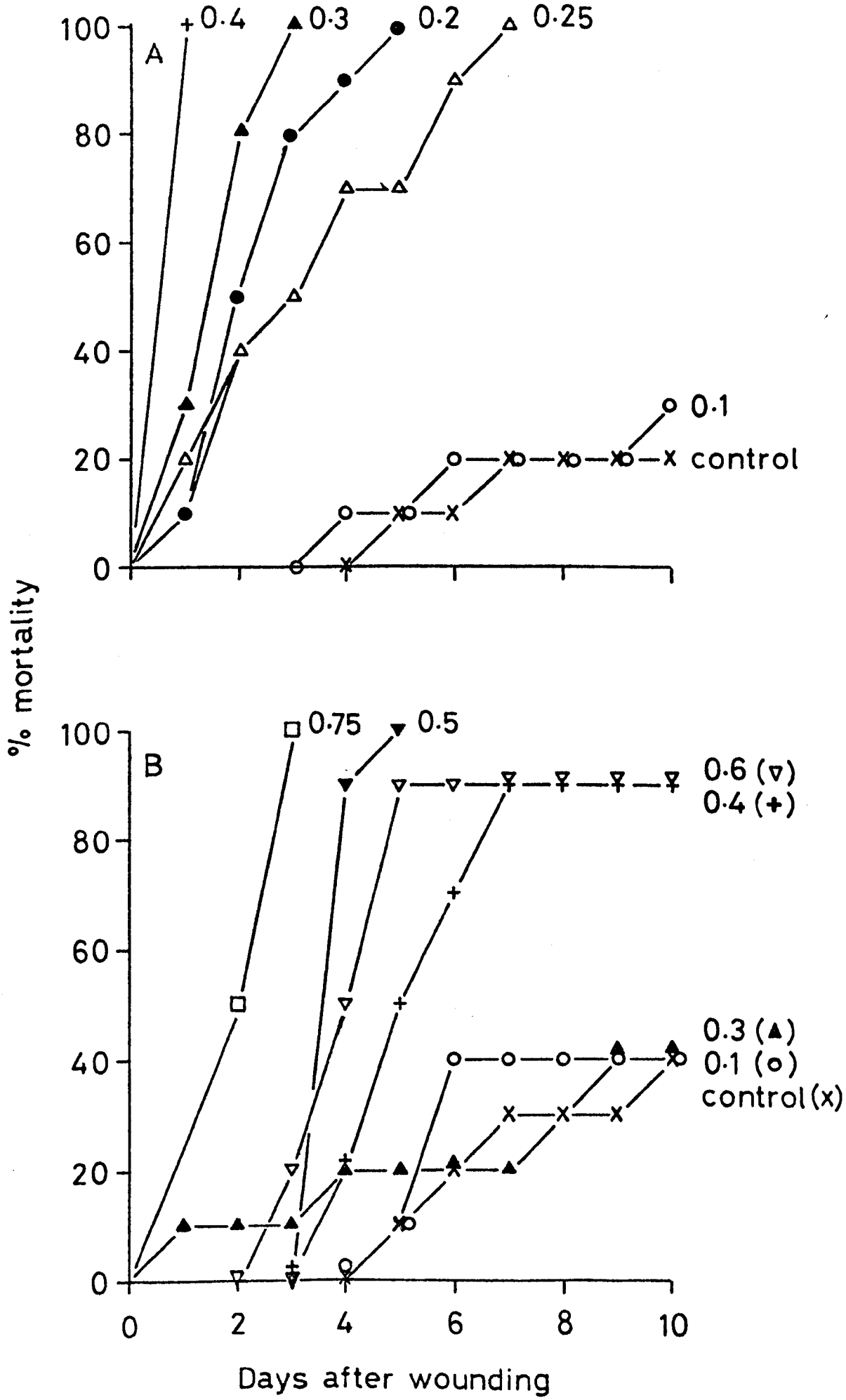

A
B
0·4
0·3
0·2
0·25
0·1
control
0·75
0·5
0·6 (▽)
0·4 (+)
0·3 (▲)
0·1 (o)
control (x)
% mortality
Days after wounding
0
20
40
60
80
100
2
4
6
8
10

Fig. 15

Effect of skin wounds of different area on survival of plaice at metamorphosis in (A) sea water and (B) 12.5‰. Cumulative percentage mortality is plotted for 10 days after wounding. The wound area in mm^2 is shown after each line. Each point represents the percentage mortality of 10 larvae. Different size groups of plaice were wounded as follows :

Wound area (mm^2)	Length plaice (mm)
0.5	10.5 - 12.7
1.8	14 - 18
6.2, 6.3	16 - 20

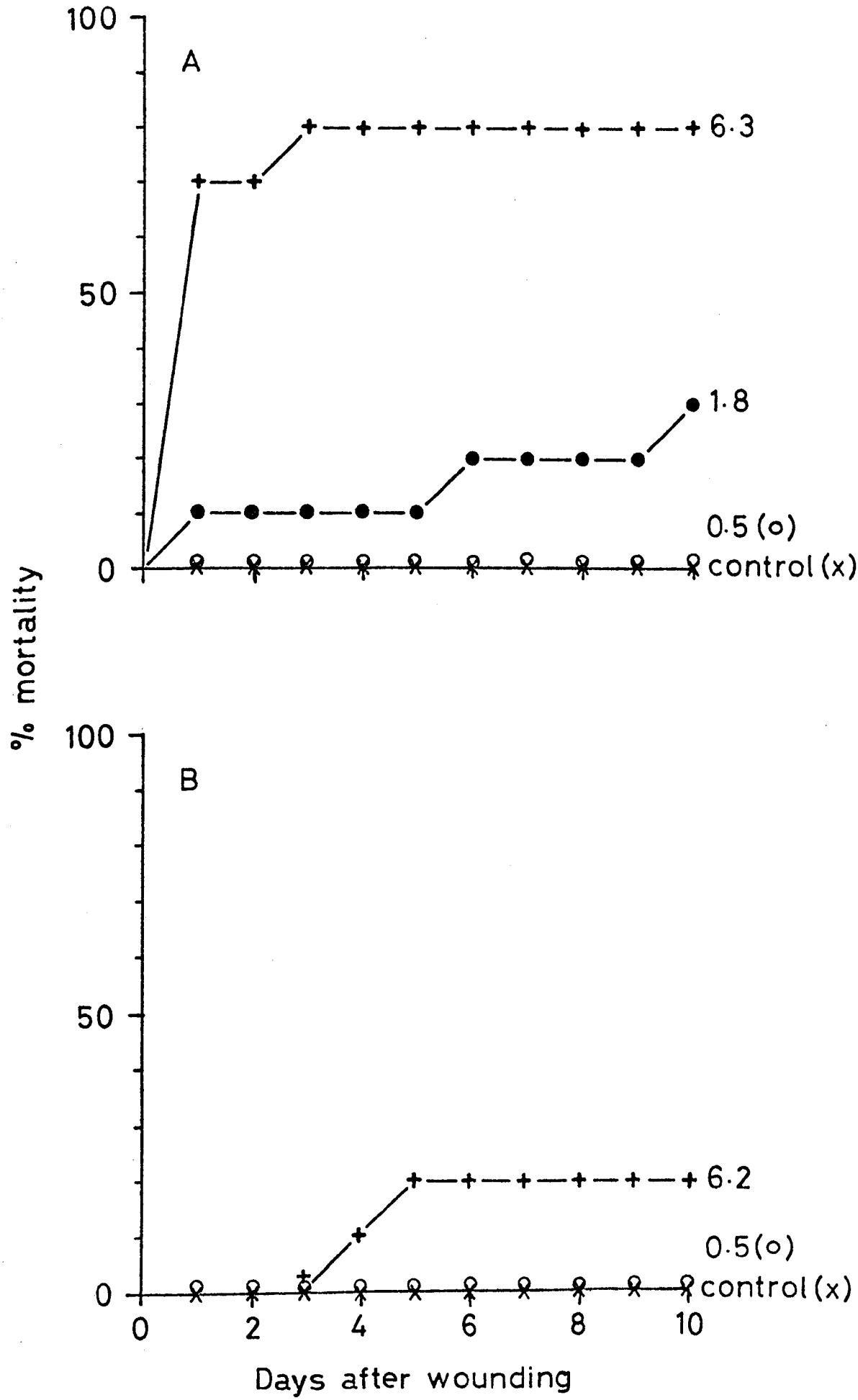
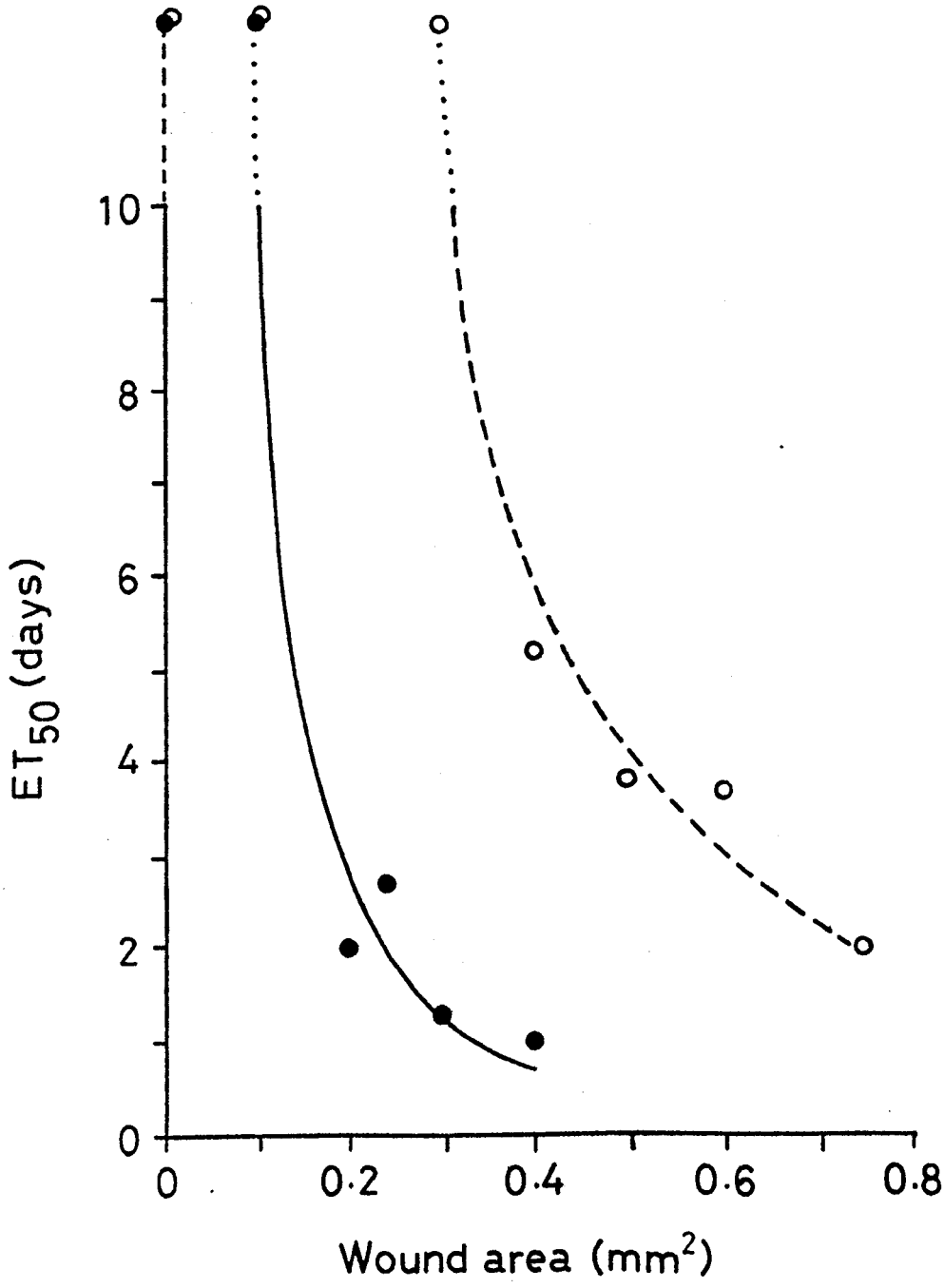

100
A
50
0
6·3
1·8
0·5 (o)
control (x)
% mortality
100
B
50
0
6·2
0·5(o)
control (x)
0
2
4
6
8
10
Days after wounding

Fig. 16

Estimated time to 50% mortality (ET_{50}) plotted against wound area for yolk sac plaice in sea water and 12.5‰ salinity. The points plotted at greater than 10 days indicate that larvae showed less than 50% mortality in 10 days after a given wound area.

● —— ●, sea water

O --- O, 12.5‰


10
8
6
4
2
0
ET_{50} (days)
0
0·2
0·4
0·6
0·8
Wound area (mm^2)

greater proportion of their body surface (Table 2). It may not be reliable to make such a comparison purely in terms of lesion area, however, since the proportion of muscle and other tissues damaged was so much greater in the yolk sac larvae. Tolerance of both groups was increased in the isosmotic salinity of 12.5‰ (Fig. 6 and Table 2). The resistance time of yolk sac larvae after a wound of a given area was also greater in 12.5‰ than in sea water; e.g., the median resistance time after a wound of 0.4 mm^2 was only 1 day in sea water but 5 days in isosmotic salinity (Fig. 16).

(c) Salmon: Results on survival of salmon alevins are shown in full in Figs. 17 and 18 and are summarised in Fig. 19 and Table 2. Alevins of 19 - 21 mm in river water survived wounds much greater than those which were lethal for yolk sac herring and plaice larvae in sea water (Table 2). This is not surprising since the total surface area of a newly-hatched 19 mm alevin is about 170 mm^2 whereas that of a newly-hatched 6.5 mm plaice larva is about 15 mm^2. On the other hand, the median lethal lesion area for salmon of 19 - 21 mm in river water was between 0.6 and 1.1% of the total body surface area, about the same as the lethal threshold (expressed as a percentage of the body surface area) for herring and plaice larvae in sea water (Table 2).

The tolerance of salmon alevins, at least in river water, increased with the size of the alevin (Fig. 19 and Table 2). Not only did older alevins survive larger wounds, but like plaice they seemed to withstand the removal of skin from a slightly greater percentage of the body surface (Table 2). Alevins of all sizes survived much greater wounds in an isosmotic salinity of 8‰ than they did in river water (Fig. 19 and Table 2). Even 24 h in 8‰ increased their tolerance; alevins of 26-27.5 mm suffered high mortality after wounds of 7 mm^2 in

Fig. 17

Effect of skin wounds of different area on survival of salmon alevins of 19 - 21 mm in (A) river water and (B) 8‰ salinity. Cumulative percentage mortality is plotted for 5 - 10 days after wounding. The wound area in mm^2 is shown after each line. Each point represents the percentage mortality of 10 larvae.

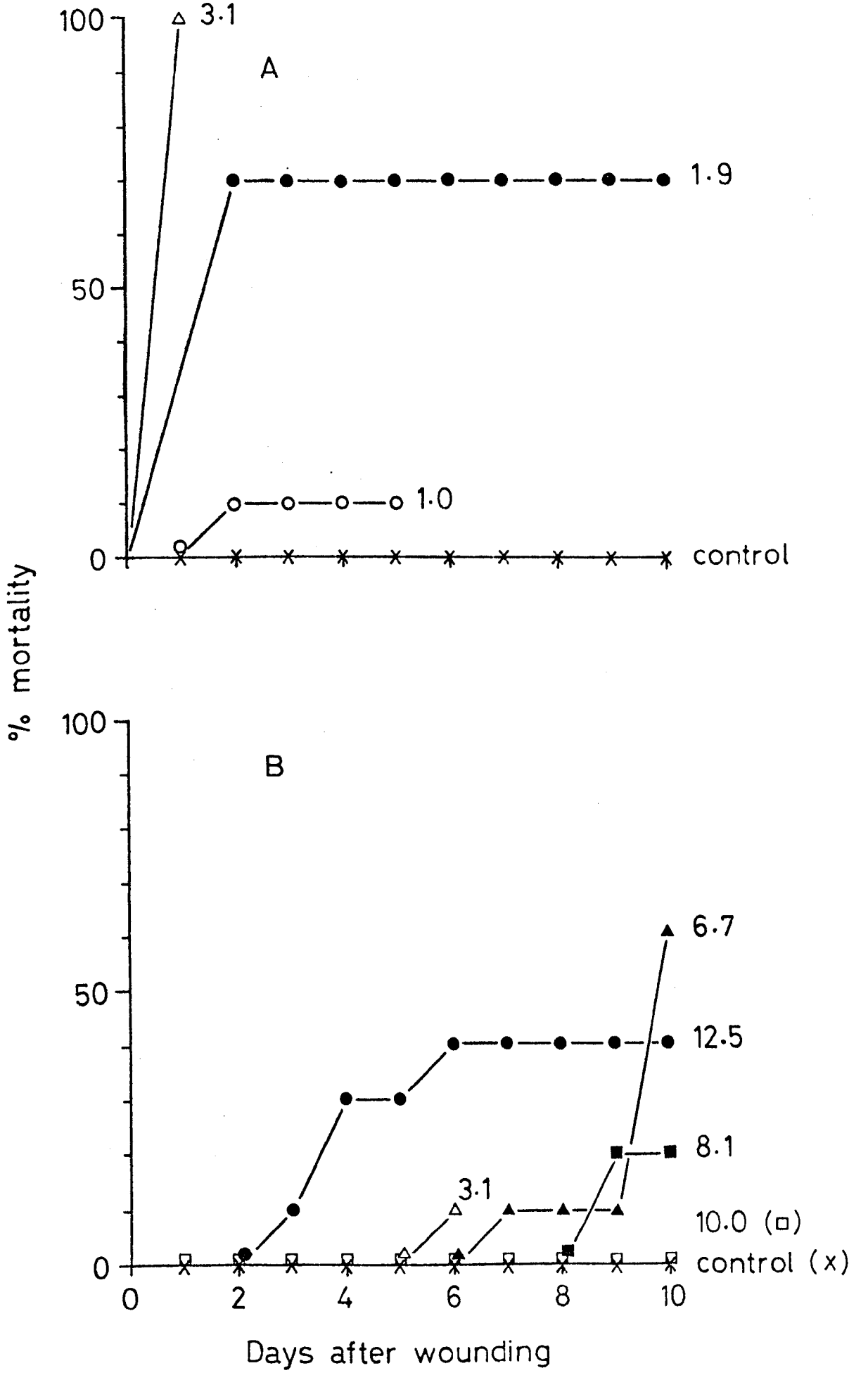

100
50
0
A
3·1
1·9
1·0
control
% mortality
100
50
0
B
6·7
12·5
8·1
3·1
10·0 (□)
control (x)
0
2
4
6
8
10
Days after wounding

Fig. 18

Effect of skin wounds of different area on survival of salmon alevins of 24 - 27.5 mm in (A) river water and (B) 8‰ salinity. In (A) 2 different size-groups of alevins were wounded: alevins of 24 - 26 mm long (dotted lines and closed symbols) and alevins of 26 - 27.5 mm (solid lines and open symbols). Cumulative percentage mortality is plotted for each day up to 10 days after wounding. The wound area in mm^2 is shown after each line. Each point represents the percentage mortality of 10 larvae, except (+) which is the percentage mortality of 5 larvae transferred to river water after 24 h in 8‰ following a lesion of 12.5 mm^2.

A.

Length of alevins (mm)	Wound area (mm^2)
24 - 26	3.1
	4.0
	5.1
26 - 27.5	5.4
	6.5
	8.2
	12.5

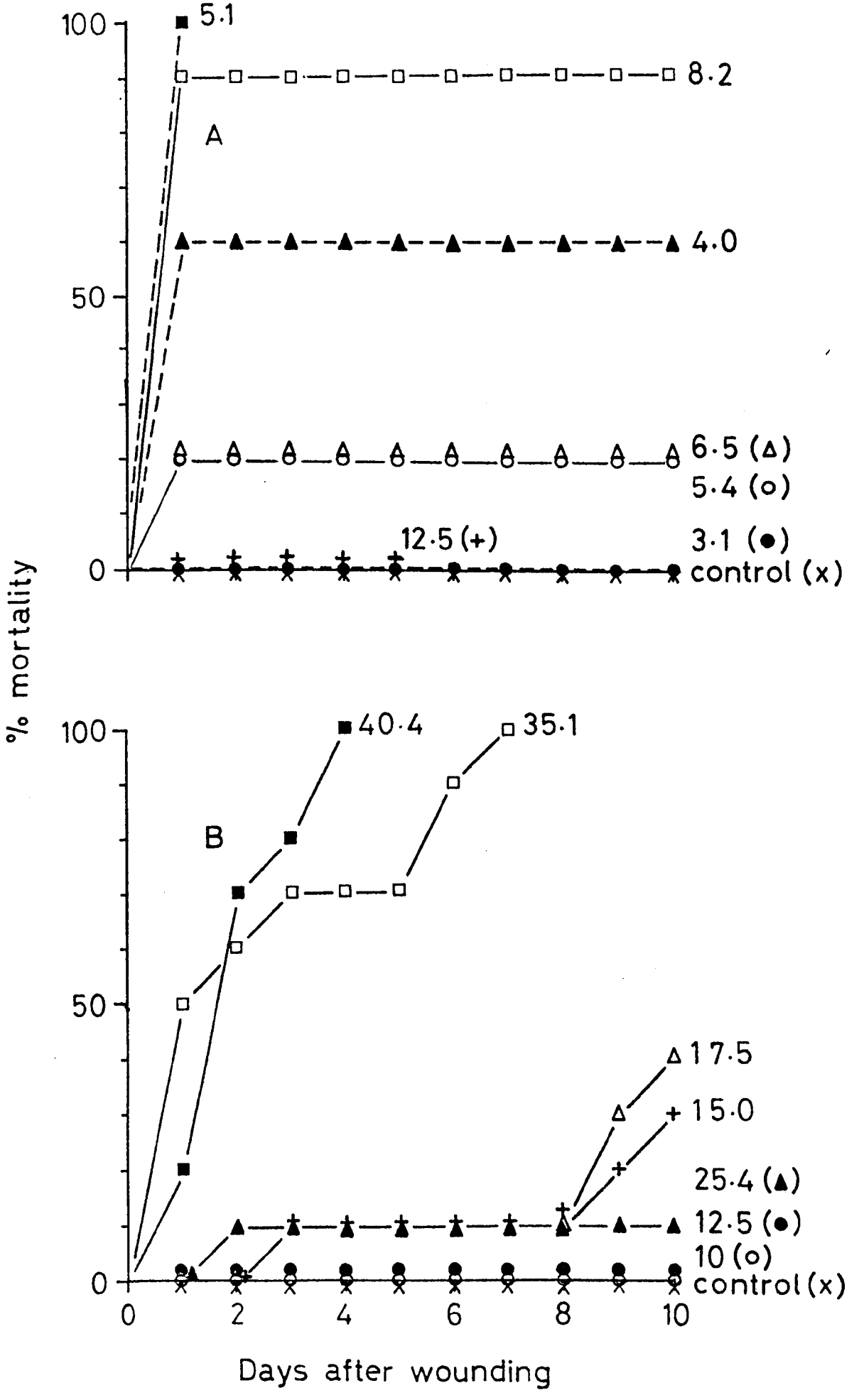

100
50
0
5·1
8·2
A
4·0
6·5 (Δ)
5·4 (o)
12·5 (+)
3·1 (●)
control (x)
% mortality
100
50
0
40·4
35·1
B
17·5
15·0
25·4 (▲)
12·5 (●)
10 (o)
control (x)
0
2
4
6
8
10
Days after wounding

Fig. 19

Estimated time to 50% mortality (ET_{50}) for salmon alevins of different sizes in river water and 8‰ salinity. The points plotted at greater than 10 days indicate that larvae showed less than 50% mortality in 10 days after a given wound area.

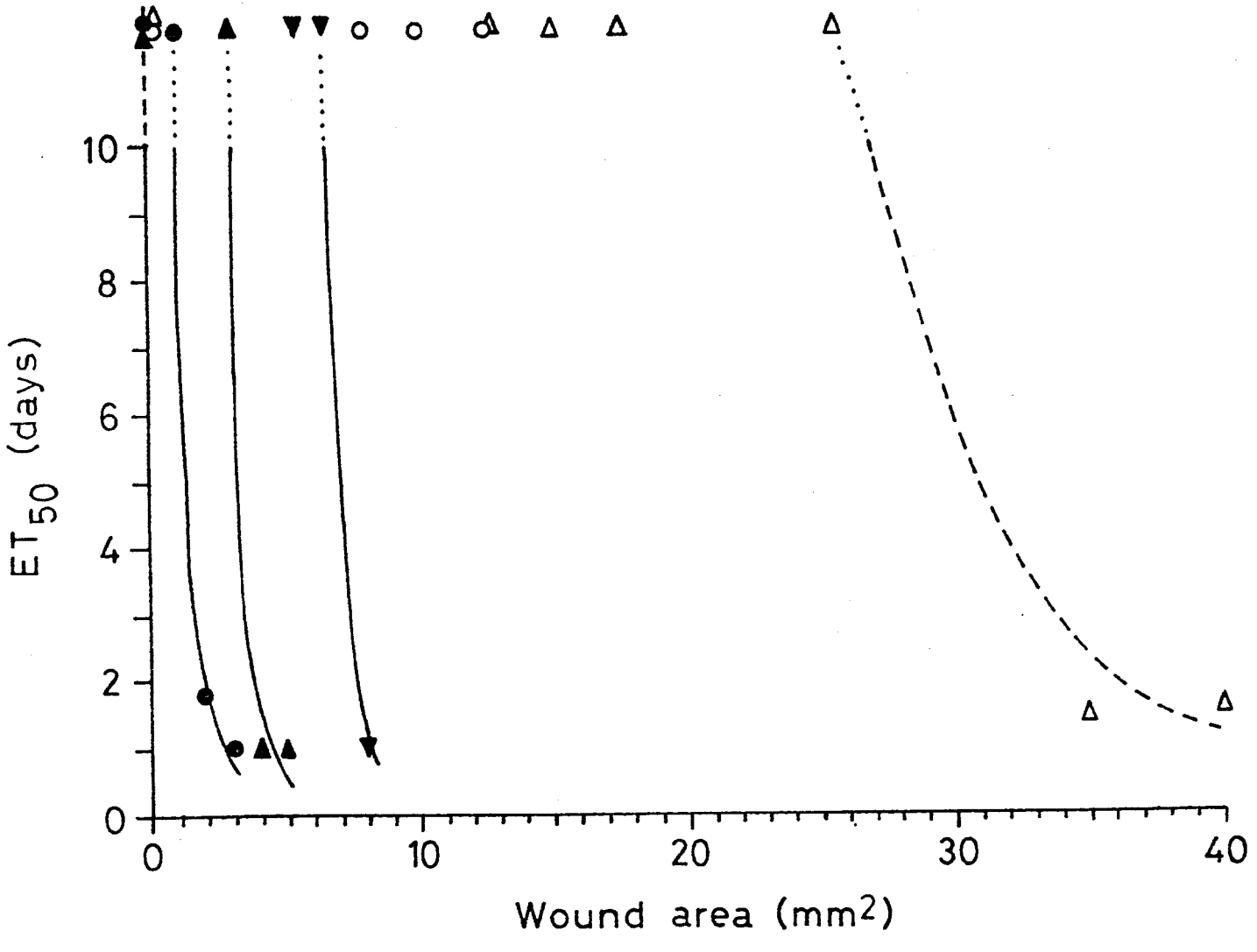
● —— ●, 19 - 21 mm alevins in river water

▲ —— ▲, 24 - 26 mm alevins in river water

▼ —— ▼, 26 - 27.5 mm alevins in river water

0 --- 0, 19 - 21 mm in 8‰

Δ --- Δ, 24 - 27.5 mm in 8‰


ET50 (days)
0
2
4
6
8
10
0
10
20
30
40
Wound area (mm2)

river water, but survived wounds of 12.5 mm^2 when held in 8‰ for 24 h before being transferred to river water (Fig. 18). The wounds were covered by epidermis before the alevins were transferred to river water.

IV CELLULAR RESPONSES OF LARVAE TO DAMAGE

This section concerns the healing of skin lesions in herring, plaice, and salmon larvae, with special emphasis on the response of the epidermis to injury. It deals briefly with repair of the dermis, the responses of inflammatory cells, and with tail fin regeneration. The structure of larval fish skin has been described previously for several species including herring and plaice. Some observations made in the present study on the structure of the epidermis of herring, plaice, and salmon larvae will be described here, however, with reference mainly to features of possible interest in wound healing.

Jones, Holliday and Dunn (1966) have shown using electron microscopy that the epidermis in newly-hatched herring larvae is composed of 2 layers of squamous epithelial cells, at least 2.3 μm in total thickness. Roberts, Bell and Young (1973) have given an account of the development of larval plaice skin from hatching to metamorphosis. They showed that in newly-hatched larvae the skin is about 5 μm in thickness and consists of an epidermis of 2 layers of epithelial cells overlying a thin delicate basement membrane, beneath which there is a fluid-filled dermal space. Together with the epithelial cells, which develop into the Malpighian cell of the adult epidermis, they observed small numbers of PAS-positive mucous cells in the epidermis of the newly-hatched larvae and also large 'chloride cells' similar to those in the epidermis of the larval sardine (Lasker and Threadgold, 1968). They showed that during development the epidermis increases in thickness, eosinophilic granule cells develop within it and the number of PAS-positive cells increases. Collagen is laid down in the dermis and later melanin-bearing cells appear above and below the collagen, so

that after metamorphosis the skin is similar in structure to that of the adult. The skin of newly-hatched larvae of other species is similar to that in herring and plaice (see Lasker and Threadgold, 1968; Wellings and Brown, 1969; Bullock, Roberts and Gordon, 1976).

A Methods

1. Surface observations of wound closure.

Wound closure was followed by direct observation in live herring, plaice, and salmon larvae, but especially in plaice because they were less fragile than herring and being easier to rear were always available through to metamorphosis. Wounds were generally made as described in the previous section by cutting out an area of skin of full thickness including epidermis and dermis down to the muscle. In a few cases with older larvae, however, only epidermis was scraped away leaving an area of intact but denuded dermis. A standard area of skin of 0.4 - 1 mm^2 (about 0.6 x 0.7 mm to 1 x 1 mm) was excised from stage 4 plaice of 10 - 12 mm, older plaice of 21 - 25 mm, stage 3 herring of 19 - 21 mm, and salmon of 24 - 27 mm in length. Epidermis only was scraped from a similar area of 0.4 mm^2 in plaice of 10 - 12 mm and herring of 19 - 21 mm. In addition, an area of skin of 0.1 - 0.2 mm^2 (about 0.3 x 0.35 mm) was cut out from yolk sac plaice of 6.5 - 7 mm and an area of 5.8 - 6.6 mm^2 (about 2.5 x 2.5 - 3 mm) from plaice of 22 - 26 mm. Wounds were made on the right side of the body just behind the body cavity in plaice (on the dorsal surface at metamorphosis), and at the anterior insertion of the dorsal fin in herring and salmon. Herring and plaice were held in dilute sea water of 10 - 11‰ ("one-third sea water") and salmon in river water.

The closure of wounds by migration of epidermal cells was observed in the live herring, early plaice, and salmon larvae by vitally staining the wounds with a 1 : 15000 solution of Nile blue sulphate during closure. The delicate epidermis of these larvae was too thin to see without staining. Twelve up to 32 larvae were wounded in a batch and 4 larvae were sampled for examination at 1- or 2-hourly intervals up to a maximum of 12 h after wounding. At each interval the larvae were anaesthetised and stained and camera lucida drawings were made of the wounds (see Fig. 20). The larvae were fixed in 10% buffered formalin after examination and a different group was observed at each interval lest the staining should have interfered with the healing process.

In older plaice, however, migration of the epidermis could be followed without staining because the epidermis was thicker and contained melanophores. Therefore only 3 of these larvae were wounded in a batch and the 3 larvae were anaesthetised and stained at 2-hourly intervals, the larvae being allowed to recover from anaesthesia between observations. A sample camera lucida drawing made from such a series of observations is shown in Fig. 20B.

Herring, plaice, and salmon larvae were generally held at 10 - 11°C after wounding but in one experiment the effect of temperature on the rate of closure of wounds of 6.3-9.8 mm^2 was investigated in plaice of 29 - 35 mm body length at 14.8 - 15.8 and 5 - 6°C.

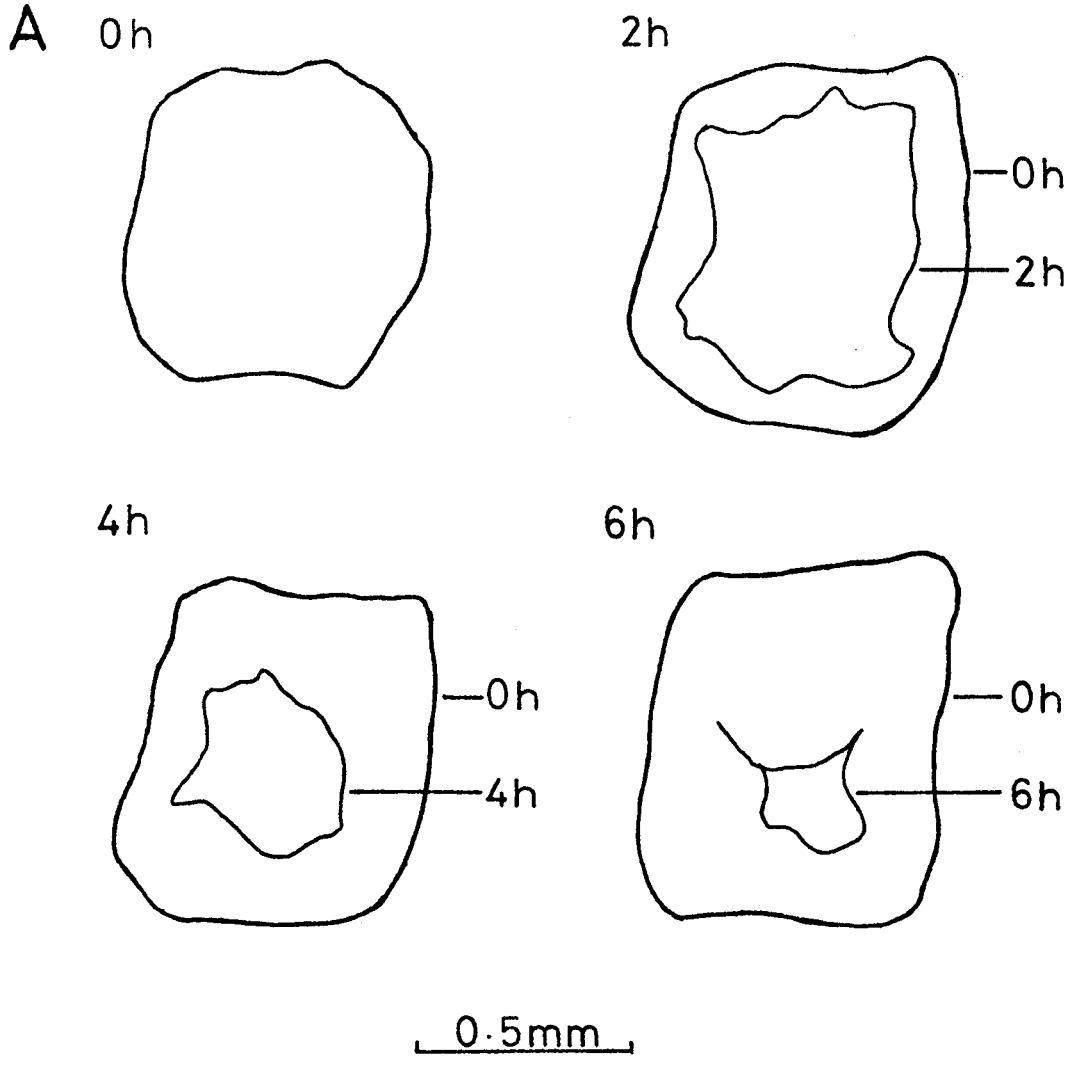
To plot the decrease in area of a wound with time, wound areas were measured by counting squares on the camera lucida drawings made on graph paper during closure. The mean distance covered by epidermis in a given time was also calculated by measuring along 8 radii of

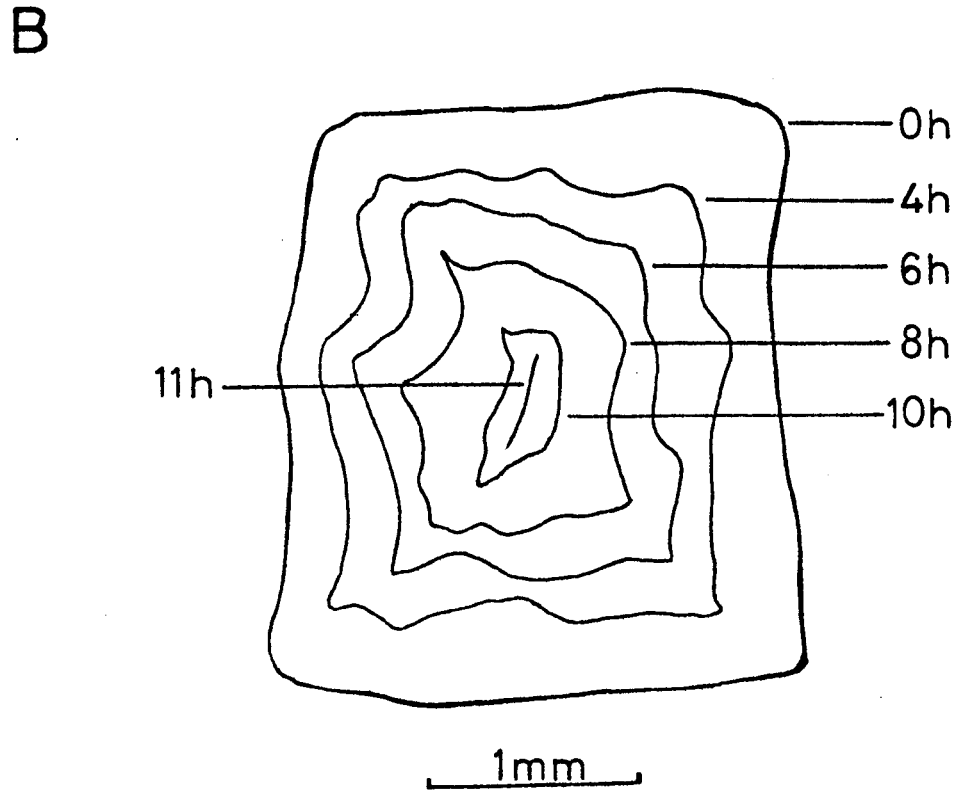
Fig. 20

Sample camera lucida drawings made during wound closure.

A. Plaice of 10 - 12 mm showing a wound immediately after skin was removed and other wounds 2, 4 and 6 h later.

B. Plaice of 22 - 26 mm showing the leading edge of the migrating epidermis at 4, 6, 8 and 10 h after skin was removed.


A
0h
2h
0h
2h
4h
6h
0h
4h
0h
6h
0·5mm


B
0h
4h
6h
8h
10h
11h
1mm

each wound the distance between the original wound edge and the edge of the epidermis spreading over the wound. The rate of movement of the epidermis was estimated from the distance covered in 4 h usually, or 10 h for the larger wounds, or from a regression of distance on time.

The larvae fixed at intervals during wound closure were superficially stained with a 50% solution of Harris' haematoxylin for 3 - 5 min to show further details of the pattern of epidermal cell movement over the wound surface. Whole mounts of the skin or of epidermis only were also prepared using a method based on that described by Gray (1973) for amphibian tail tips. The skin surrounding a wound, together with the epidermis spreading over the wound, was peeled off and allowed to dry for several hours on an albumenised slide. When stuck firmly to the slide, the skin was stained for 3 - 5 min in Harris' haematoxylin, blued in an alkaline solution of Scott's Tap Water Substitute, dehydrated in alcohols, cleared in xylene and mounted in D.P.X. Whole mounts of undamaged skin were prepared in the same way to show the structure of the normal epidermis. Some whole mounts were stained with Periodic Acid-Schiff (PAS) to show mucous cells in the epidermis. In salmon, and in plaice at metamorphosis, epidermal cell detail could not be seen if the pigmented dermis was present. Hence in these larvae a small area of epidermis only was peeled off and mounted.

2. Histological studies.

Together with a histological study of the wounds observed in living larvae during closure, an entirely histological study was made of wound closure, subsequent skin repair, and the cellular inflammatory response in yolk sac, stage 2, 4 and 5 plaice larvae. Wounds

were made by peeling back skin from an area of about 0.6 x 0.8 mm on the right side of the body just posterior to the body cavity. At least 20 larvae of each stage were wounded, held in one-third sea water at 10 - 11°C and 2 larvae fixed at intervals of 1, 2, 4, 8, 16, 32 h and 3, 5, 11 and 22 days after wounding.

Yolk sac and stage 3 herring larvae were also fixed for histological examination at intervals up to 11 days after amputation of the caudal fin, and some observations were made on the response in herring larvae to a scratch of 0.6 mm long through the epidermis.

Larvae were embedded in paraffin wax, serially sectioned at 5 - 7 μm and stained with haematoxylin and eosin and Periodic Acid-Schiff.

3. Blood cell development and the cellular inflammatory response to injury in live early stages of herring and plaice.

Using bright field illumination on a Wild M20 research microscope or a Wild M40 inverted microscope, observations were made of cells in the circulatory system of dechorionated herring embryos and of the transparent early larvae of herring and plaice. The response of inflammatory cells to injury was also observed in live newly-hatched herring and plaice larvae. An incision of about 0.15 mm long was made through the body just posterior to the yolk sac, and the larvae were examined at intervals afterwards to observe the cells in the transparent fin lumen at the site of injury. The larvae were examined in a small amount of water in a depression slide or, at higher magnification, under a glass cover slip supported by pieces of cover slip to avoid crushing the larvae.

4. Gross observations on tail fin regeneration in herring larvae.

The tail fin of yolk sac (about 8 mm long) and stage 2 herring larvae (11 - 12 mm) was cut off along a line about 0.9 mm from the tip of the fin, leaving some of the hypural fin mesenchyme intact. Larvae were held in sea water throughout the experiment. Camera lucida drawings were made and photographs taken at intervals to record the regeneration of the fin. Observations were made on 7 larvae at one and 2 days and then at 5-day intervals up to 36 days after amputation. Less frequent observations were made on 12 other larvae, one of which survived until 48 days after amputation.

B Results

1. Wound closure.

(i) The normal integument in herring, plaice, and salmon larvae.

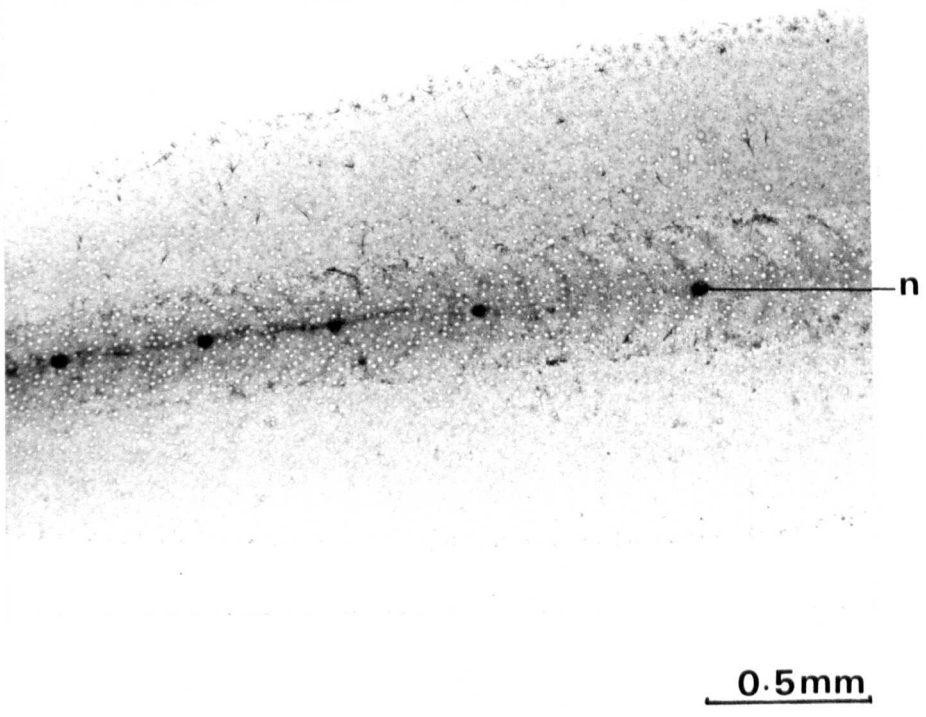
(a) Plaice: In the epidermis of 2 cells thick in yolk sac plaice larvae the epithelial cells are roughly polygonal in shape, about 7 - 15 µm in diameter over the main part of the body, and up to 35 µm in diameter where very flattened in the fin. A surface view of the skin is shown in Plate I but it is difficult to see the cell outlines. Clear round cells with eccentric elliptical nuclei, which can be seen as clear spaces in Plate I, appear to be mainly immature mucous cells; a small proportion of these cells stain PAS-positive in yolk sac larvae but the proportion staining positive increases with development. The surface of the skin is covered by strands of PAS-positive material, probably mucus. Large irregular cells, 20 - 40 µm in diameter, which usually have long extensions of cytoplasm and stain weakly with PAS are also present in the larval epidermis in the

Plate I

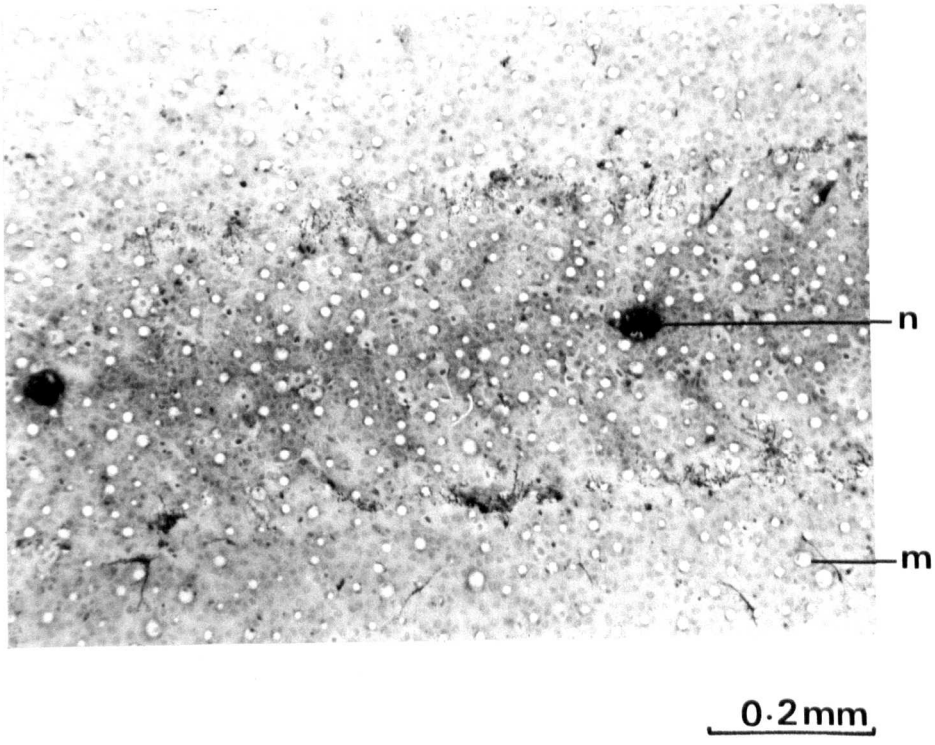
A. Whole mount of undamaged skin from one side of an early yolk sac plaice larva. Stained with haematoxylin. n, neuromast organ.

B. Higher magnification of part of (A). m, mucous cell.

A



B



yolk sac stage. They may be a different type of mucous cell or possibly the 'chloride cell' described by Roberts et al. (1973).

The epidermis increases in thickness to become 3 - 7 μm and about 3 cells thick in stage 4 larvae of 10 - 12 mm, and 7 - 14 μm, 4 - 6 cells, thick at a body length of 22 - 26 mm (Pl. IIA). Melanophores develop in the epidermis and scales in the dermis at a body length of around 20 mm.

(b) Herring: In whole mounts of the skin of yolk sac herring the epithelial cells are similar to the polygonal cells of plaice; they are 10 - 20 μm in diameter over muscle and up to 50 μm in diameter towards the edge of the primordial fin where the skin is as thin as 1.2 μm in some areas. There are no round clear cells in yolk sac herring as there are in plaice (see Pl. IIB), but there are large irregular cells of 25 x 25 μm to 40 x 30 μm containing vesicles of various sizes. They stain weakly with PAS but their nature was not investigated further. These cells are concentrated in the epidermis over the gut and also along the myotomes. There is very little mucus on the surface of yolk sac herring compared with yolk sac plaice larvae. The epidermis is up to 3.5 μm thick where only epithelial cells are present but is 6 - 8.5 μm where there are large vacuolar cells. There is no dermal space in yolk sac herring larvae, the thin basement membrane being in close contact with the muscle.

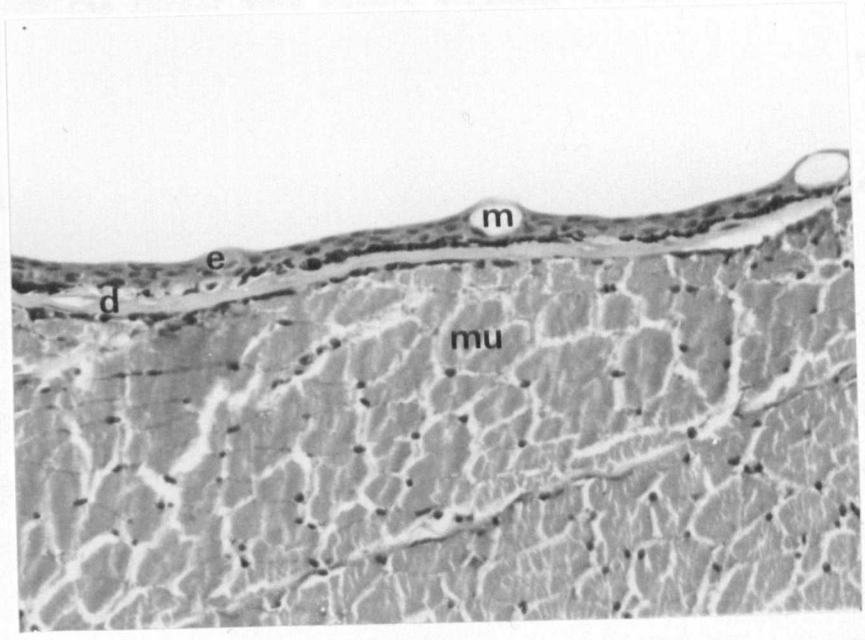
The epidermis increases in thickness during development but the epithelial cells remain very flattened parallel to the basement membrane compared with those in plaice and salmon. By stage 2 a small number of intensely PAS-positive mucous cells of about 8 - 15 μm in diameter may be seen in the epidermis, and their numbers increase

Plate II

A. Transverse section of the skin of a 22 - 26 mm plaice showing general structure. Stained with haematoxylin and eosin. e, epidermis; d, dermis; m, mucous cell; mu, muscle.

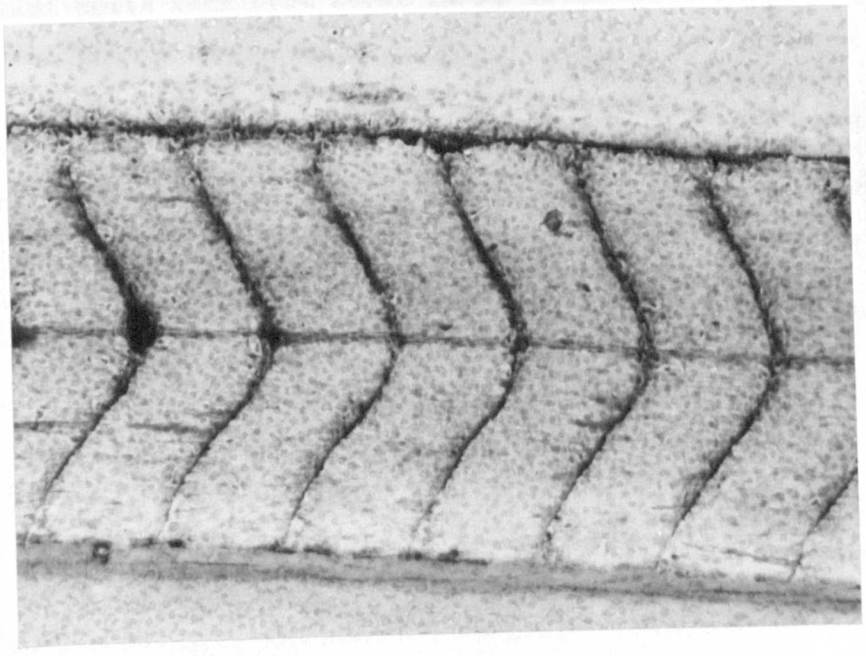
B. Whole mount of skin of a yolk sac herring larva. Stained with haematoxylin.

A



0·1mm

B



0·2 mm

with development. The mucous cells are present mainly over the gut together with the larger more weakly staining PAS-positive cells. In stage 3 larvae of 19 - 21 mm the epidermis is 3 - 4 cell layers and 3.5 - 7 μm thick. It increases to up to 35 μm in thickness by a body length of 40 mm at which stage the dermis is also well developed and about 15 μm thick. The skin remains transparent until metamorphosis when it begins to silver and scales develop.

(c) Salmon: The skin of newly-hatched salmon alevins was not examined in section, but surface staining with PAS and also with Nile blue sulphate shows that there are very large numbers of mucous cells of about 9 - 18 μm in diameter, similar to those in plaice but nearly all containing mucous. In salmon of 24 - 27 mm the epidermis is 4 - 5 cell layers and 7 - 12 μm thick and there is a dermis about 6 μm in thickness with a pigment layer in the hypodermis. There is no basal columnar layer of epithelial cells as seen in the mature epidermis. The epithelial cells vary from about 14 μm to 40 μm in diameter depending on how flattened they are. Frequent irregular shaped nuclei may be seen which are probably blood leucocytes migrating between the epidermal cells, as seen in adult salmon epidermis (Roberts, Shearer, Elson and Munro, 1970). There were no scales in the alevins studied up to a body length of 27.5 mm, and melanophores were not seen in the epidermis as in older plaice.

(ii) Extent of damage and its immediate effects.

As soon as an area of skin was removed, herring, plaice, and salmon larvae, especially the earlier stages, contracted their bodies becoming bent concavely on the wounded side so as to bring the edges of the wound closer together. The larvae became straighter, however, after recovery from anaesthesia. In the transparent early stages of

herring and plaice, the damaged area immediately became white and opaque and remained so for several days. Exposed muscle was damaged most severely at the wound edges where incisions were made while removing the skin. Wounds in yolk sac plaice and in plaice at metamorphosis are shown in Plate III to illustrate the extent of damage caused by a lesion of about 0.6 x 0.8 mm in larvae of different sizes.

Bleeding was not obvious in early herring and plaice larvae, since the blood is colourless, but the removal of skin did cause some bleeding in salmon of all stages and in plaice at metamorphosis, which have red blood. The blood clotted within a minute usually so there was never a serious bleeding. As well as cellular clots on the wound surface, congestion of cells was sometimes seen within vessels in the wound region, especially in salmon. In the later plaice and in salmon, the damaged muscle was usually covered by a thin exudate, probably of coagulated blood, and possibly containing fibrin (see Anderson and Roberts, 1975). An exudate was not obvious in early plaice but occasionally could be seen in herring of 19 - 21 mm. When only epidermis was scraped away the dermis was not penetrated so there was no muscle damage or bleeding. It was not known if the basement membrane was removed in these wounds and so the wound surface consisted of either intact dermis or of basement membrane.

(iii) Mechanism of wound closure.

The healing response of the epidermis was basically similar in all herring, plaice, and salmon larvae studied. When skin or epidermis was removed, epidermis from the wound edges spread over the wound until cells from all sides converged and the wound was completely covered. The process is easiest to illustrate in plaice at metamorphosis. Plate IV shows a wound of about 6 mm^2 in

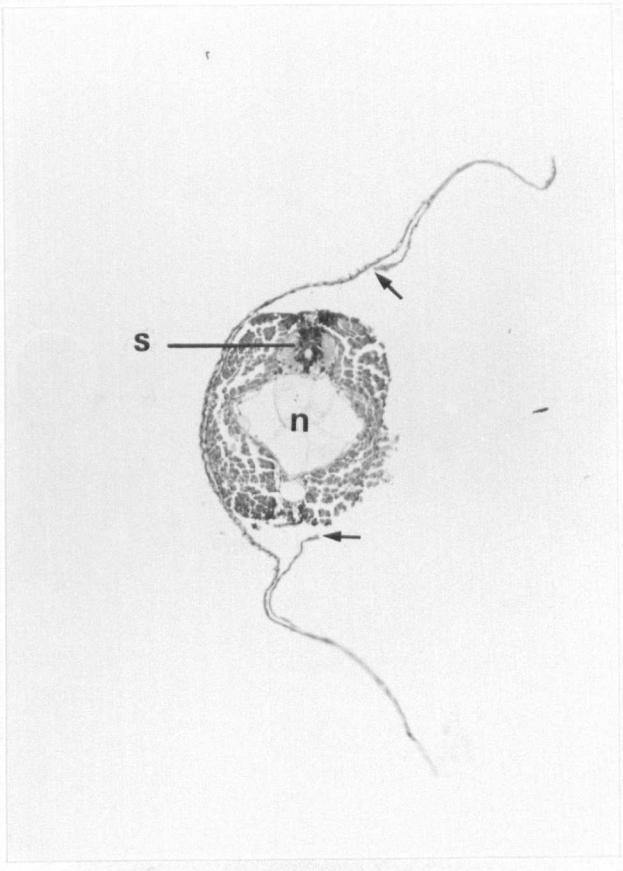
Plate III

A. Transverse section showing the extent of damage in a yolk sac plaice larva of 6.4 - 6.9 mm after skin was peeled back (from the left hand side in this case) to expose an area of muscle of about 0.5 mm^2 (0.6 x 0.8 mm).

B. Traverse section showing a similar wound of about 0.6 x 0.8 mm on the dorsal surface of a plaice of 20 - 21 mm. N.B. The fish has metamorphosed so the right hand side has become the dorsal surface.

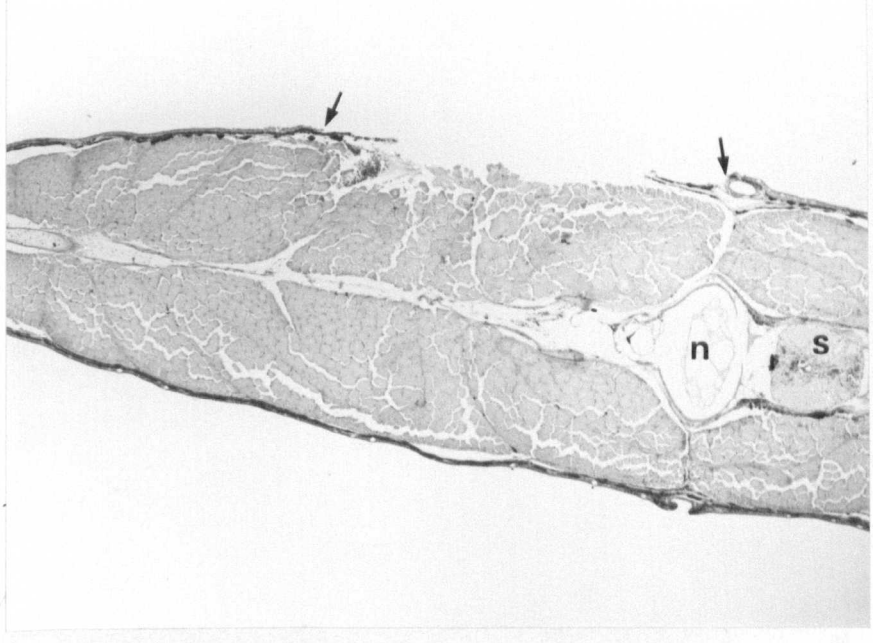
Stained with haematoxylin and eosin. The edges of the cut epidermis are arrowed. n, notochord; s, spinal cord.

A



0·2 mm

B



0·5 mm

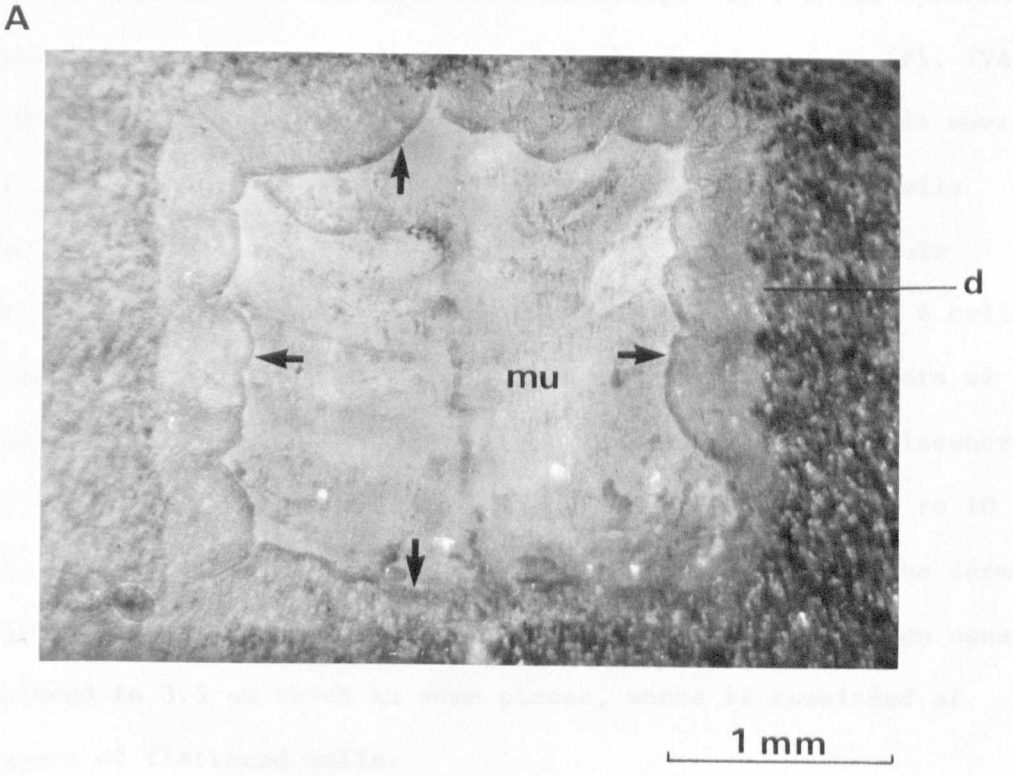
Plate IV

Surface view of a wound of about 6 mm^2 (2.5 x 2.5 - 3 mm) in plaice of 22 - 26 mm.

A. Two h after wounding. Epidermis from the wound edges has started to advance over the exposed muscle.

B. Ten h after wounding. The wound is almost covered by the migrating epidermis. Melanophores and mucous cells can be seen in the migrating sheet.

Whole larvae superficially stained with haematoxylin. Leading edges of the migrating epidermis are arrowed. d, edge of cut dermis which marks the original wound edge; mu, exposed muscle; me, melanophore.


A
d
mu
1 mm
B
d
mu
me
1 mm

plaice of 22 - 26 mm at 2 and 10 h after wounding. By 2 h the epidermis had already advanced for some distance over the wound surface (Pl. IVA), and by 10 h the wound was almost covered (Pl. IVB). The epidermis moved across a smooth wound exudate (Pl. VA) as a cohesive sheet of cells which included melanophores, mucous cells and eosinophilic granule cells as well as epithelial cells. The normal epidermis of 4 - 6 cells thick spread out in some areas of the wound to become 2 - 3 layers of flattened cells, about 2 - 3 μm in total thickness (Pl. VB). Elsewhere over the wound it was often thicker than normal, ranging from 4 to 10 cells, 5 - 30 μm thick (see Pl. VA). Behind the cut edges of the dermis in the area surrounding the wound, the epidermis was thinner than usual, being reduced to 3.5 μm thick in some places, where it consisted of 3 - 4 layers of flattened cells.

At the leading edge of the migrating epidermis, some epithelial cells spread out delicate extensions of cytoplasm onto the wound exudate (Pl. VIA); others were aligned parallel to the advancing edge and tended to pile up in folds several cells deep. Further back from the advancing edge most cells were elongated and radially aligned. They were often about 2 - 5 times as long as broad but in some areas even more streamlined, reaching lengths of up to 35 μm, by 2.5 - 5 μm wide (Pl. VIB). Normal epithelial cells measured about 9 - 17 μm in diameter. Melanophores in the migrating epidermis were also radially arranged, especially where there was streamlining of epithelial cells. Darkly staining elongated ridges could be seen on the surface of many of the elongated epithelial cells. They appeared to be associated with the change in shape of cells during cell movement.

Plate V

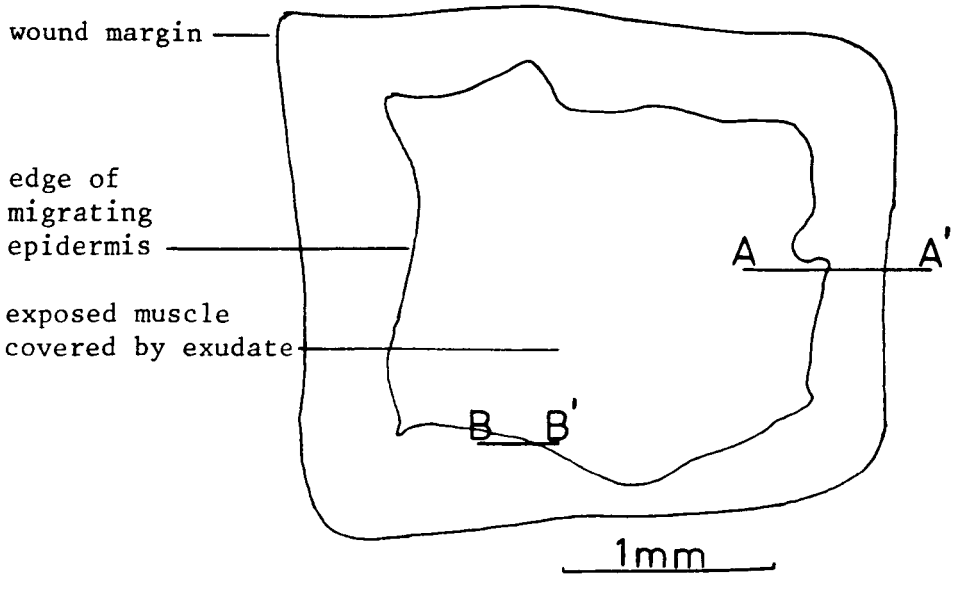
Transverse sections through a wound similar to that shown in Plate IV in plaice of 22 - 26 mm at 4 h after wounding. The drawing below shows where the sections were made. Stained with haematoxylin and eosin.

A. Epidermis of several cell layers has migrated from the edge of the wound across an exudate. Note that the epidermis migrated down into the incision at the wound edge. The leading edge of the migrating epidermis is arrowed. As shown below the right hand margin of the wound is to the right of the area shown in the Plate.

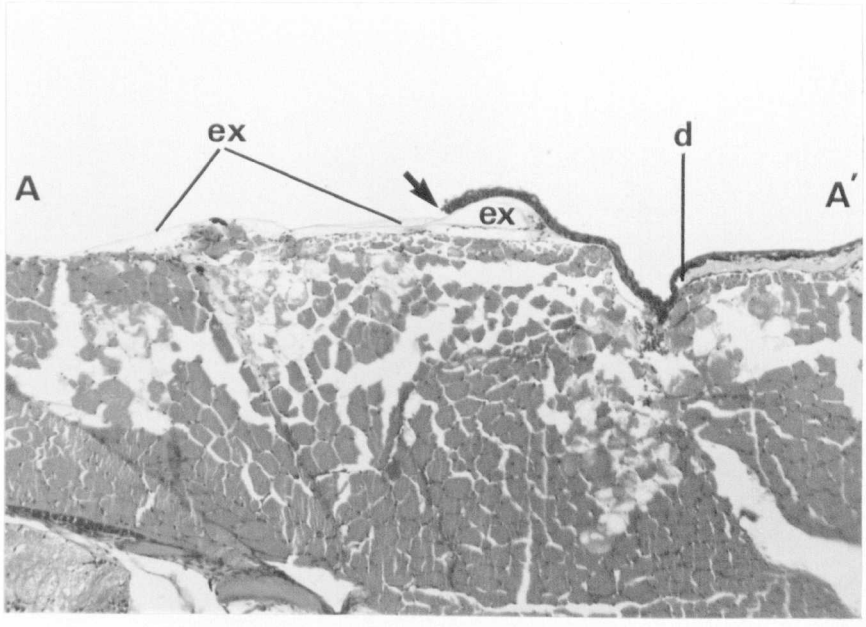
B. Shows thin epidermis of about 2 cell layers also migrating over an exudate.

ex, exudate; d, edge of cut dermis

Diagram showing surface view of whole wound. The line marked A shows where the section in Plate VA was cut, and that marked B shows where the section in Plate VB was cut.

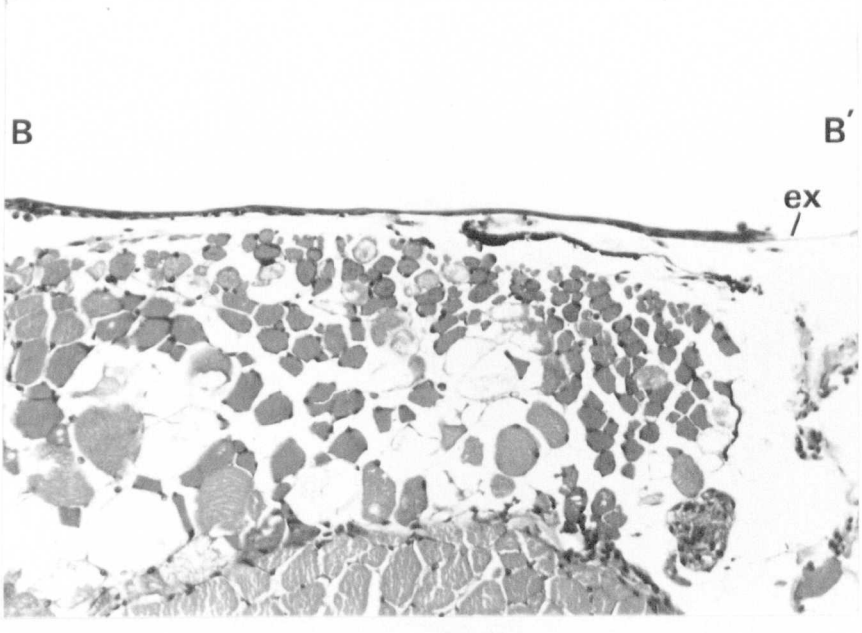


A



0·2 mm

B



0·1 mm

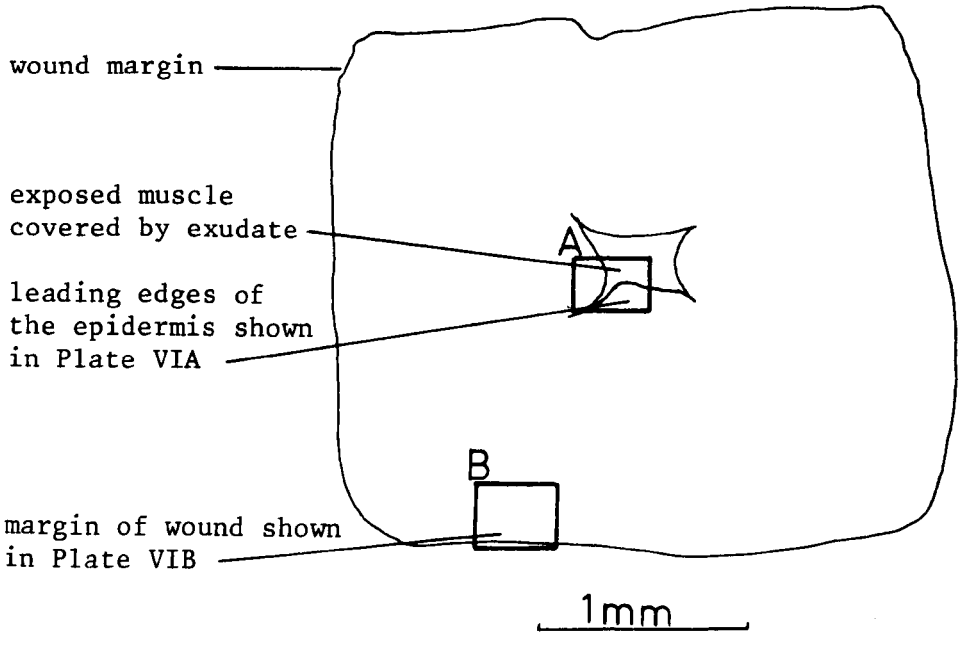
Plate VI

Surface view (whole mount) of epidermis migrating over a wound similar to that shown in Plate IV in plaice of 22 - 26 mm at 10 h after wounding. Stained with haematoxylin.

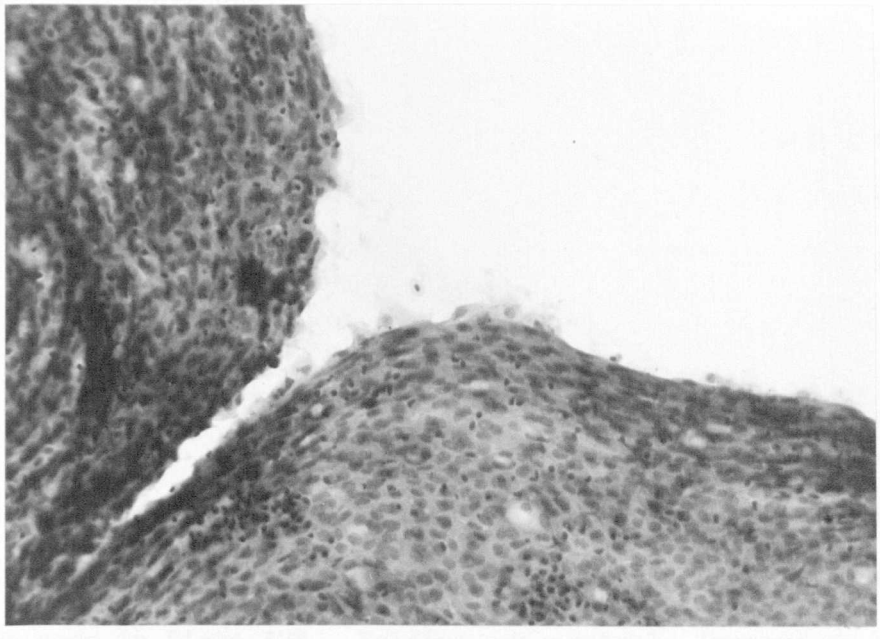
A. Leading edges (see below) of the migrating epidermis showing flattened epithelial cells (possibly phagocytic) with cytoplasm extended onto the wound exudate. The exudate was disrupted when the whole mount was prepared.

B. Epidermis near the original edge of the wound showing streaming of migrating cells. m, mucous cell.

Diagram showing surface view of whole wound from which Plates VIA and B were taken.

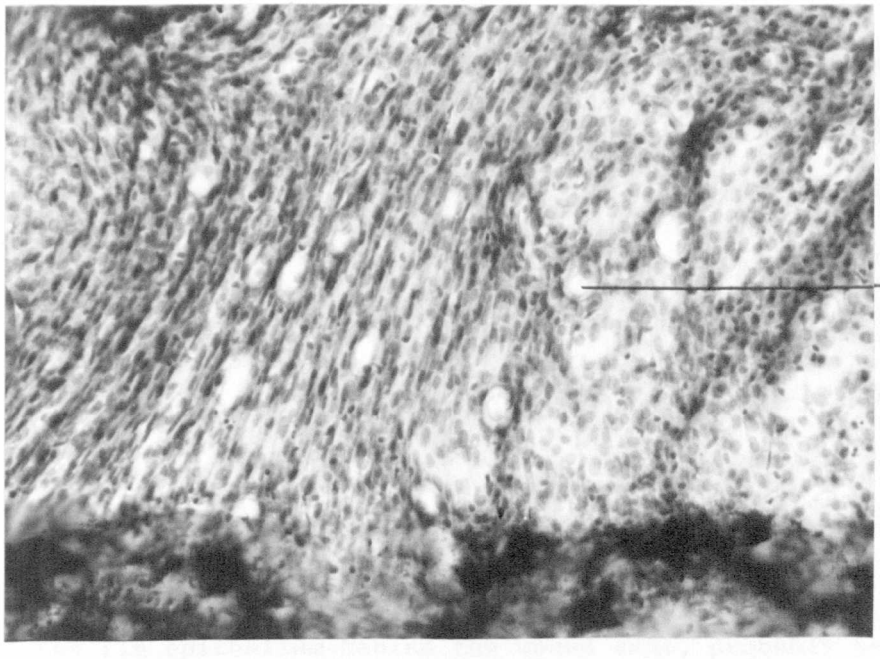


A



0·1 mm

B



0·1 mm

In whole mounts it was difficult to see cell detail in the epidermal cells surrounding wounds because of the thickness of the dermis in plaice of 22 - 26 mm. It was clear, however, that some cells behind the wound edge were just as elongated as those migrating over the wound surface, especially behind the most elongated cells over the wound. The epidermis surrounding wounds was not examined for mitotic figures in plaice of this size. Mitoses were only rarely seen in the sheet of cells which migrated over the wound surface. In wounds in plaice of 22 - 26 mm, and in all other wounds observed in whole mounts, it was only possible to look for mitoses in epidermis which was spread out and not contracted into ridges and folds such as may be seen in Plate IVB. Folding was probably imposed on the migrating epidermis by the reduction in length of the perimeter of the wound with the decrease in wound area during closure.

The pattern of epidermal healing was similar in small wounds of about 0.1 mm^2 in yolk sac plaice larvae (Pl. VII) but here the migrating epithelium was thinner, often only 1 cell thick. Examination of whole mounts of skin of 5 larvae during wound closure (at 2, 3 and 4 h) showed that there was, at most, 2 obvious mitotic figures in the whole area of epidermis within a radius of about 0.5 mm from the edge of wounds of 0.1 mm^2. The wounds were thus covered almost entirely by cells which spread from the existing epidermis around the wound margin rather than by cells provided by a 'mitotic burst'. The stretching and radial orientation of these cells are shown in Plate VIII. The changes in cell shape were most marked in the fin epithelium behind the wound edge, probably because even in the normal epithelium cells were especially flattened and the epidermis was very thin in this region.

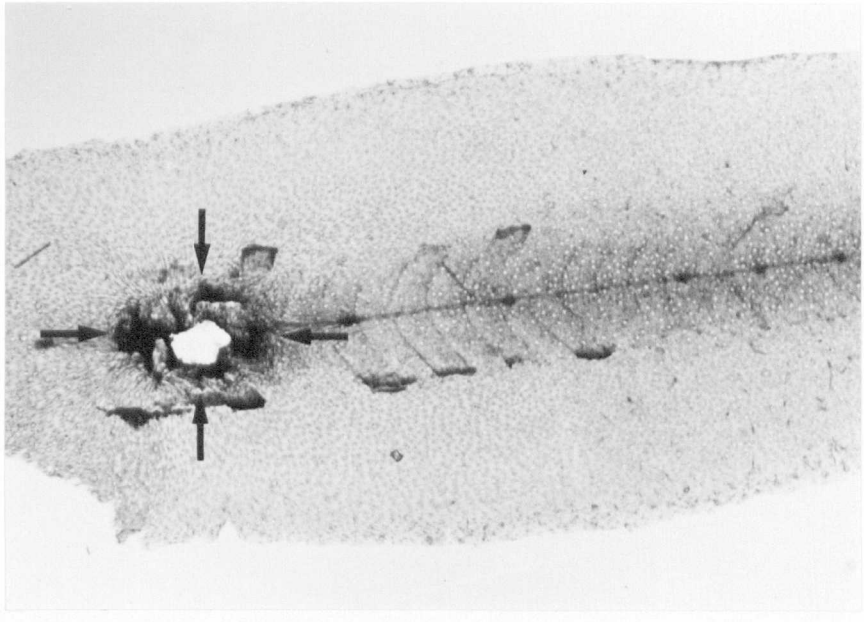
Plate VII

Whole mounts of the skin of yolk sac plaice larvae showing a wound of about 0.1 mm^2. Stained with haematoxylin.

A. After 2 h. The dark zone surrounding the open area is the epidermis that has migrated over the wound. Approximate edges of the cut dermis are arrowed.

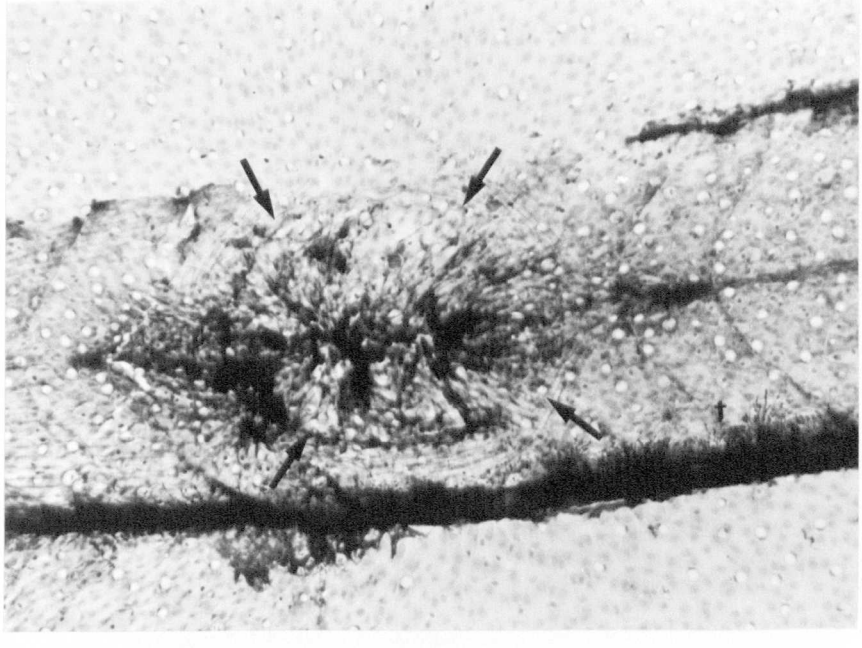
B. After 4 h at a higher magnification. The wound is closed. Note the concentric rings which appear to be due to retraction of the dermis after wounding; they mark the original edge of the wound.

A



0·5mm

B



0·2mm

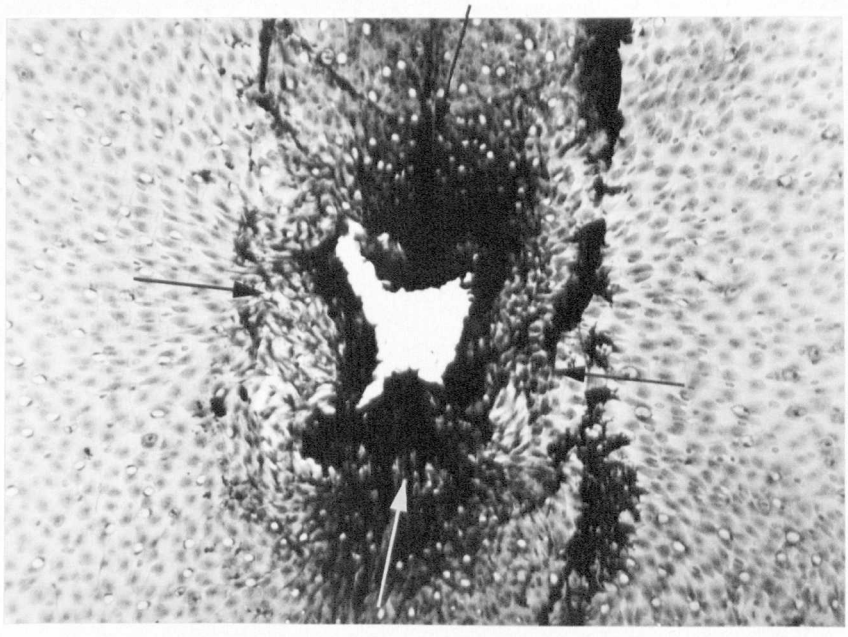
Plate VIII

A. Whole mount similar to those in Plate VII at 3 h after wounding in yolk sac plaice, showing the recruitment of cells from the epidermis surrounding the wound.

B. Higher magnification of migrating epithelial cells in the area surrounding the wound.

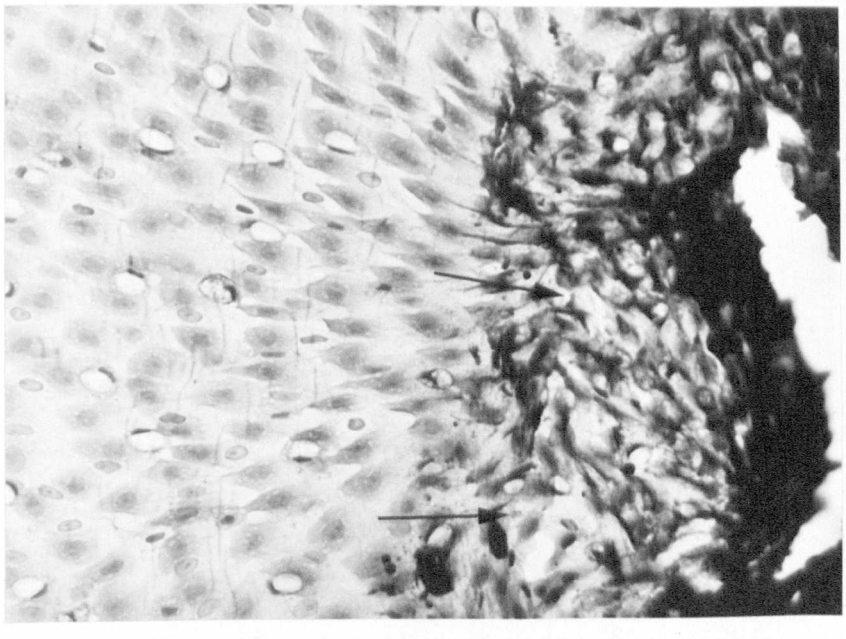
Stained with haematoxylin. Approximate original edges of the wound are arrowed.

A



0·2 mm

B



0·1 mm

Examination of whole mounts of skin of plaice of 10 - 12 mm and herring of 19 - 21 mm also showed that there were not enough mitoses, either in the epidermis migrating over the wound surface or in that at the wound margins, to provide new cells to cover wounds. In plaice of 10 - 12 mm, when the epidermis was scraped away from an area of about 0.4 mm^2 (0.6 x 0.6 mm) the denuded area was covered within an hour by cells which flowed in from the wound edges (Pl.IXA). A decrease in density of cells in the epidermis surrounding the wound indicated that the cells which covered the wound had migrated from within a radius of 0.6 mm from the wound edge. It was not possible to count numbers of cells per unit area because the epidermis was several cells thick, but the area from which cells appeared to have migrated into the wound was lightly stained compared with the undisturbed epidermis. In herring larvae, cells surrounding wounds of 0.5 - 1 mm^2 lost their normal orderly arrangement for distances of up to 1.4 mm from the wound edge during closure (see Pl. IXB). The cells were not usually orientated in any special direction but there was a densely staining concentration of cytoplasm around the nucleus. It could be seen from sections of such wounds that the surface layer of cells seemed to become detached from the lower layer, and there were only thin cytoplasmic connections between cells within each layer. Although such changes in cell shape and organisation could have been caused by osmotic changes or some other direct effect of damage, it seems likely that the zone of reorganisation around wounds in herring marked the area from which cells were "recruited" to cover the wounds.

The observations on the migration of cells from a wide area around wounds are in keeping with similar observations on the closure of wounds in amphibian larvae. Herrick (1932), by looking at the elongation of

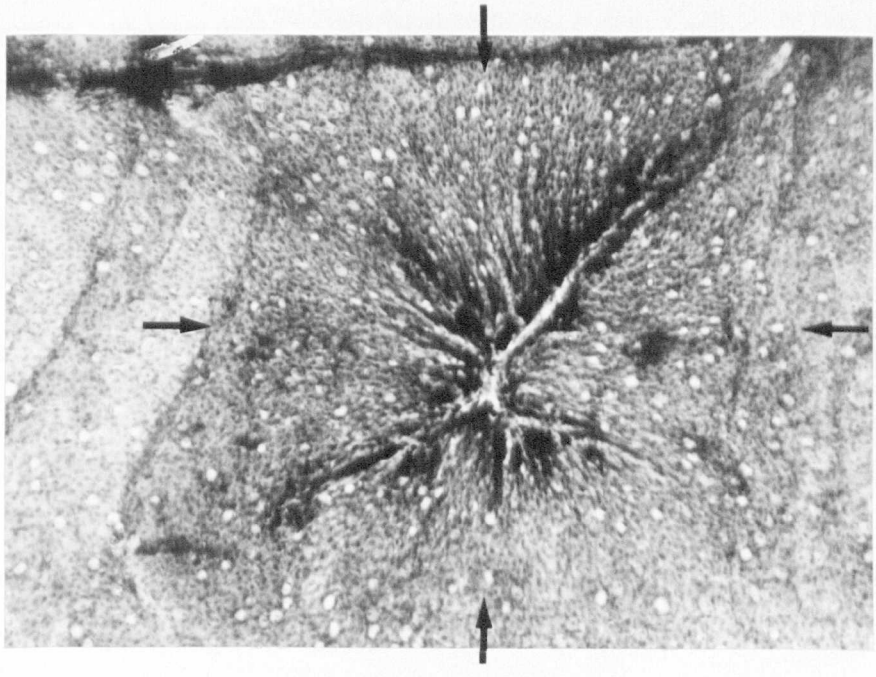
Plate IX

A. Whole mount of the skin of plaice of 10 - 12 mm showing a closed wound 1 h after epidermis was scraped from an area of about 0.4 mm^2. The original edges of the wound are arrowed. The lightly staining area outside the wound is the area from which cells have emigrated into the wound.

B. Whole mount of skin of herring of 19 - 21 mm showing the reorganisation of epidermal cells around a wound after 4 h. (The wound is not shown because it was not possible to remove the wound epidermis without tearing it.) Approximate wound margin is arrowed. t, torn epidermal edge; r, recruitment zone of reorganised cells; n, normal undisturbed epidermis.

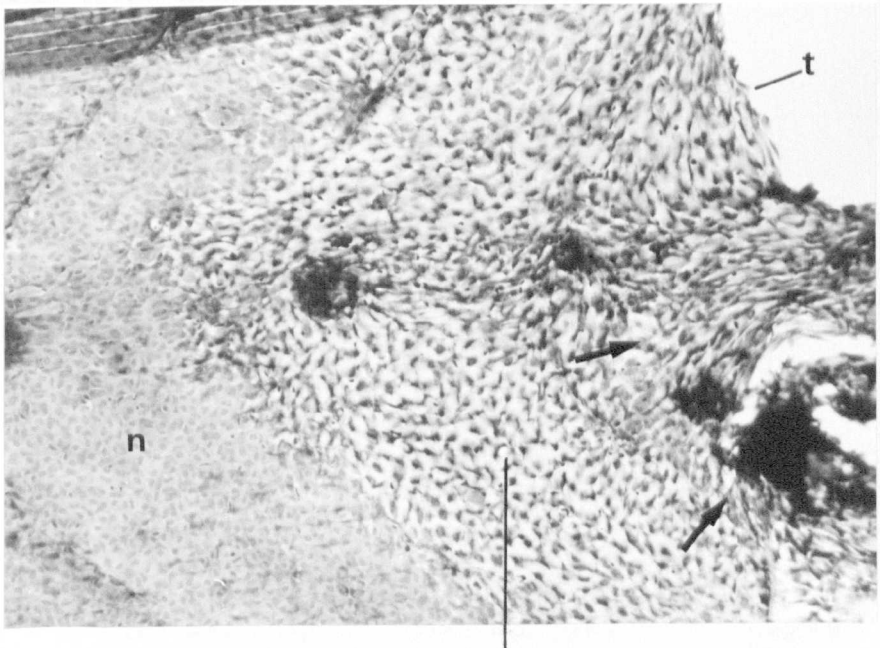
Stained with haematoxylin.

A



0·2mm

B



0·2mm

cells, thinning of epidermis and radial arrangement of melanophores around wounds, showed that cells migrated from distances of up to 5 mm to cover wounds of 5 - 10 mm across in frog (Rana clamitans) tadpoles. Lash (1955) marked individual cells in the epidermis of urodele larvae and followed their movements to cover wounds of 1 mm^2. He found that by the second hour after wounding cells had become detached from the basement membrane for distances of 0.21 - 2.80 mm from the wound edge, indicating that cells migrated towards the wound from these distances.

Most observations, such as those described so far, were made on the healing response of the epidermis in non-lethal wounds. Wounds which were extensive enough to be lethal did not heal. This was especially noticeable in moribund salmon after lethal wounds were inflicted in river water; the alevins became pale and the epidermis formed a thick rim at the wound edge instead of migrating across the wound. Some large wounds of 35 - 40 mm^2 which were lethal for salmon in 8‰ salinity were partially covered by epidermis when alevins died at 4 days after wounding, but other lethal wounds in this salinity did not heal at all.

(iv) Rate of wound closure.

Typical results are plotted in Figs. 21 and 22 in which the advance of epidermis over the wound surface and the decrease in wound area with time are shown for plaice of 22 - 26 mm (see Pl. IV) and salmon of 24 - 27 mm. In Fig. 21, the slower advance of epidermis in the first 4 h after wounding may have been because the epidermis migrated down into the incisions at the wound edge rather than because there was a lag before migration began. The results of most observations of wound closure are summarised in Table 3 in which healing

Fig. 21

Wound closure in plaice of 22 - 26 mm.

A. Decrease in wound area with time.

B. Movement of epidermis over the wound surface.

The different symbols represent the area of wound in 3 different fish and the mean distance covered by the epidermis in the same 3 fish. The curves are drawn through the mean for the 3 points.

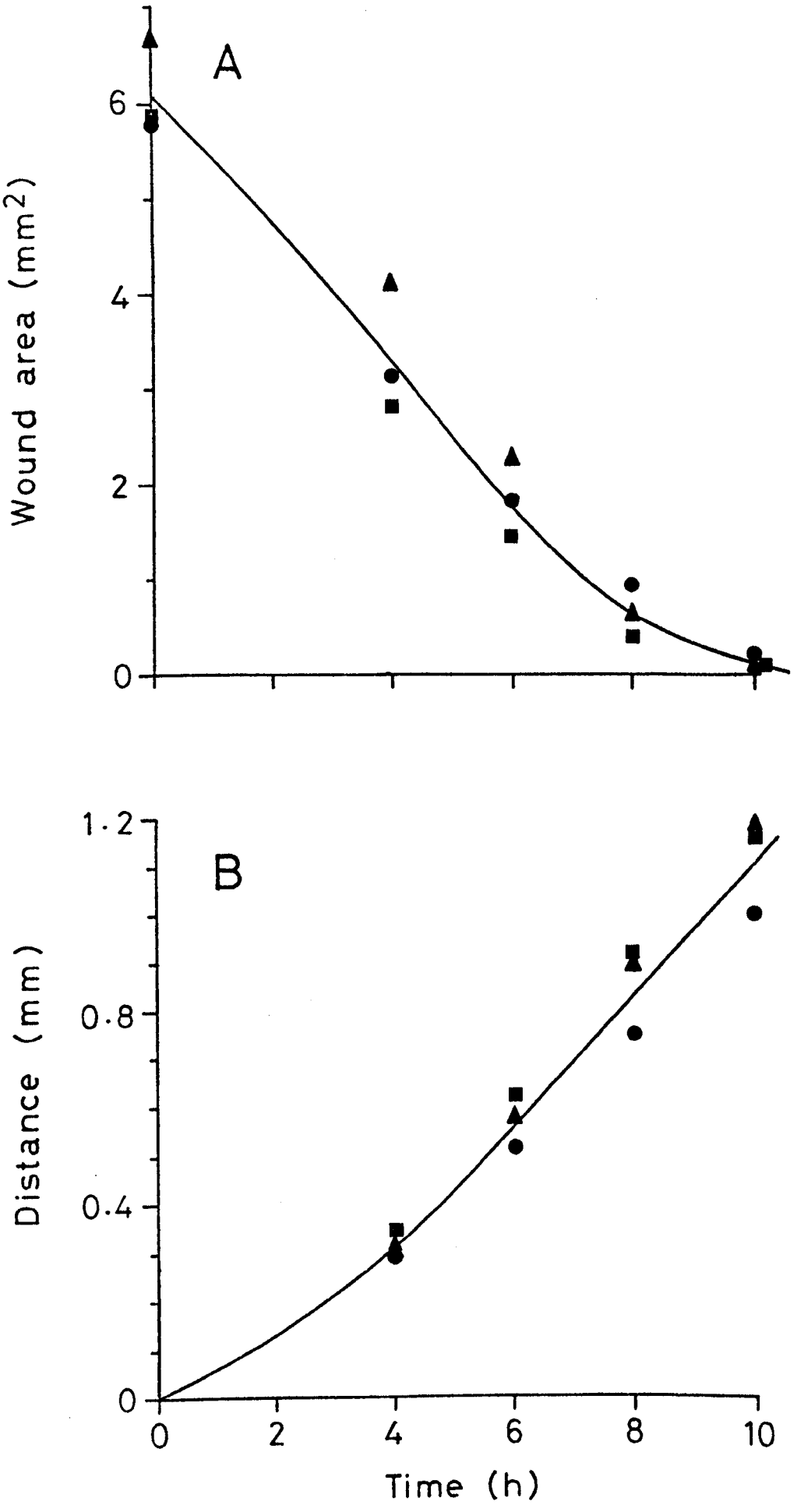

A
Wound area (mm²)
6
4
2
0
B
Distance (mm)
1·2
0·8
0.4
0
0
2
4
6
8
10
Time (h)

Fig. 22

Wound closure in salmon of 24 - 27 mm.

A. Decrease in wound area with time.

B. Movement of epidermis over the wound.

Each symbol represents the area of one wound and the mean distance covered by epidermis in that wound in a given time, except ▼ which is the mean area of 3 wounds at 0 and 5 h and the mean distance covered for each of the 3 wounds in 5 h.

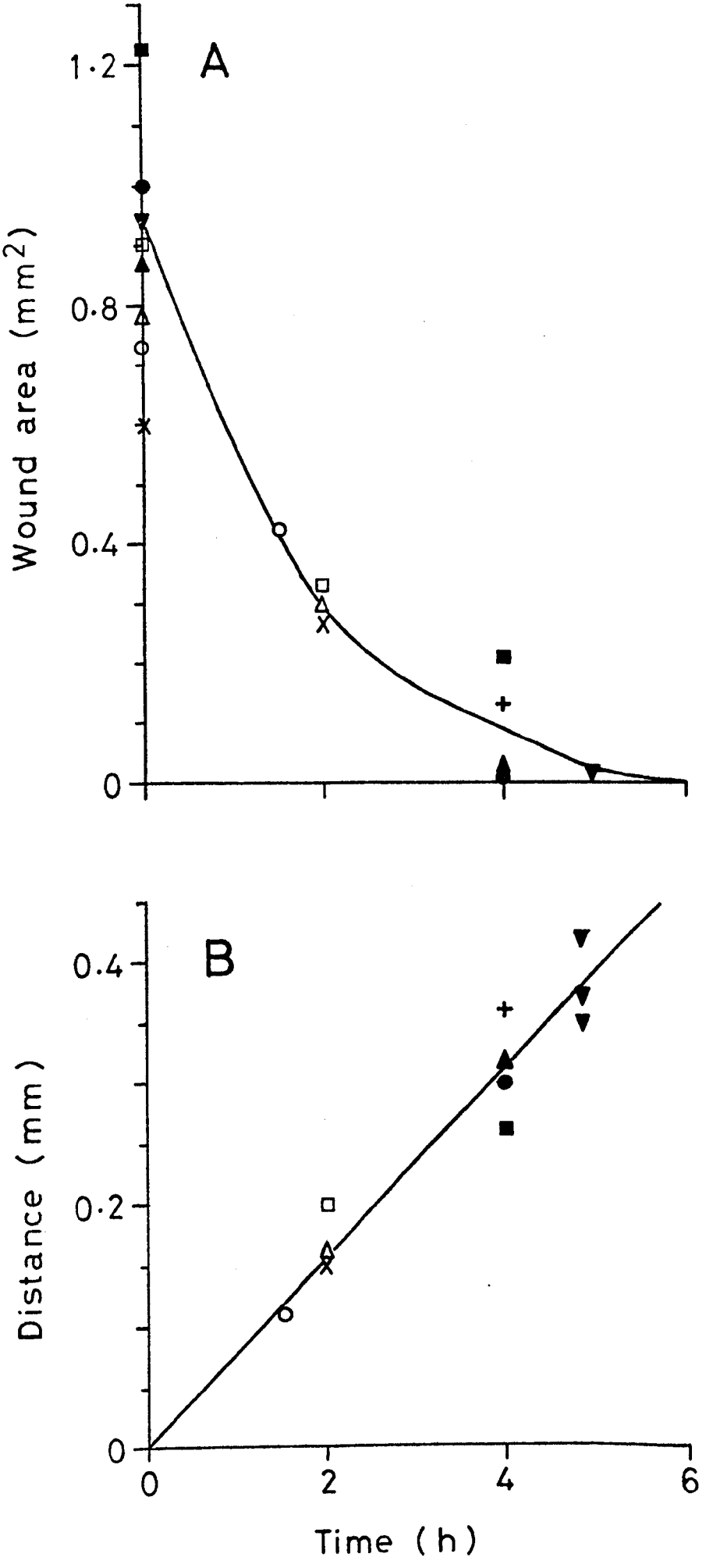

A
1·2
0·8
0·4
0
Wound area (mm²)
B
0·4
0·2
0
Distance (mm)
0
2
4
6
Time (h)

Table 3. Rate of wound closure in plaice, herring, and salmon larvae.

	Wound type	Temp. °C	Total no. of observations*	Wound area (mm^2)	Healing time (h)	Rate of epidermal movement: No. of observations**	Mean µm/h	Range µm/h
Plaice								
6.5-7 mm	skin	10-11	12	0.1-0.2	4	-	-	-
10-12 mm	skin	10-11	32	0.4-0.6	6-10	4	60	20-80
	epidermal	10-11	1	0.4	1	1	310	260-360
21-25 mm	skin	10-11	16	0.5-1.0	4-5	4	90	50-130
22-26 mm	skin	10-11	18(a)	5.8-6.6	11-12	3(b)	110	80-150
29-35 mm	skin	5-6	18(a)	7.3-9.8	-	18(c)	70	30-80
	skin	14.8-15.8	17(a)	6.3-8.3	-	17(c)	130	70-160
Herring								
19-21 mm	skin	10-11	32	0.4-0.6	4-6	4	70	30-130
	epidermal	10-11	1	0.4	-	1	290	260-330
Salmon								
24-27 mm	skin	10-11	32	0.4-0.6	4-8	4	80	50-120

* Each observation was made on a different fish except in cases marked (a) in which 3 fish were examined at 5 or 6 intervals (see Fig. 20).

** Rate of movement in skin wounds was usually estimated from distance covered in 4 h (in 4 fish), but from distance covered in 10 h (in 3 fish) in the case marked (b) and from a regression of distance on time (for 3 fish) in the cases marked (c). The distance covered in 1 h was measured in epidermal wounds. In all cases 8 measurements were made for each observation and the mean and total range of these is given.

times and rates of epidermal migration are given for plaice of different ages and for herring and salmon larvae. The range in healing time shown is the time at which at least one of the wounds sampled was closed to the time at which all the wounds sampled were closed. Variation in the time to complete closure within a particular group of larvae and in the rate of epidermal migration even within one wound was caused mainly by irregularities in the wound surface. For example, deep incisions, damaged muscle fibres and possibly blood clots tended to obstruct the advance of epidermis from one or more sides of the wound. Wounds made by cutting out an area of skin of full thickness are referred to as "skin" wounds, and those made by removing only epidermis are termed "epidermal" wounds. During closure of the skin wounds the epidermis migrated over exposed muscle or wound exudate and during closure of epidermal wounds it migrated over basement membrane or the surface of the dermis.

(a) Effect of larval age and species: It may be seen from Table 3 that skin wounds of 0.4 - 1 mm^2 closed somewhat more rapidly in older than in younger plaice, the epidermis migrating at an average rate of 60 µm/h (range 20 - 80 µm/h) in plaice of 10 - 12 mm, and at 90 µm/h (range 50 - 130 µm/h) in plaice of 21 - 25 mm at 10 - 11°C. In yolk sac plaice of 6.5 - 7 mm, smaller skin wounds of 0.1 - 0.2 mm^2 closed in 4 h at 10 - 11°C. Camera lucida drawings were not made of these wounds during closure and so the rate of epidermal migration was not calculated in the usual way. It can be estimated, however, that to cover the wounds which were 0.3 mm wide the epidermis migrated for 0.15 mm from each side of the wound at about 40 µm/h. This rate cannot be directly compared with the rates shown for older larvae, however, since wound size may influence the rate of epidermal advance. The

faster rate of closure in older larvae may have been due to several factors. First, since the normal epidermis increases in thickness with age there was a greater reserve of cells available for migration from the wound edges in the older larvae. Secondly, the wound surface on which the cells moved was probably more irregular in the younger larvae, because the removal of skin usually caused more severe muscle damage and there appeared to be relatively little wound exudate in these larvae. In plaice of 22 - 26 mm at 10 - 11°C the epidermis seemed to advance at a slightly faster rate (average 110 µm/h) over skin wounds of 5.8 - 6.6 mm^2 than over smaller wounds of 0.5 - 1 mm^2, possibly because the muscle in the centre of the larger wounds was hardly damaged at all and thus the wound surface was smoother.

There were no major differences between rates of healing in the different species. The rate of epidermal advance at 10 - 11°C in herring of 19 - 21 mm, at an average of 70 µm/h (range 30 - 130 µm/h), was intermediate between that in plaice of 10 - 12 mm and plaice of 21 - 25 mm. The rate in salmon of 24 - 27 mm at 10 - 11°C, an average of 80 µm/h (range 50 - 120 µm/h), was about the same as that in plaice of 21 - 25 mm.

(b) Effect of temperature: In plaice of 29 - 35 mm, the rate of epidermal migration over wounds of 6.3 - 9.8 mm^2 was almost doubled by a rise in temperature of about 10°C; the average speed at 5 - 6°C was 70 µm/h while that at 14.8 - 15.8°C was 130 µm/h (Fig. 23 and Table 3). In this experiment wounds were observed only for 10 h and not until complete closure. Healing times are therefore not given in Table 3. Lash (1955 and 1956) observed a similar effect of temperature on the rate of closure of wounds made by removing skin in urodele larvae. He

Fig. 23

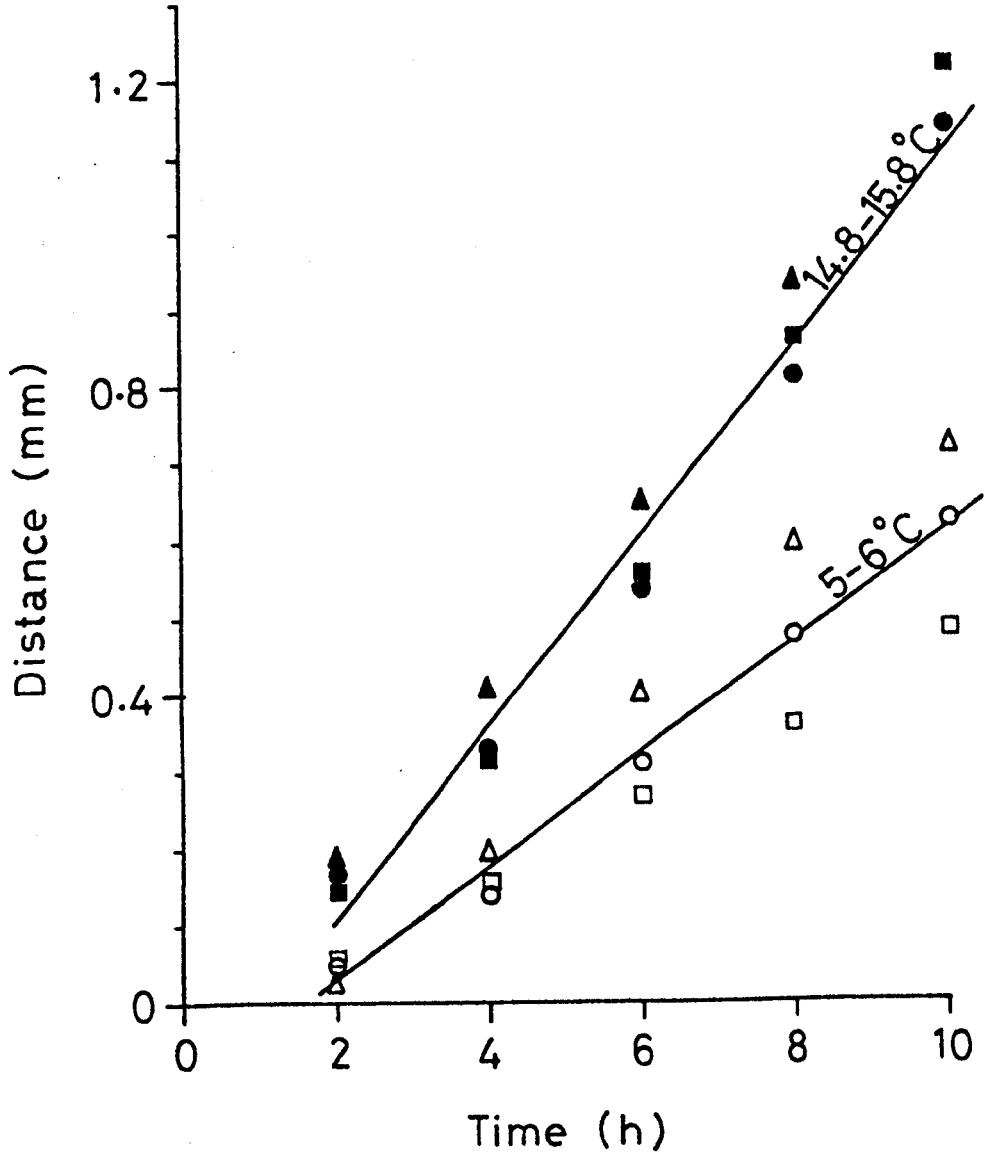
Effect of temperature on rate of wound closure in plaice of 29 - 35 mm. Movement of epidermis is plotted for 3 different fish at 5 - 6°C and 14.8 - 15.8°C. A different symbol represents the mean distance covered by epidermis in each fish. Regressions of distance on time were significant ($p < 0.001$). Equations for the lines in the form $\hat{y} = a + bx$ and standard errors of the slopes ($S.E._b$) are given.

At 5 - 6°C $\hat{y} = -0.066 + 0.067x$, $S.E._b = 0.007$

Rate of cell migration = 0.067 ± 0.007 mm/h

At 14.8 - 15.8°C $\hat{y} = -0.124 + 0.126x$, $S.E._b = 0.006$

Rate of cell migration = 0.126 ± 0.006 mm/h


1·2
0·8
0·4
0
Distance (mm)
0
2
4
6
8
10
Time (h)
14.8–15.8°C
5–6°C

found that the average rate of epidermal movement over wound coagulum was 20 µm/h at 10°C; 100 µm/h at 20°C; 120 µm/h at 25°C; and 220 µm/h at 30°C. Anderson and Roberts (1975) have also reported an increase in the rate of wound closure with increasing temperature in salmon (Salmo salar) and in the tropical white mountain cloud minnow (Tanichthyes albonubes). Incisions 1 mm deep and 4.5 mm long in the skin of salmon closed within 3 h at 23°C and within 24 h at 5°C. Bullock, Marks and Roberts (in press) found that incisions of 5 mm x 1 mm in juvenile plaice closed in 9 h at 10°C and 12 h at 5°C.

(c) Effect of wound substratum: Although few wounds were made by removing epidermis only the results in Table 3 suggest that the most important factor influencing the rate of epidermal migration was the type of substratum over which the cells moved. When epidermis only was scraped from an area of about 0.4 mm^2 in plaice of 10 - 12 mm and herring of 19 - 21 mm, the epidermis from the wound edges spread over the denuded dermis or basement membrane at an average rate of 290 µm/h in herring and 310 µm/h in plaice, roughly 4 and 5 times as fast as over exposed muscle in herring and plaice respectively. Lash (1955) also found that in urodele larvae at 25°C, epidermis moved at an average rate of 250 µm/h over denuded basement membrane, twice as fast as over the coagulum which filled wounds when skin was removed. Cells can probably spread rapidly over basement membrane because it provides a substratum free of obstructions to cell movement.

Apart from the results shown in Table 3, some observations were also made on epidermal healing in larger skin wounds in salmon during experiments on survival (Section III). In alevins of 24 - 27.5 mm held in 8‰ salinity, even wounds of 25 mm^2 (about 12.5 x 2 mm) were

covered by epidermis within a day after skin was removed. The time to healing was probably determined mainly by the breadth of the wound, epidermis migrating about 1 mm from each side to cover a wound 2 mm wide. In river water it seemed that healing was sometimes delayed in wounds just smaller that the lethal area. In salmon of 26 - 27.5 mm, wounds of 5 mm^2 (about 4 x 1.25 mm) healed within 16 h and all 10 wounded alevins survived (see Fig. 18) and swam actively. Wounds of 6 mm^2 (about 4.8 x 1.25 mm) killed 2 out of 10 alevins, however, and at 16 h in the 8 surviving larvae, 4 wounds were closed and 4 wounds were almost but not fully closed. The alevins with healed wounds were normal in colour and swam actively, whereas those with only partially healed wounds were darker than normal and could not swim properly. A dark colour is often a sign of osmotic imbalance. All the 8 alevins with lesions of 6 mm^2 did recover, however, and survived for 10 days. Wounds of 7 mm^2 (about 5.6 x 1.25 mm) killed 9 out of 10 alevins within a day (see Fig. 18) and the wounds never closed. It seemed that healing of the wounds of 6 mm^2, together with osmotic regulation, allowed most of the alevins to recover from the osmotic stress, but the removal of 7 mm^2 of skin probably caused a greater dilution of the body fluids than the tissues could tolerate and therefore the alevins became moribund and the wounds could not heal.

The rates of wound closure seen in larvae in the present study are within the range of rates which can be calculated from data on epidermal migration in adult fish. Berlin (1951) found that in the loach (Misgurnis fossilis) wounds made by cutting out an area of skin of 5 x 5 mm to 10 x 10 mm were covered by epidermis within 24 - 36 h (no temperature cited). The possible range in the rate of epidermal movement was therefore around 70 - 200 µm/h, assuming that epidermis

from each edge migrated over about half the width of the wound. Kearn (1967) observed that when scales were removed from the common sole (Solea solea) and kept in sea water, epidermal cells which were removed with the scale began to spread from the tip of the scale over the scale lamina in a way typical of the response to wounding. At 17°C the epidermis migrated across the scale surface in a linear direction at about 300 - 400 μm/h (my calculation); this was similar to the rate of movement of larval epidermis over denuded dermis or basement membrane at 10 - 11°C in the present study.

(v) Repair of skin after wound closure.

Some observations were made on the surface of healing wounds after they had been covered by epidermis, mainly in herring and plaice larvae kept for some time after areas of skin had been removed for the experiments on survival described in Section III. Wounds in yolk sac and stage 2 herring larvae could still be distinguished under a dissecting microscope 20 days after wounding because the damaged area appeared "granular" compared with the surrounding translucent tissues, and also because the notochord in the damaged area was either slightly thickened or filled in by tissue and was therefore opaque. Wounds in yolk sac plaice, on the other hand, were not at all obvious even by 10 days after wounding, but there were usually some scattered melanin granules in the wound region, and in both herring and plaice larvae there were often 3 or 4 melanophores or melanocytes on the surface of the notochord. Such melanin-containing cells are often found in fish lesions (see Roberts, 1975). The skin of salmon was pigmented even in newly-hatched alevins and so removal of skin left an unpigmented area which was still visible at 10 days; later wounds were not examined.

When skin was removed from plaice of 10.5 - 12 mm before the dermis was pigmented, the wound area developed pigment at the same time as the surrounding skin so that by 23 days the wound was superficially indistinguishable from the normal skin except that there was no lateral line. On the other hand, when skin was removed from plaice of 14 - 18 mm after pigment had developed in the dermis, the wound area was unpigmented after 23 days. It had developed pigment by 30 days but even at 60 days the lateral line did not appear to have regenerated.

Surface observations showed nodules of dark pigment (probably melanin) in many wounds after 20 - 30 days in plaice of 10.5 - 12 mm and 14 - 20 mm. Superficial staining of whole larvae with haematoxylin showed that most pigment was in the area where cells had converged during closure and so the melanin may have been associated with necrosis of epidermal cells which had accumulated there. Melanin is often associated with lipid residues from the breakdown of cell membranes (Edelstein, 1971). Surface staining with PAS also showed that mucous cells were concentrated in the area where the epidermal sheets had converged.

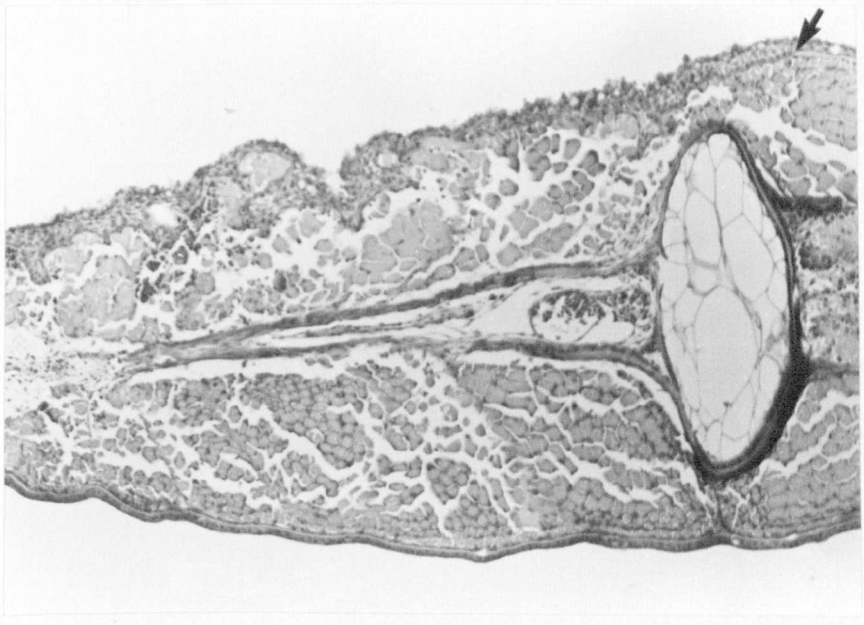
Some histological observations were also made in plaice of 7 - 8.5 mm, 10.5 - 12.5 mm and 17 - 25 mm on the restoration of normal skin structure after wounds were covered by epidermis. The wounds were made by peeling away skin from an area of about 0.5 mm^2. In larvae of all stages, the epidermis covering the wound was thicker than normal and very irregular for at least 5 days after wounding, extensions of epidermis often penetrating deep between the damaged muscle fibres (see Pl. X). After 5 days there were no signs that regeneration of basement membrane or dermis had started. By 11 days the epidermis was still thick and irregular but repair of basement membrane and dermis

Plate X

A. Transverse section showing thick irregular epidermis covering a wound 3 days after skin was removed in plaice of 10 - 12 mm. The right edge of the wound is arrowed; the other edge is just to the left of the area photographed.

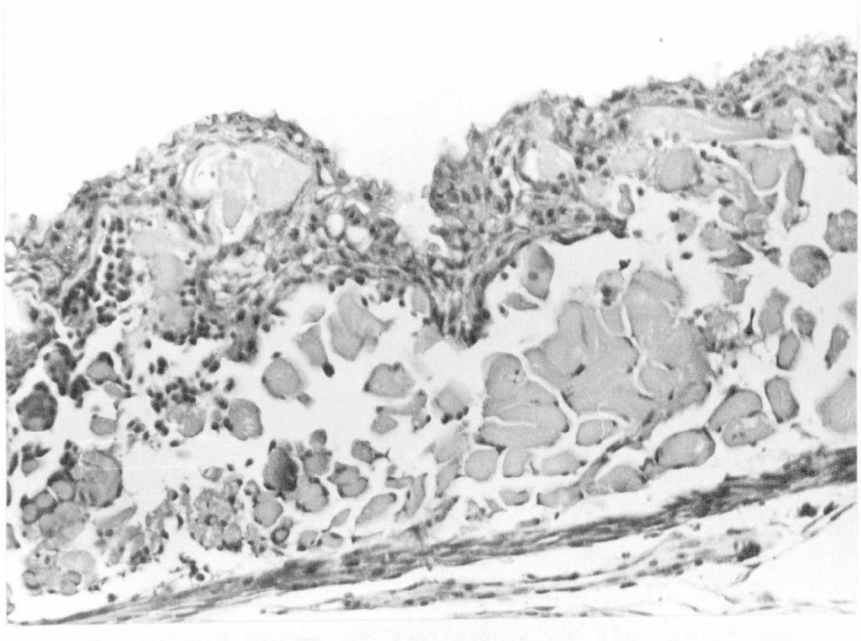
B. Higher magnification of part of the wound epidermis.

A



0·2 mm

B



0·05mm

was in progress. In larvae of 7 - 8.5 and 10.5 - 12.5 mm, some fibres, probably of collagen, had been laid down beneath the epidermis by this time, and in larvae of 17 - 25 mm there was a layer about 5 μm in thickness of basophilic vesicular cells (probably fibroblasts) bridging the gap between the cut edges of the old dermis. Melanin granules scattered throughout the wound epidermis in the later stages of healing were possibly contained within macrophages migrating through the epidermis to release the melanin at the surface (see McQueen, Mac Kenzie, Roberts and Young, 1973). It has been reported, however, that fish epithelial cells become phagocytic during wound healing (Phromsuthirak, 1977). Eosinophilic granule cells were present in the wound epidermis of plaice of 17 - 25 mm at least as frequently as in the normal epidermis, being most numerous where the epidermis was especially thickened.

Only wounds in larvae of 10.5 - 12.5 and 17 - 25 mm were examined at 22 days after wounding. The epidermis was smooth and almost normal in structure but, as the surface observations had shown, there were some thickened areas with nodules of melanin within the epidermis, and there were high concentrations of mucous cells in some areas of the wound. The surface observations suggest that such mucous cells were due to movement of cells into the wound rather than to increased production of mucous cells. The basement membrane and dermis were almost fully restored by 22 days, but the new dermis was less compact than the original dermis, and there were more fibroblasts visible between the fibres. One larva wounded at a body length of 17 - 25 mm was examined at 62 days after wounding; epidermis and dermis were normal in structure but there were no melanophores at the edges of the wound where incisions had been made.

2. The cellular inflammatory response to injury in herring and plaice larvae.

(i) The development of the circulation.

Observations on living herring embryos showed that the heart began to pulsate slowly 8 days after fertilisation in eggs incubated at around 6.5°C. By 9 days several large cells were seen circulating through a dorsal aorta, sub-intestinal vein and yolk sac sinus and then re-entering the heart. The cells measured about 14 - 22 μm in diameter, were very irregular in outline with short pseudopodia and they contained refractile granules or vacuoles. There were also some smaller round cells about 6 μm in diameter. Embryos at 8 - 9°C were not examined until 8 days after fertilisation when there were already cells circulating in the vessels. By 13 days at 6.5°C there was a forward circulation through a dorsal vein as well as in the sub-intestinal vein (see Shearer, 1930; Balakrishnan and Devi, 1970 for an account of the development of the circulation in other teleosts).

With differentiation of muscle and expansion of the notochord, the lumen of the blood vessels became narrower and it was more difficult to see cells in the circulation of newly-hatched larvae. In both herring and plaice, there were cells in the vessels of the newly-hatched larvae but many of them seemed to be stationary, adhering to the vessel walls. The cells were mainly 8 - 14 μm in diameter with short pseudopodia. By the end of the yolk sac stage there was a considerable number of cells in the circulation of both species. It was not possible to identify most of the cells seen in the living embryos and larvae, but in stage 2 herring and plaice, thrombocytes could be distinguished by their characteristic shape (see Ellis, 1976). There were many

thrombocytes of the "spiked" form, rounded at one end with a long slender extension at the other, the cells measuring about 16 μm in length. There were also "spindle" cells which were tapered at both ends (see Boyar, 1962).

Sections through the heart of yolk sac herring and plaice confirmed that there were nucleated blood corpuscles in circulation, but even in stained preparations identification of cells was difficult. They were mainly round cells with darkly stained nuclei and basophilic cytoplasm which could have been either immature leucocytes or erythrocytes. The number of cells seen in the heart in sections increased with development and by metamorphosis in both species there were large numbers of mature red blood cells (see de Silva, 1974). Attempts to make blood smears during early development were unsuccessful because of the small volume of blood available.

(ii) Cellular response to injury.

Observations on the transparent fin of newly-hatched herring and plaice larvae (less than 24 h after hatching) showed that there was some cellular inflammatory response to injury even in these early larvae with few circulating blood cells. When an incision was made through the body just ventral to the notochord in both species, cells accumulated at the site of injury in the fin lumen, and sometimes in the dermal space above the muscle in plaice larvae. In the plaice larvae the incision caused the extrusion into the fin lumen of quantities of cellular debris containing melanin. As early as 4 h after injury, cells in the lumen contained melanin granules which had presumably been phagocytosed. The phagocytic cells were 14 - 25 μm in diameter, usually had long slender pseudopodia and often contained vacuoles. By 8 days

these wandering cells were packed with melanin and most of the cellular debris had been removed. Similar cells were seen in the fin lumen of herring larvae after injury although there was initially less debris in the fin space of herring than in that of plaice and fewer of the cells which accumulated contained melanin (Pl. XIA). The wandering phagocytic cells seen in both species could not be identified.

A histological study of the cellular responses to the peeling back of skin in plaice larvae of different ages showed that the cellular inflammatory response was never as intense in yolk sac and stage 2 larvae as in stage 4 and 5 larvae. In yolk sac larvae the relatively weak cellular response may have been because the skin was peeled back with little associated muscle damage since the skin was separated from the muscle by a dermal space. Although amoeboid cells which were probably local mesenchymal cells were observed in wounds of yolk sac and stage 2 larvae, there were also some cells which may have been blood-derived macrophages. Both types of cell sometimes contained melanin. In one stage 2 larva at 5 days after wounding there was a fluid-filled vesicle which seemed to contain mainly local mesenchymal cells.

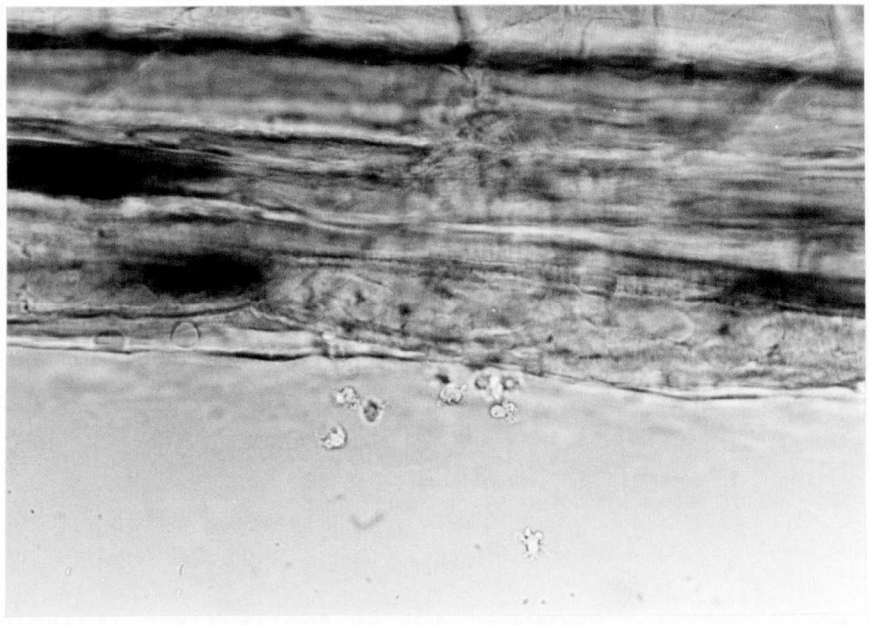
At 16 h there was very little cellular response in larvae of all stages. Later the response varied somewhat, being most intense where muscle was damaged or wound exudate had accumulated. At 32 h in stage 4 larvae there were large numbers of round vacuolate cells which seemed to phagocytose necrotic muscle and exudate (Pl. XIB). Most of the inflammatory cells were probably macrophages but there may have been some granulocytes present. After 5 days in both stage 4 and 5 larvae, many macrophage-like cells with "foamy" cytoplasm, often containing

Plate XI

A. Transparent fin lumen of a living yolk sac herring larva showing phagocytic cells near an incision after 48 h.

B. Transverse section through a wound in plaice of 10 - 12 mm after 32 h, showing leucocytes that have accumulated in the damaged muscle. The dermis is intact because the area shown is at the edge of the wound. n, remains of necrotic muscle; mu, intact muscle fibre.

A



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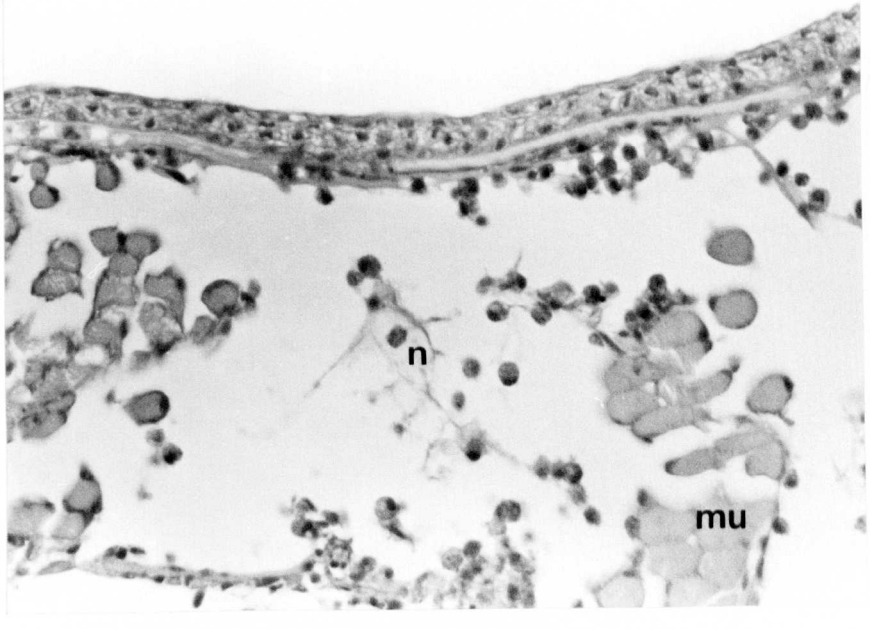
B



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melanin, could be seen among the degenerating muscle fibres. Inflammatory cells were also sometimes seen in the epidermis, especially where there was an accumulation of epidermal cells in one area. By 11 days there were still some macrophage-like cells in the wounds but most of the cells resembled fibroblasts or other connective tissue cells; they were usually elongated and tended to fuse together to form a network of cells especially in areas which had been cleared of necrotic muscle. After 22 days in stage 5 larvae there were some small eosinophilic muscle fibres which were probably regenerating fibres (see Anderson and Roberts, 1975).

Histological observations on the leucocyte response to damage in herring larvae were very limited. In one herring of 55 mm body length a shallow incision in the skin became inflamed (red and swollen). Sectioning of the wound at 14 days showed a very intense inflammatory response, with an exudate beneath the skin containing many round cells of 4.5 - 6 μm with granules which stained neither eosinophilic or basophilic with Giemsa. These inflammatory cells were possibly neutrophils.

3. Regeneration of the caudal fin in herring larvae.

Plate XII A - F shows some stages in the regeneration of the caudal fin of early herring larvae (not all the same larva). After amputation the posterior end of the body became white. Colourless blood could be seen flowing from the cut end of the dorsal aorta but it coagulated rapidly (Pl. XIIB). In older larvae of about 18 mm aggregation of colourless blood cells was seen within the clot. The notochord sometimes protruded but not in the larva shown in Pl. XIIB. In 16 mm larvae the cut end was covered by epidermis of one to several layers

Plate XII

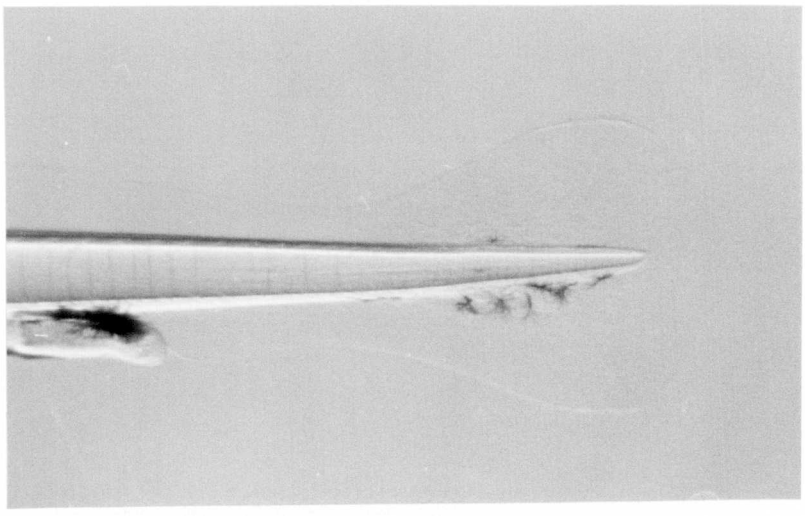
Stages in the regeneration of the caudal fin of herring larvae of 9 - 12.5 mm.

A. Before amputation.

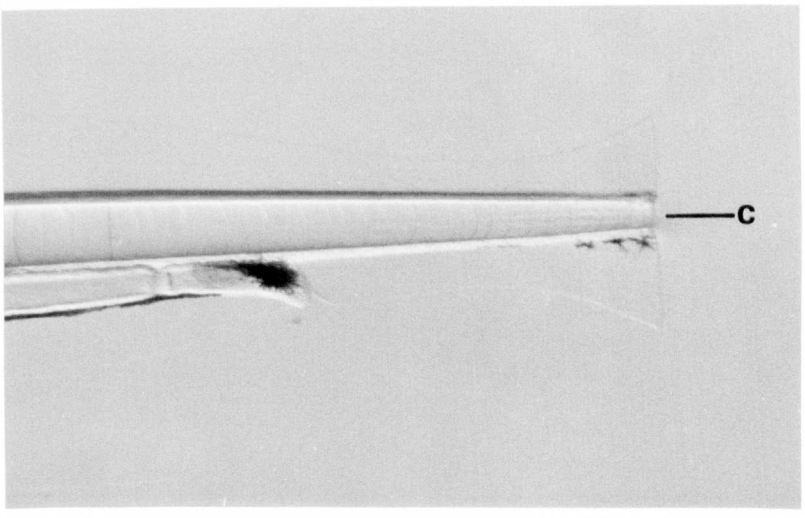
B. Immediately after amputation showing a clot of colourless blood. c, clot.

C. After 48 h. The protruding notochord is covered by epidermis.

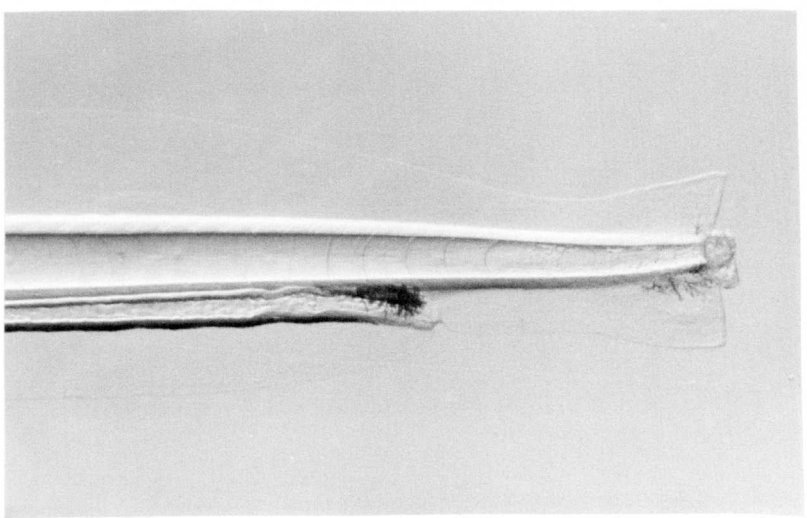
A



B



C



1 mm

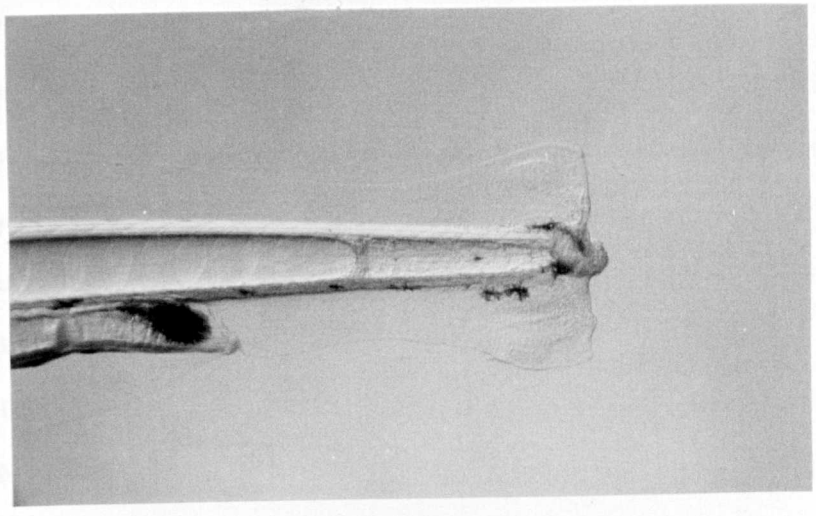
Plate XII contd.

D. After 10 days.

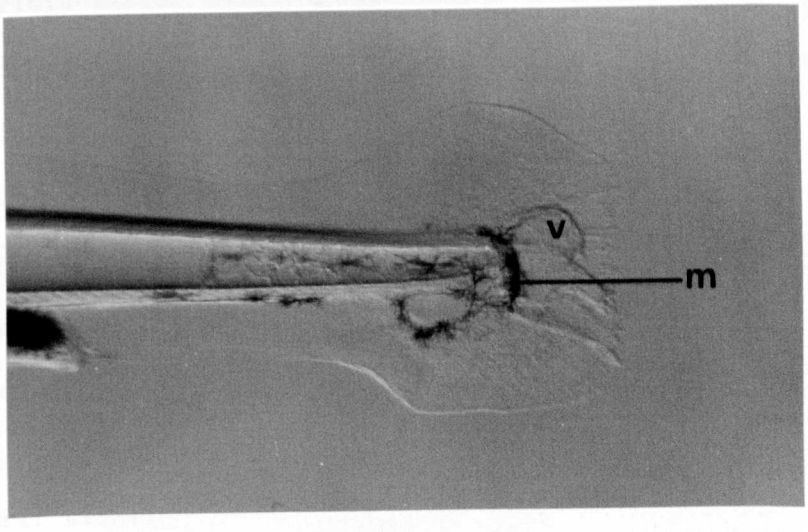
E. After 25 days showing an almost complete fin outline. v, fluid-filled vesicle; m, melanophores.

F. After 30 days.

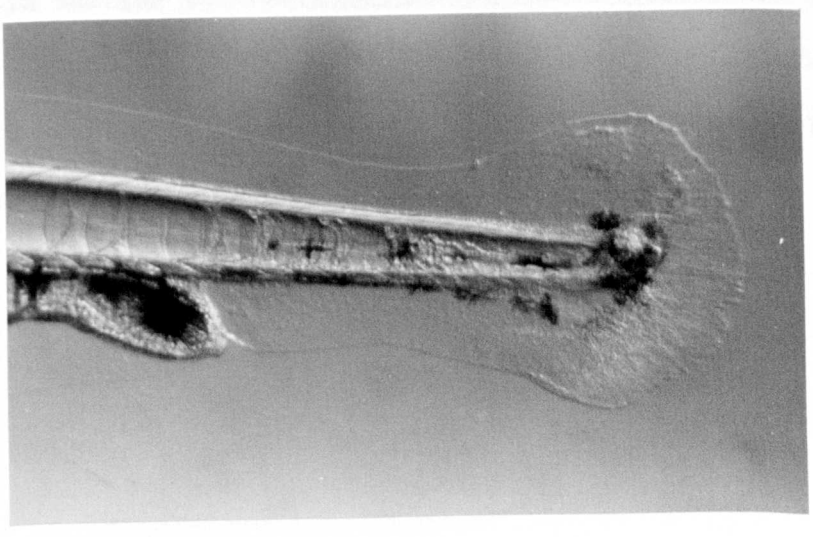
D



E



F



1 mm

within 8 h after amputation and cells in the epidermis anterior to the cut were reorganised as already described for the area surrounding skin wounds during closure. After 48 h (Pl. XIIC) the notochord was covered by several layers of cells, and later a plug of epithelial tissue developed that was probably partly epidermal and partly notochord epithelium. The fin gradually grew until by 25 days (Pl. XIIE) and 30 days (Pl. XIIF) the normal shape of the caudal fin was almost restored although the edge was more ragged than usual. As shown in Plate XIIE, melanophores often accumulated on the tip of the cut notochord, and a fluid-filled space, which seemed to be associated with the regenerating notochord, usually developed in the new fin. This vesicle usually contained fibres of no special orientation and wandering cells which sometimes had melanin granules in their vacuolar cytoplasm. The notochord often became divided into small compartments by irregular septa, longitudinal as well as transverse (see Pl. XIIE). During this process the larvae increased in length and in developmental stage, the hypural plate developing from the rudiment which had been left intact (Pl. XIIE).

As noted in Section III there was no apparent regeneration after a 1 mm length of the notochord was cut off, removing the whole fin. It may be that some part of the fin rudiment must be intact before the fin will regenerate.

V DISCUSSION

Since larvae could withstand larger skin lesions in an isosmotic salinity than in river water or sea water it seems that mortality after skin damage in river water or sea water is caused by osmotic disturbance as in adult teleosts. Lewis (1971) showed a marked decrease in sodium and total osmolality in the body fluids of the golden shiner (Notemigonus crysoleucas) after mucus and scales were removed in freshwater, the decrease probably being caused by an inflow of water. Damaged fish died when the osmolality fell from the normal level of about 285 mOsm to below 240 mOsm. Both the drop in osmolality and the mortality were prevented by holding the damaged fish in a 0.35% solution of NaCl. Wedemeyer (1972) also found a decrease in plasma chloride levels in juvenile coho salmon (Oncorhynchus kisutch) and steelhead trout (Salmo gairdneri) after the fish were handled in freshwater, but there was no decrease when they were held in a solution of 0.3% NaCl. Krogh (1939) had earlier shown an increased flux of water and ions across damaged skin in the roach (Leuciscus rutilus) and in eels (Anguilla vulgaris) in freshwater; the fish died in freshwater but survived in 5 mM and 11.5 mM Ringer solutions respectively.

The flow of salts and water across a wound appears to be related to the size of the wound since mortality of larvae increased with wound area. Recovery after skin damage is probably based on a combination of rapid epidermal healing and osmoregulation, together with subsequent tissue repair. It would seem that in the present study wounds up to the lethal area in sea water and river water could usually be completely covered by epidermis within 4 to 16 h after skin was removed. During this time the flux of water and ions is probably reduced as the

area of the wound decreases, until eventually at complete closure the flux should be almost normal and osmoregulatory mechanisms can begin to restore normal body fluid osmolarity. Lewis (1971) found that in golden shiners held in freshwater osmotic concentration decreased during the first 12 - 22 h after skin damage, after which it began to increase. The damaged area had probably been covered by epidermis within 12 - 22 h. It must be noted that in herring and plaice the gills and kidney are not fully functional in the early stages of development and it is believed that the epidermis may be responsible for osmoregulation (see Holliday and Blaxter, 1960; Holliday, 1965; Holliday and Jones, 1965; 1967; Roberts, Bell and Young, 1973).

The present findings indicate that when larvae are subject to mechanical damage under cultured conditions, survival could be improved by altering the salinity of the medium so as to reduce the osmotic gradient between it and the internal body fluids, at least until wounds are completely covered by epithelium. Long-term holding in an isosmotic salinity may not always be advisable, however (see also Lewis, 1971).

Larvae can withstand the removal of only up to about 3% of the total area of the skin in sea water or river water. It has not been shown how much similar damage adult teleosts can tolerate, but it seems that they can survive quite extensive areas of descaling. Loss of scales indicates the loss of some epidermis and sometimes dermal damage since scales are dermal structures which usually protrude to the surface where they are covered by epidermis and also by a thin layer of dermis in some fish. Holliday and Blaxter (1961) suggested that herring need at least 50% of the scales intact in order to maintain osmotic equilibrium. Black and Tredwell (1967) noted that 1½ year old rainbow trout could withstand the removal of scales from 17% or 25% of the total

body surface and could survive after mucus was scraped from the body posterior to the pectoral fins. Lewis (1971) found that in the spring and summer, golden shiners survived when mucus and scales were scraped from the whole of one side in freshwater, although this caused 98% mortality in the spawning season.

Skin lesions in fish are analogous to burns in mammals since in both there is an increased flux of water across the damaged surface. The removal of about 30% of the skin of a terrestrial mammal in dry air at a temperature of less than 28^{o}C will cause death, because at this critical area, heat loss as a result of evaporation exceeds the animal's capacity to compensate by generating heat from exothermic chemical reactions (Moyer and Butcher, 1967). It seems, therefore, that terrestrial mammals can survive the loss of roughly ten times as great a proportion of their skin as can fish larvae in their natural salinity. Rapid epidermal healing must be of especial importance in a hypo-osmotic or hyper-osmotic environment where the integrity of the skin is so important in maintaining osmotic equilibrium.

In herring, plaice, and salmon larvae skin lesions appear to be covered initially by a redistribution of existing epidermal cells and so it is of interest that wound closure was rapid even in yolk sac herring and plaice larvae when the epidermis was only 2 cells thick. The high rate of cell division which must take place in larval epidermis may have been useful in restoring normal epidermal thickness after wound closure, but this was not examined. The strategy for rapid wound closure in fish larvae seems to be similar to that in older fish and other aquatic groups. Harabath (1928), Arey (1932, 1936), Berlin (1951), Mittal and Munshi (1974) and Bullock *et al.* (in press) have all shown

that in adult teleosts skin wounds are covered by a rapid mass migration of epidermis from the surrounding area without cell division. Berlin (1951) did observe a wave of mitosis spreading towards the wound 3 - 4 days after complete closure. Phromsuthirak (1977) also described the migration of epidermal sheets into incisions in the skin of the stickleback (Gasterosteus aculeatus), the Malpighian cells becoming elongated and phagocytic during wound closure. It seems that the presence of scales does not alter the basic healing response of the epidermis. This was also noted in plaice at metamorphosis when scales were developing only along the mid-line, although it seemed that the epidermis sometimes migrated more freely from the sides of the wound where there were no scales.

Infection of wounds did not appear to be responsible for acute mortality of larvae, and it seems that the immaturity of the circulatory system and the inflammatory response to injury was not a major disadvantage for early larvae under the conditions of the present study. Embryonic mammals may show only a local mesenchymal response to injury before their haemopoietic tissues are mature and wounds can then become infected and cause mortality. Later mammalian stages which show a mature inflammatory response with infiltration of wounds by white blood cells can resist such infections (see Block, 1960; Dixon, 1960). Wounds in larvae may be more susceptible to infection in the wild, and recovery could then be affected by the immaturity of the defensive system in the early larval stages.

The phagocytic response to injury found in early herring and plaice larvae appears similar to the type of simple primitive response to injury and foreign material found throughout the invertebrates (see Sparks, 1972). It seems that by metamorphosis at least (and probably

sooner) the haemopoietic tissues of herring and plaice larvae have developed sufficiently for damage to elicit an inflammatory response similar to the response of mature fish to damage (see Roberts, Mac Queen, Shearer and Young, 1973; Mittal and Munshi, 1974; Anderson and Roberts, 1975; Phromsuthirak, 1977). The role of coagulated blood in wounds as a barrier to water and ions has not been investigated here, but the wound exudate does seem to play an important part in providing a suitable substratum for epidermal migration. Therefore early larvae with little blood and no peripheral blood vessels may be at a disadvantage if there is a lack of exudate after damage to the skin. On the other hand, blood clotted rapidly after amputation of part of the caudal fin of yolk sac herring and thrombocytes were present early in development in the circulation of both herring and plaice larvae.

While it seems that the early stages in the life history of herring, plaice and salmon do have protective mechanisms which will allow survival after various injuries, it is difficult to assess the importance of such defences for survival under natural conditions. First, although herring and plaice embryos are protected by a tough chorion, forces much less than those needed to burst the chorion will destroy the embryo. The chorion of the demersal herring egg may protect against mechanical shock such as movement of gravel, and possibly against hazards such as hydroids which may be present in the spawning grounds (Russell, 1976), but it seems that the chorion could be easily punctured by the sharp spines and mouth parts of predators. Petipa (1965) found that although rockling larvae were eaten intensively by Calanus helgolandicus, rockling eggs were not eaten because they were too large and awkward for the copepods to catch. Nevertheless, the copepods burst large numbers of eggs while trying to grasp them, and

the results of my experiments suggest that such damage would be lethal at least for herring embryos.

In general, larvae seem to be most vulnerable and sensitive to damage in the earliest stages of development, though herring larvae may go through a second sensitive period after they have reached about 25 mm. The present findings suggest that in the wild larvae could survive some damage inflicted by organisms such as small browsing copepods (see Garstang, 1900; Davis, 1959; Rosenthal, 1967). In the earliest stages, however, the number of delicate marine larvae recovering from attacks of predators or parasites may be very insignificant compared with the number eaten or fatally injured by larger predators such as copepods (Lillelund and Lasker, 1971), medusae (see Fraser, 1969), chaetognaths (Lebour, 1922; 1923), euphausiids (Theilacker and Lasker, 1974), ctenophores (Lebour, 1922; 1923; Stevenson, 1947) and amphipods (Westernhagen and Rosenthal, 1976). Healing and regeneration may be a more significant survival mechanism for older herring and plaice larvae and for the more robust salmon alevins. It will, perhaps, be easier to assess the importance of wound healing and recovery from damage for larval survival and subsequent stock recruitment when more information is obtained on the predators and parasites of fish larvae.

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APPENDIX 1

Staging system for plaice larvae (Ryland, 1966)

Stage	Developmental criteria	Mean body length (mm)*	Mean age (days)*
1	Yolk sac present	7.1	7
2	Yolk resorbed; notochord straight; hypural fin rudiment developing	7.7 - 8.4	20 - 28
3	Notochord turns dorsally at caudal end; marginal fin rays developing; eye symmetrical	8.9 - 10.1	36 - 48
4	Eyes asymmetrical; flat fish shape develops	10.5 - 11.4	52 - 60
5	Pupil of left eye visible on dorsal surface; metamorphosis complete when eye reaches its final position		

* The range given is mean length and age at substage (a) to mean length and age at substage (b) or (c).

APPENDIX 2

Staging system for herring larvae (Doyle, 1977)

Stage	Developmental criteria	Mean notochord length (mm)*	Age (days)
1	Post-hatching; yolk sac present	7.8 - 9.3	0 - 11
2	Yolk sac absent; notochord straight; dorsal fin differentiating	10.4 - 13.3	12 - 48
3	Notochord turns dorsally at caudal end	14.8 - 17.6	49 - 85
4	Pelvic fin begins to protrude ventrally; the gut is shortening relative to body length.†	21.9 - 39.8	-

* The range given is the mean length at substage (a) to the mean length at substage (c) or (d), obtained from Doyle 1975, M.Sc. Thesis, University of Stirling.

† Metamorphosis, the gradual transition from larval to juvenile body proportions, takes place towards the end of stage 4.