

PRELIMINARY REPORT ON THE EFFECTS OF TEMPERATURE ON THE DEVELOPMENT OF EGGS AND LARVAE OF HALIBUT (*Hippoglossus hippoglossus*) AND ON THE BACTERIAL POPULATION IN THE INCUBATORS

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ABSTRACT

Eggs were stripped from one female of the halibut broodstock at Austevoll Aquaculture Station, and fertilised with sperm from two males immediately before incubation. Eggs were held in nine open-circulation 250 l incubators at either 3°, 6° or 9°C with three incubators at each temperature. When hatched, the larvae were transferred to fifteen similar incubators, with five incubators at each temperature. The timing of developmental events in the eggs and larvae was monitored, mortality in the egg and larval stages recorded, growth and yolk absorption measured in the larvae, RNA and DNA content and RNA/DNA ratios determined for each temperature group and samples taken for embryonal and larval histology. Total and viable count of free-living bacteria in the incubators was monitored from hatching until termination of the experiment. Flow rate, temperature, oxygen and ammonia were recorded.

Differences in development rates were apparent from the first cell divisions. The mean number of Kupffer's vesicles was most in the 9°C groups and least in the 6°C groups. At hatching, relative protein synthesis and yolk sac size was best at 3°C but there was no difference in standard length between the groups. At 9°C, larvae grew faster, but developed abnormalities associated with sublethal stressors. A rise in mortalities occurred at the same stage of development at 6° and 9°C. An increase in larval mortalities lead to an increase in bacteria which preceded an increase in ammonia levels. There was no significant difference in bacterial numbers between groups. The experiment was terminated due to uncontrolled temperature fluctuations.

INTRODUCTION

Hatchery temperatures for incubating eggs have an effect on the rate of embryo development and on the condition of the emergent larva (Rosenthal & Alderdice, 1976). Subsequently, temperatures experienced by the primitive halibut larva during the yolk sac stage have been shown to affect growth and yolk conversion efficiencies as well as rates of deformity (Pittman et al. 1988). However it is clear that temperature also influences bacterial activity and the impact of abiotic parameters such as oxygen tension and ammonia (Wedermeyer, 1976). These in turn may also affect larval mortality. This experiment was conducted in order to monitor correlations among abiotic factors, egg and larval development and the bacterial populations in the incubators. The preliminary results are presented here and the conclusions regarded as tentative pending further analysis. Additional work is being carried out in histology, histopathology, free amino acids and protein conversion. The experiment was terminated when temperature control was lost.

MATERIALS AND METHODS

Water treatment

Sand-filtered water from about 40 m depth was piped to the incubators through mixing valves, which blended two "stock" temperatures (3° and 10°C). The mixing valves were Satchwell Linear Activator, type ALM 1601, and the heat pump aggregator was an EUW 20 F (Daikin). Cascade filters were used to aerate the water and drive off excess nitrogen.

Incubators

A total of 24 incubators were used, nine for the egg stage and fifteen for the larval stage. These were arranged on line so that the same stock water was used for each temperature group. The incubators at 3° and 9°C were wrapped in rockwool insulation to prevent variation in temperature (only 3° was insulated during the egg stage), while 6°C was close to "room temperature".

Each incubator was a semiconical, 250 l fibreglass unit with a split inlet valve on the bottom for introduction of new water and extraction of sedimented material and larvae, and a sleeve filter for overflowing water at the top. A fitted lid on the top prevented light from reaching the developing halibut (Figure 1).

Egg source and incubation

One female and two males from the broodstock of the Austevoll Aquaculture Station were stripped for eggs and sperm. The eggs (1.4 liters) and sperm were transported separately from the broodstock tank, where the temperature was 6-7°C, to the nine egg incubators (Fig. 1). Sand-filtered 6°C seawater was added to the eggs, which were then fertilised. About 1.6 dl of the fertilised eggs were placed into each of nine 1 liter beakers and one beaker placed in each of the incubators to acclimatise for half an hour to 3°, 6° or 9°C. A control group had 93% fertilisation of the eggs.

When hatched, the eggs were transferred from the three egg incubators at a given temperature and distributed randomly among five similar larva incubators at the same temperature. The emergent larvae from one egg incubator at 6°C and one at 3°C were not mixed with the others in order to counteract a possible infection.

Husbandry

Flow rates were recorded daily. Flow was maintained between 0.5 and 2 liters per minute during the experiment, and adjusted using a manual valve.

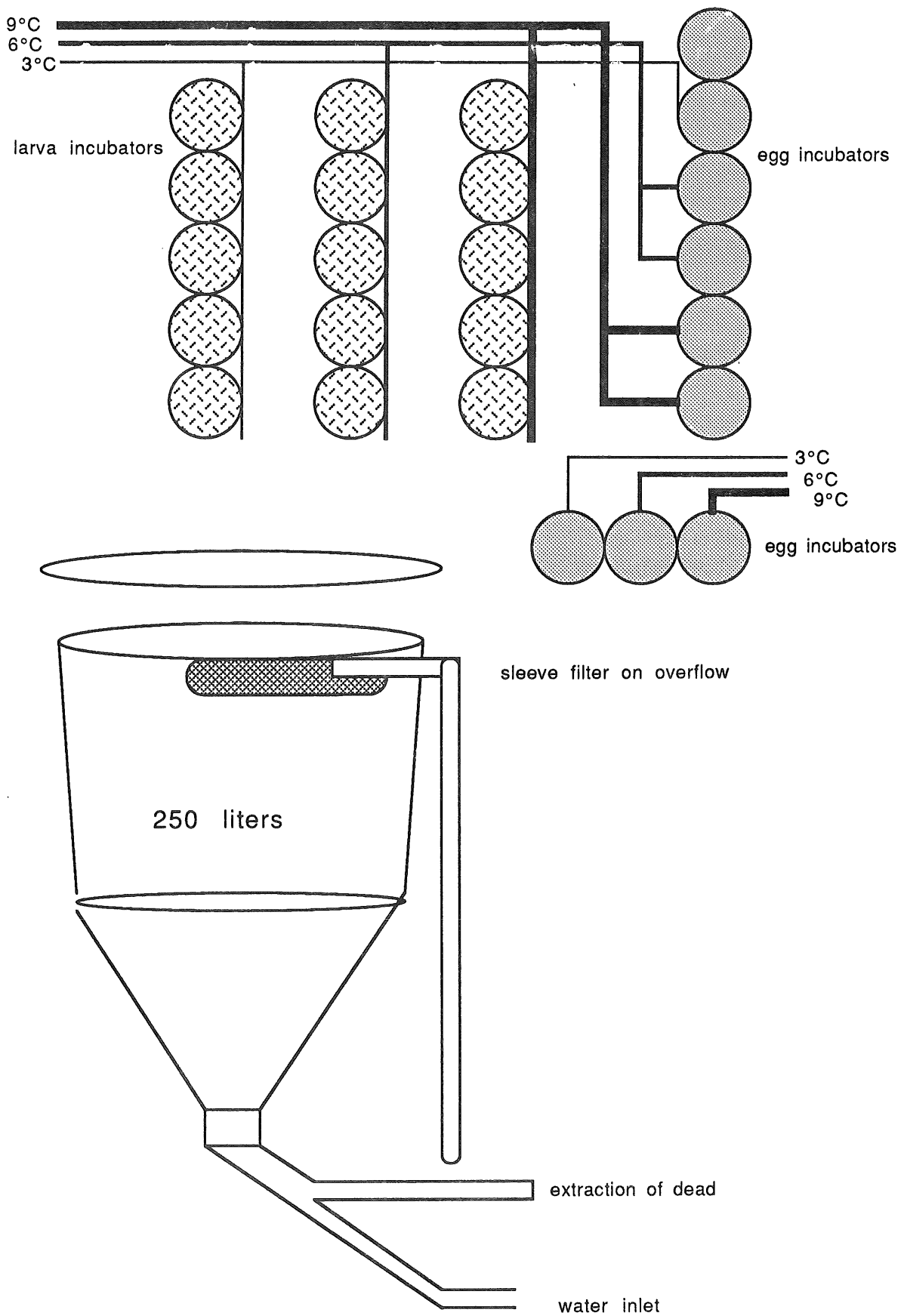


FIG. 1. Schematic diagram of incubator arrangement (top) and construction of incubators (bottom).

Dead eggs and larvae were removed from the incubators every second day by stopping the inflow and introducing ten liters of salted water (about 45 ppt) for about 10 minutes. Material sedimented down into the cone and was removed via the bottom valve along with the ten liters of salted water. The material was then filtered and preserved in formalin for mortality counts. Inflow was restarted and adjusted according to the apparent density of the eggs or larvae.

Egg samples

About twenty eggs were taken every day from each of the incubators, using a sterilized glass pipette and red light for illumination, for development rates (more often during the first cell divisions). These were observed live and preserved in glutaraldehyde. Thirty eggs from each group were taken every third day, measured for diameter and put in a drying oven for 48 hours at 60°C for dry weight.

Larva samples

Larvae were removed every third day from hatching to the end of the experiment. Ten to fifteen larvae were taken from each incubator for live morphometric measurements. Five of these larvae were preserved in cacodylated glutaraldehyde for later examination by scanning electron microscopy and histological and volumetric analyses; and five to ten frozen for dry weight and free amino acid and protein content analyses. In addition eight larvae were extracted from three incubators in each temperature group once a week for RNA and DNA analysis, according to the procedure in Raae et al. 1988.

Bacterial counts

For total counts of free-living bacteria, sample volumes of 3-5 ml were filtered on Nucleopore filters and stained with DAPI (Porter and Feig 1980). Support filters were soaked in the 10 µg/ml stain solution, according to Hoff (1988). Counting was carried out using a Nikon epifluorescence microscope at 600x. At least 200 cells were counted in each sample. Total counts were performed twice a week, at the same time as the other samplings.

In addition, viable counts of bacteria were conducted once a week on three different media, Tryptone Soya Broth (TSA, Difco), Tryptone Citrate Bile Salt (TCBS, Difco) and Cytophaga Agar (Frerichs 1984). All media were made on 70% seawater. Petri dishes were incubated at 10°C for at least 10 days.

Water Quality

Samples were taken for ammonia and oxygen about twice a week during the larval stage. Water was siphoned from below the surface directly into the sample bottles. Triplicate samples were taken for ammonia and analysed spectrophotometrically on a Shimadzu spectrophotometer according to Koroleff (1969). Oxygen was measured by Winkler method. Temperature of the outgoing water from each incubator was recorded daily.

RESULTS

Water Quality

Temperature varied near the desired level until uncontrolled fluctuations necessitated terminating the experiment (Figure 2, egg incubators, and Figure 3, larva incubators). Flow rate varied from around 0.5 to 2.0 liters per minute during the egg and larval stages (Figs. 4 and 5), and oxygen was fairly stable at around 7.3 ml/l (3°C), 6.6 ml/l (6°C) and 6.4 ml/l (9°C) as shown in Fig. 6. This was below saturation but not near any critical level. Total ammonia was generally below 1 µmol/l except after increased mortality when it went over 3.5 µmol/l in 4 incubators (Fig. 7). Salinity of the deepwater, recorded daily at the station, was 33.7 ppt +/- 0.06 during the season.

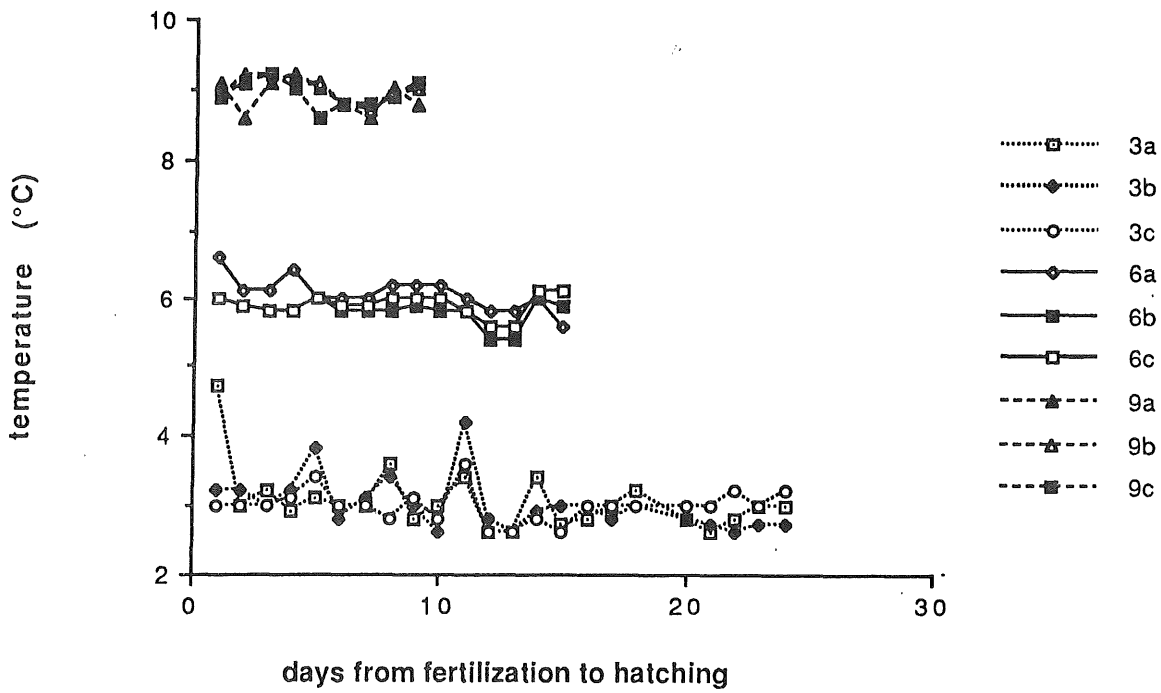


FIG. 2. Temperature in egg incubators.

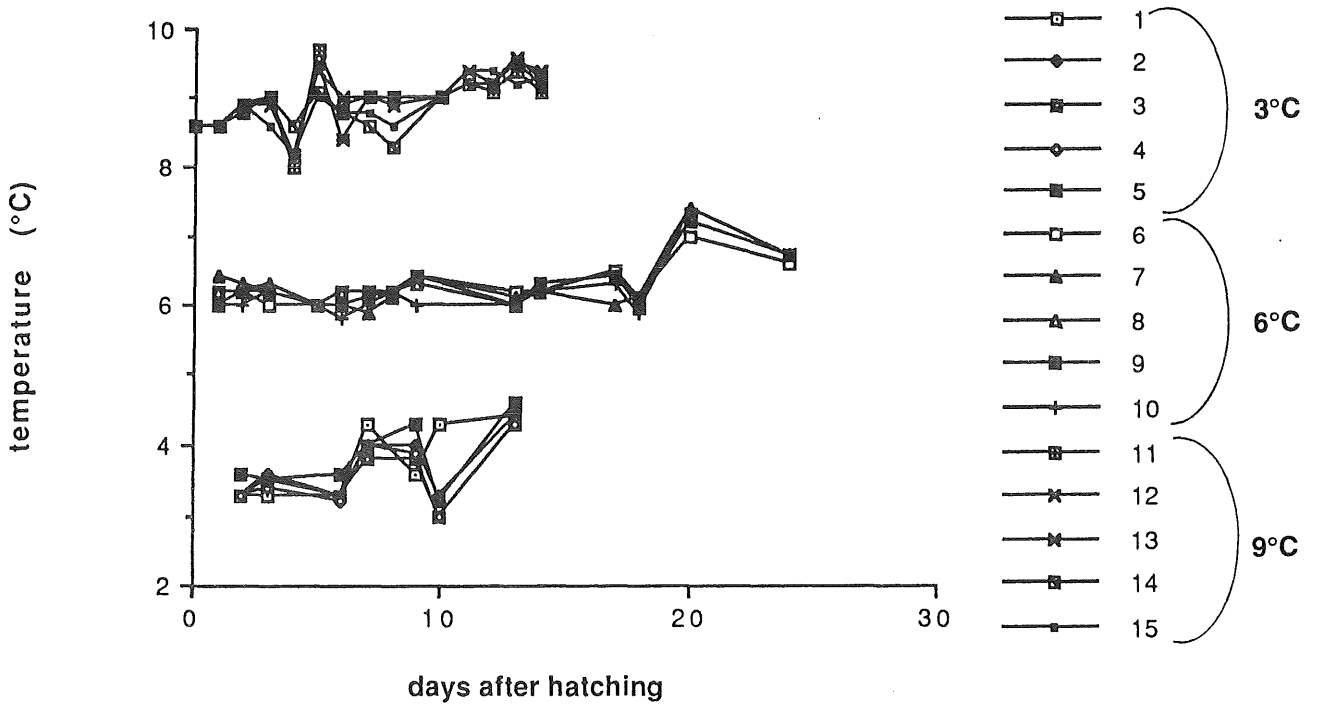


FIG. 3. Temperature in larva incubators.

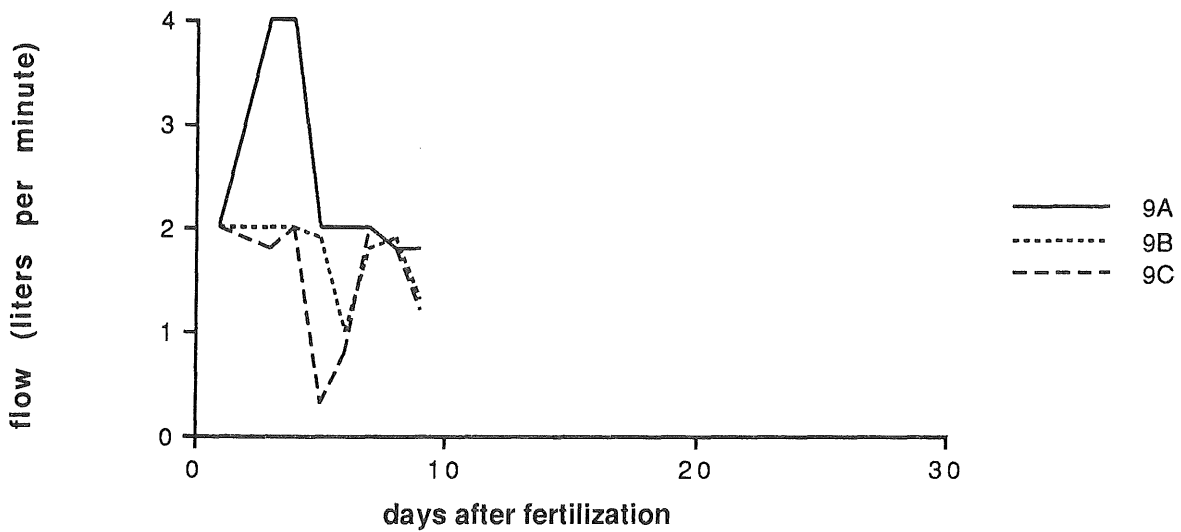
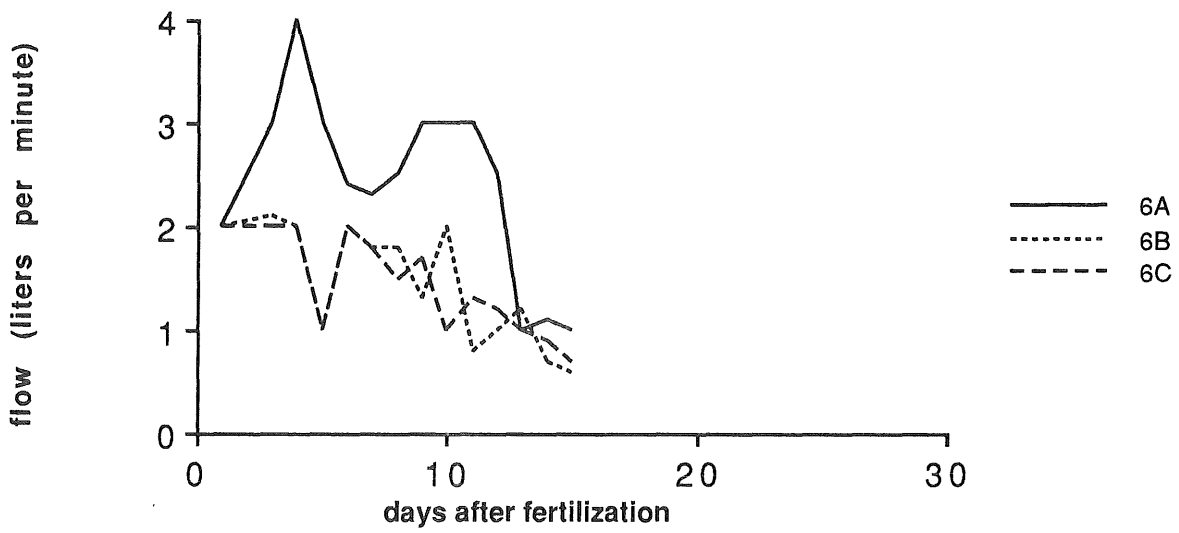
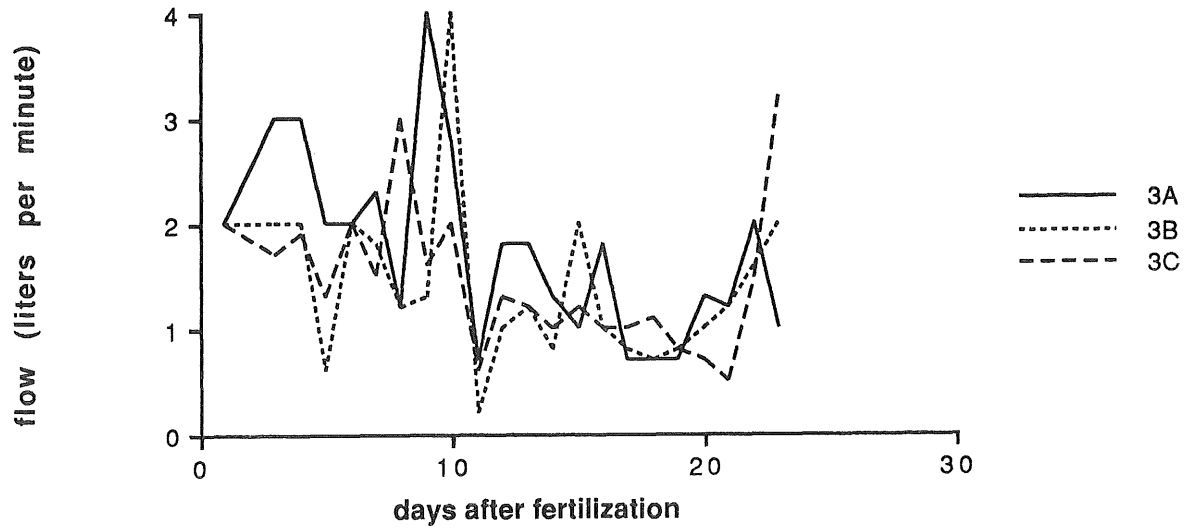


FIG. 4. Flow rates in egg incubators at 3°, 6° and 9°C (top to bottom)

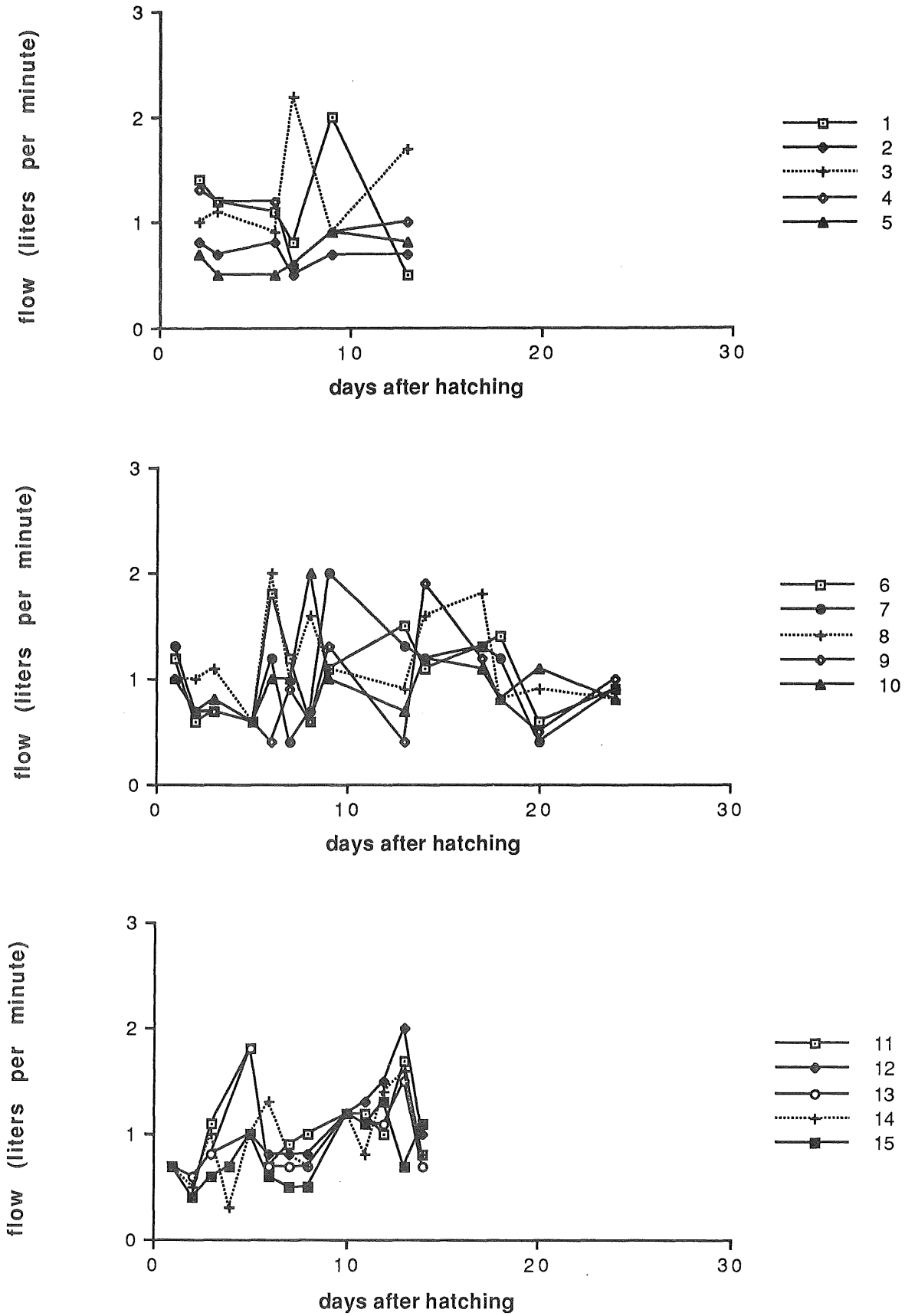


FIG. 5. Flow rates in larva incubators at 3°, 6° and 9°C (top to bottom)

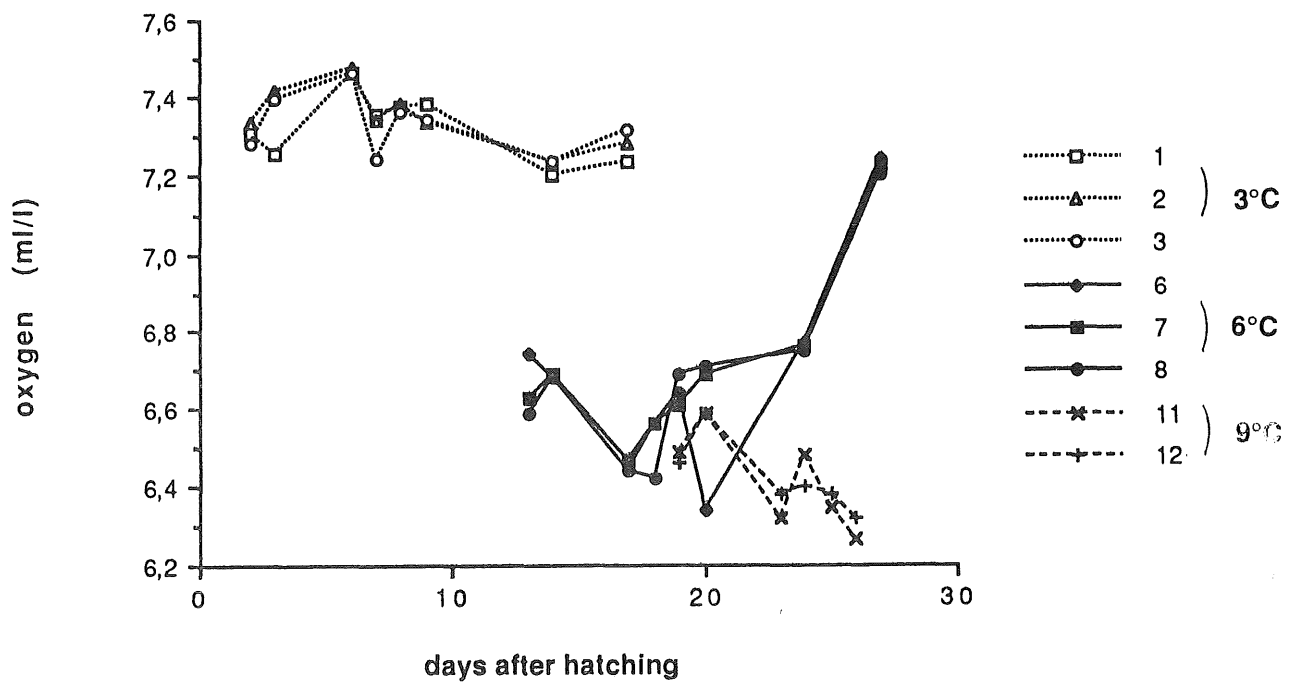


FIG. 6. Oxygen in larva incubators.

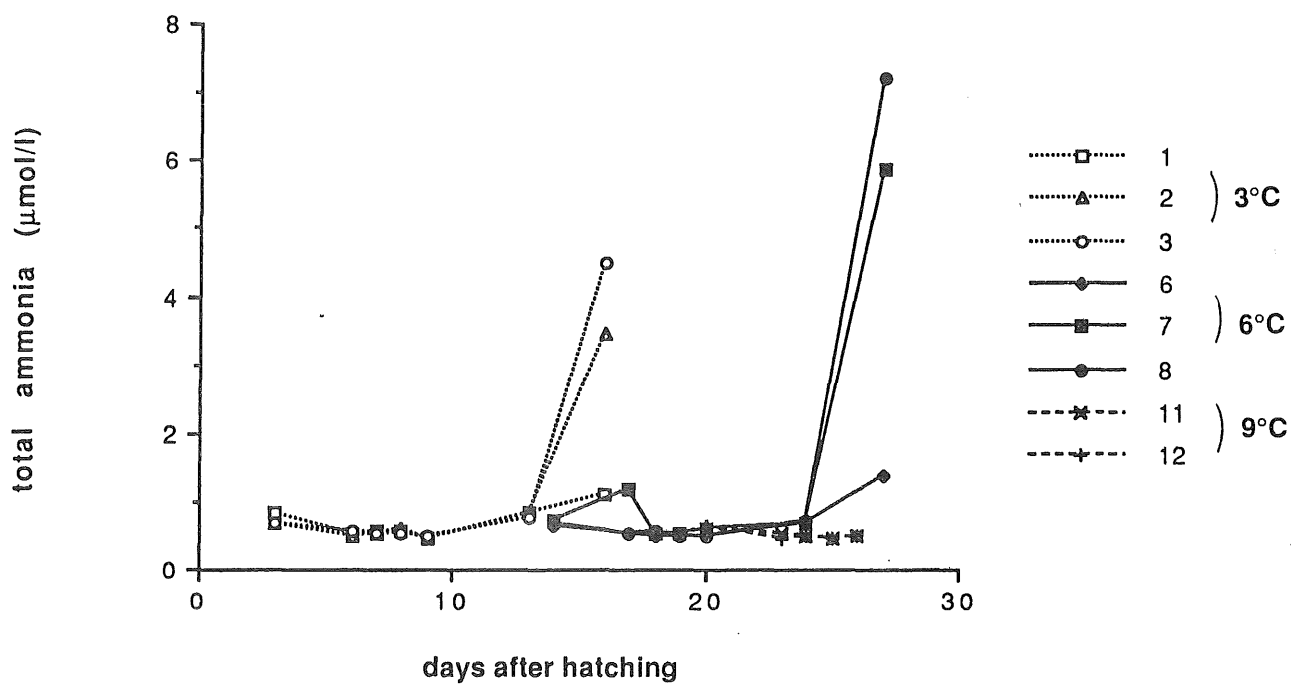


FIG. 7. Total ammonia in larva incubators.

TABLE 1 EGG DEVELOPMENT (IN HOURS) FROM FERTILIZATION TO HATCHING IN HALIBUT EGGS INCUBATED AT 3°, 6° AND 9°C.

STAGE	3a	3b	3c	6a	6b	6c	9a	9b	9c
2-cell	8.1	8.8	8.8	6.8	6.8	6.8	4.3	5.5	5.1
4-cell	13.6	13.9	13.9	9.3	10.8	10.8	6.8	7.8	7.5
8-cell	17.9	19.6	19.9	12.6	13.6	13.6	8.4	9.3	9.3
16-cell	24.6	23.5	24.1	15.6	16.8	16.8	10.8	11.8	11.8
32-cell	28.6	28.6	28.6	-	-	-	12.6	13.6	13.6
germring formed	125	118	125	74	74	74	49.5	49.5	49.5
blastopore closing	330	295	295	144	145	145	103	106	107
heartbeat	536	524	536	297	297	297	191	191	191
hatching	600	600	600	336	336	336	211	211	211

Growth and Mortality

Developmental events in eggs at 3°, 6° and 9°C are listed in Table 1. The effect of temperature on development rate was seen from the time of the first cell divisions, when eggs at 3°C took about 60% more hours to reach the 2 cell stage. By the 8 cell stage the time difference was over 100% relative to the warmest groups. Hatching took almost three times as many hours at 3° as at 9°C. Kupffer's vesicles, small inclusions of unknown function posterior to the yolk which have been observed since Rollefsen (1934), arose before the closing of the blastopore in all groups, but the number of these was different in each group. The mean total number of vesicles (counted from 20 eggs from each incubator) was 1.5 at 3°C, 0.3 at 6°C and 5 at 9°C. An abnormality was often seen in the 3°C groups during embryo development where the caudal fin fold was not completely expanded and the caudal end of the notochord resembled a small clump.

There were visible differences between the temperature groups at hatching. Yolk sac size was larger at hatching in larvae from eggs raised at 3°C, although there was no significant difference in standard length between the groups (Figs. 8 and 9). At 3°C, the yolk sacs filled the peritoneal cavity whereas those at 9°C often had fairly large spaces between the sac membrane and the outer membrane. At 6°C, the space was smaller but still visible. In Figure 8, the mean standard length of larvae is plotted versus yolk sac length for each of the three temperature groups. Slope was -3.48 (9°C), -3.31 (6°C) and -2.24 (3°C), and correlation coefficients (*r*) were -0.92, -0.973 and -0.908 for 9°, 6° and 3°C respectively.

Development of larvae in the three temperature groups is shown in Figure 10. All larvae in a temperature group reached the same stage of development at the same time, as opposed to those larvae from previous years raised in larger systems subject to internal temperature variations. In the 9°C groups, the liver developed quickly into a tight, dark cell mass high on the yolk sac and the intestines were generally curved at the colon, rather than making the normally distinct right angle. As the hearts developed, it was apparent that the pericardial cavity was larger and the hearts smaller in the 9°C groups. This trend decreased with decreasing temperature.

The DNA and RNA content of larvae is displayed in Figures 11 and 12, respectively. RNA/DNA ratios are plotted in Figure 13. RNA generally increases in all groups from hatching onward, whereas DNA increases from hatching only in the 3°C group. In the 6° and 9°C groups, DNA remains almost constant during the first 12 days. The ratio of RNA/DNA for all groups seems to follow a general bell curve, reaching the same levels as at hatching about twenty to twenty five days posthatching. The curve for the 9°C groups is lower than for the others, while the RNA/DNA of the 3°C groups is higher from about one day after hatching.

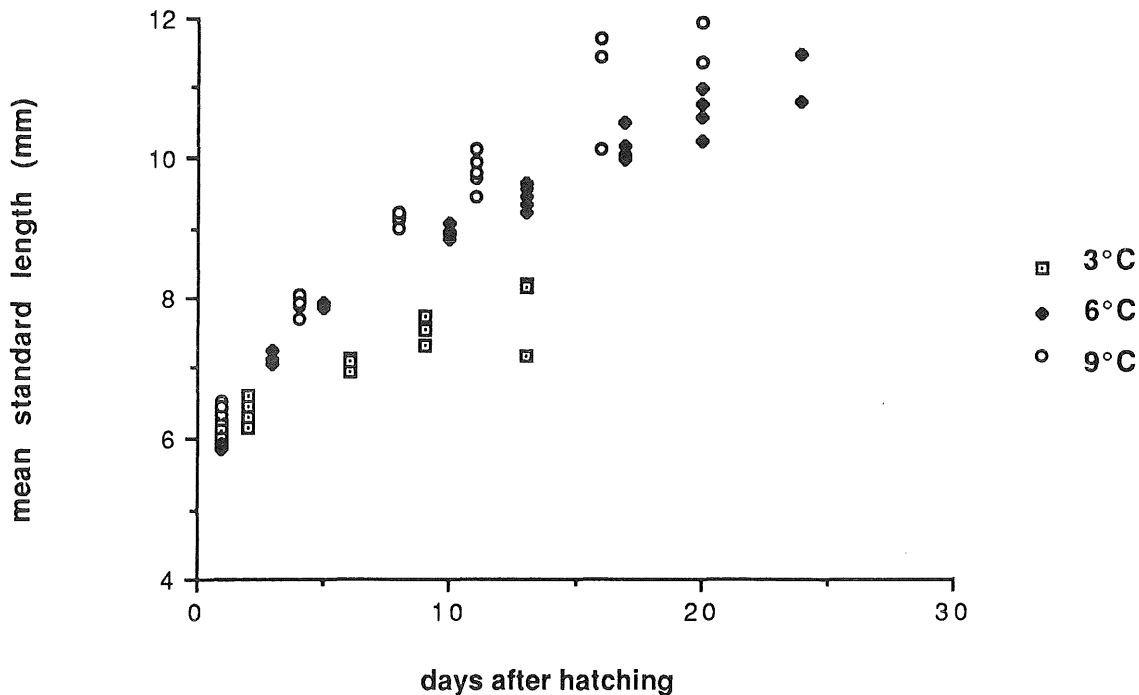


FIG. 9 Mean standard length of larvae. Each point represents the mean of 10-15 larvae from one incubator.

Cumulative mortality for the three temperature groups is displayed in Figure 14 (egg incubators) and Figure 15 (larva incubators). Egg mortality was fairly constant throughout embryo development except in one incubator. The time lag between the increase in larval mortality at 9° and 6° corresponds with the time at which the head lifts from the yolk sac and there is a groove for the mouth. The increase in mortality at 3°C corresponds to the time at which temperature fluctuated nearly 2°C on a daily basis.

Bacterial Counts

Total count of bacteria is shown in Fig. 16. As a general tendency, total counts increased from about 5×10^5 cells/ml short after hatching to $1.5-2.5 \times 10^6$ cells/ml when experiments were terminated. Two of the 9°C incubators were atypical, with high total counts 10 days after hatching, followed by a decrease. The 3°C incubators were terminated at an earlier stage in larval development, and total counts did not exceed 1.5×10^6 cells/ml.

Viable counts of bacteria on the TSA and Cytophaga agar were grossly correlated with total counts, and were in the order of $1-4 \times 10^4 \text{ ml}^{-1}$ when experiments were terminated. Counts on the TCBS medium were low ($0-100 \text{ ml}^{-1}$), indicating low numbers of *Vibrio sp.* The characteristic "spreading growth"-type colonies on the Cytophaga agar were rare ($0-200 \text{ ml}^{-1}$) throughout the experiment. Three of the 6°C incubators showed higher values day 1 after hatching ($500-2200 \text{ ml}^{-1}$, but values were in the same order as the other incubators from the next measurement (day 6 after hatching) until termination of the experiments.

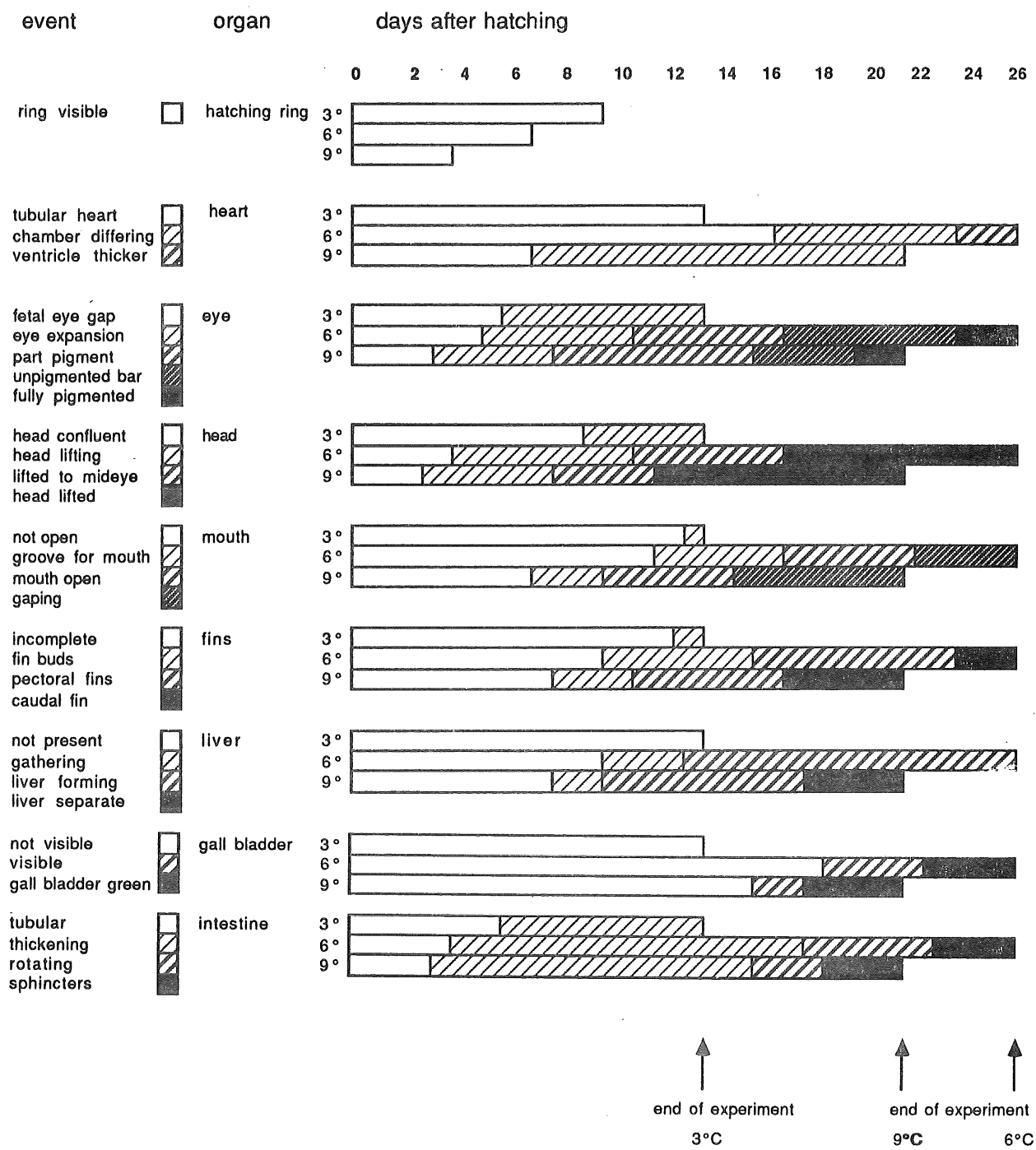


FIG. 10. Morphological development (in days to event) of halibut larvae at 3°, 6° and 9°C

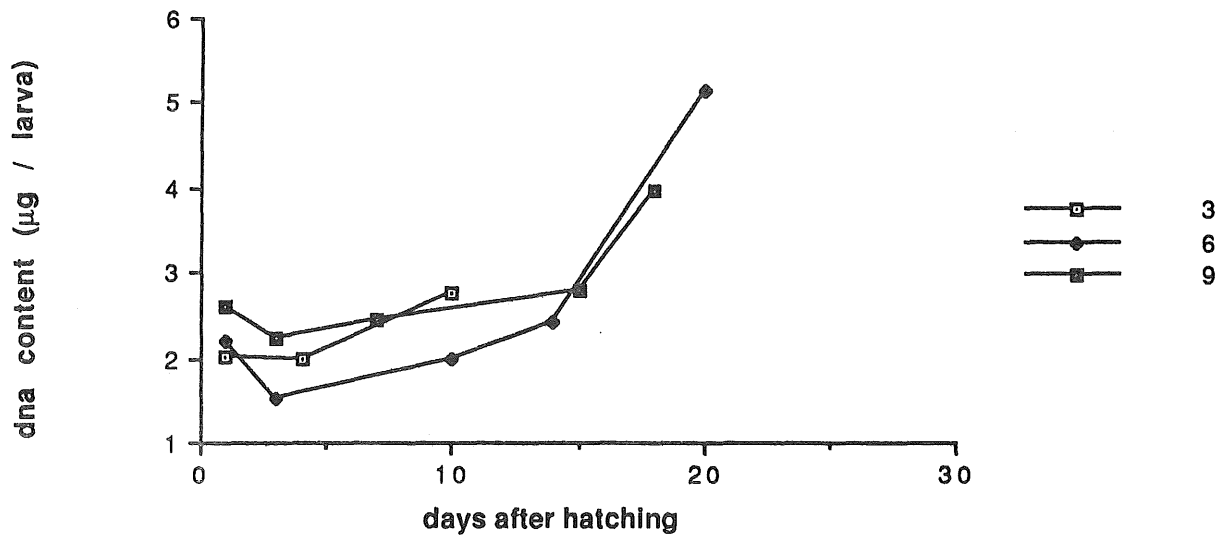


FIG. 11. DNA content of halibut larvae.

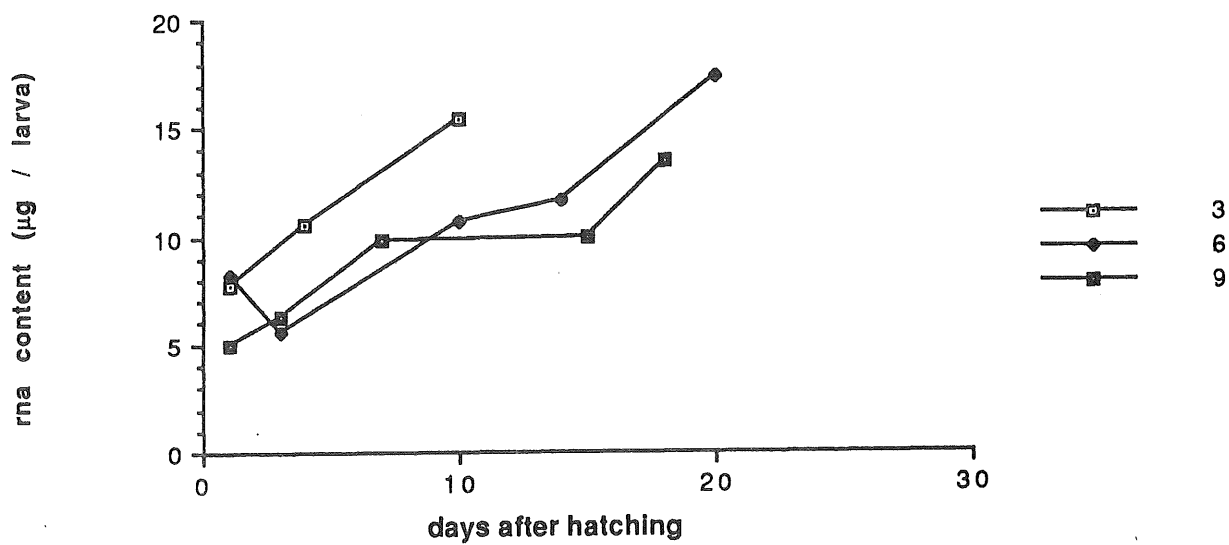


FIG. 12. RNA content of halibut larvae.

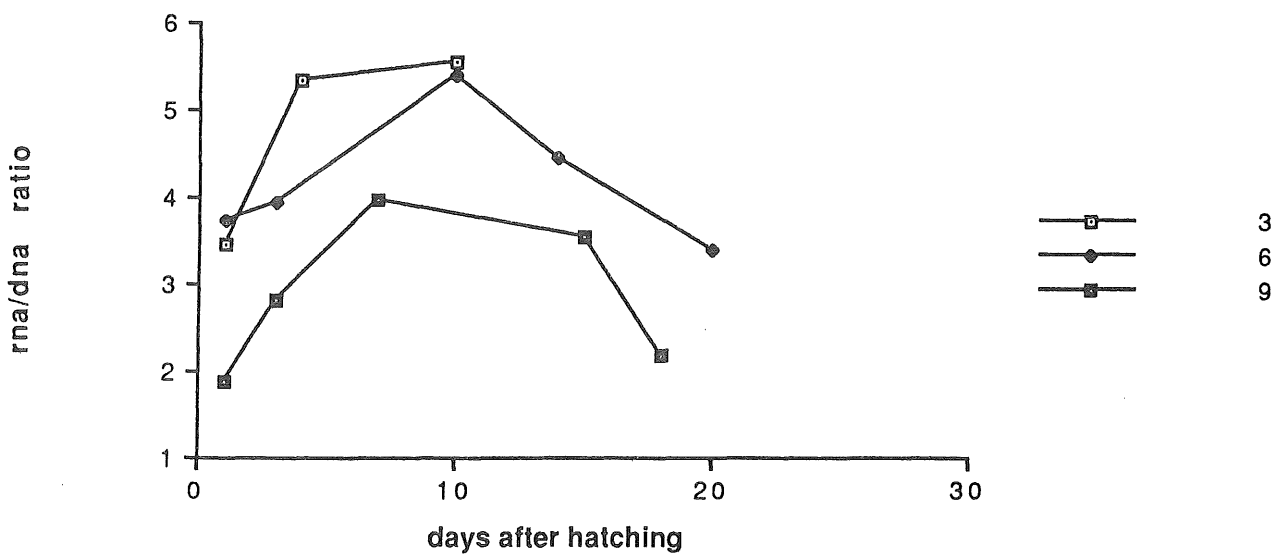


FIG. 13. RNA/DNA ratio of halibut larvae.

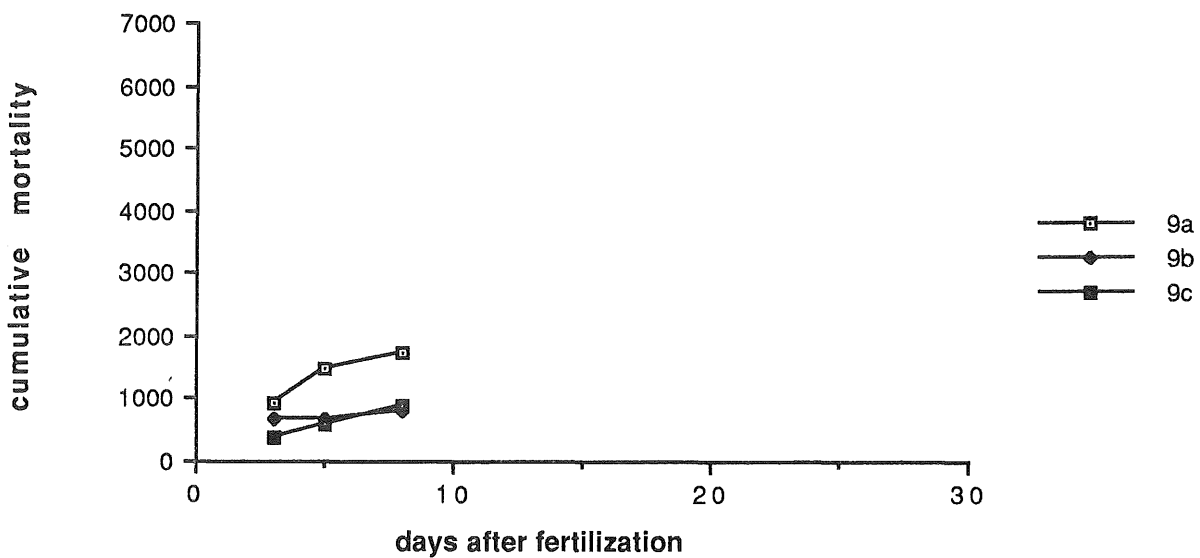
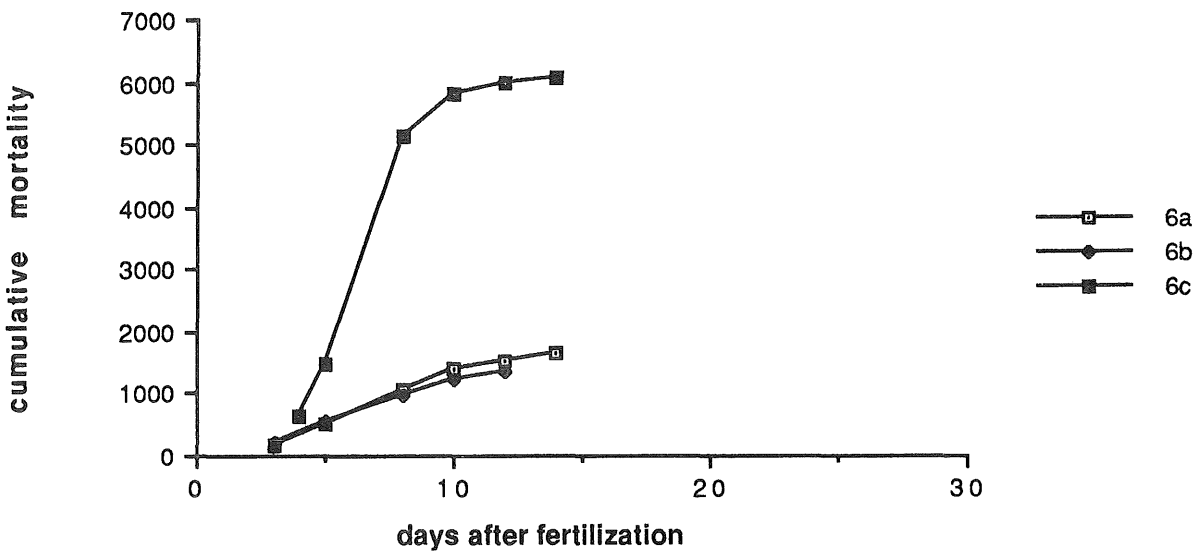
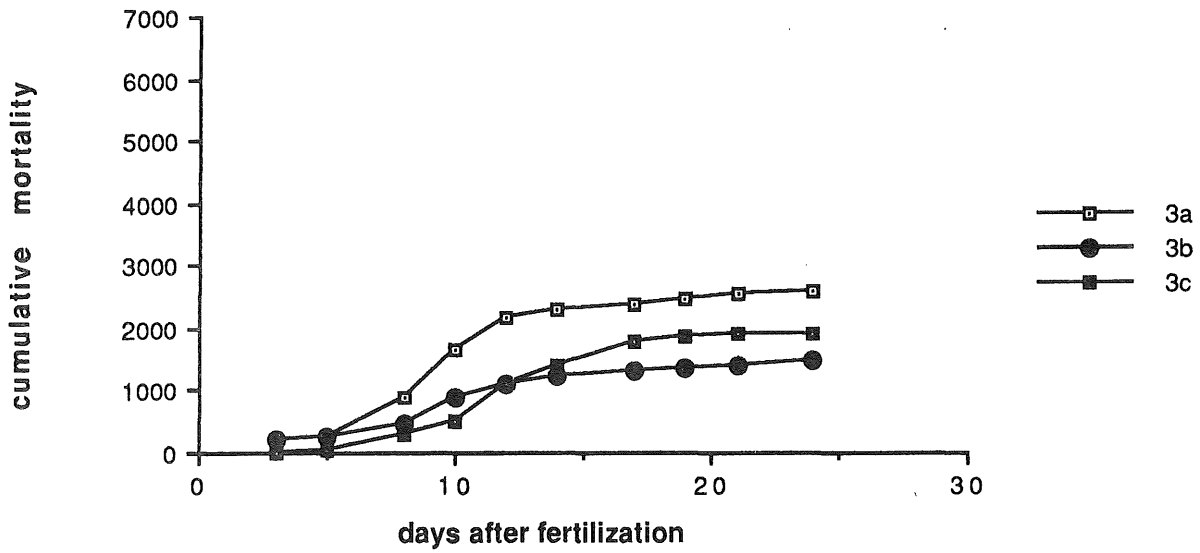


FIG. 14. Cumulative mortality of halibut eggs incubated at 3°, 6° and 9°C (top to bottom).

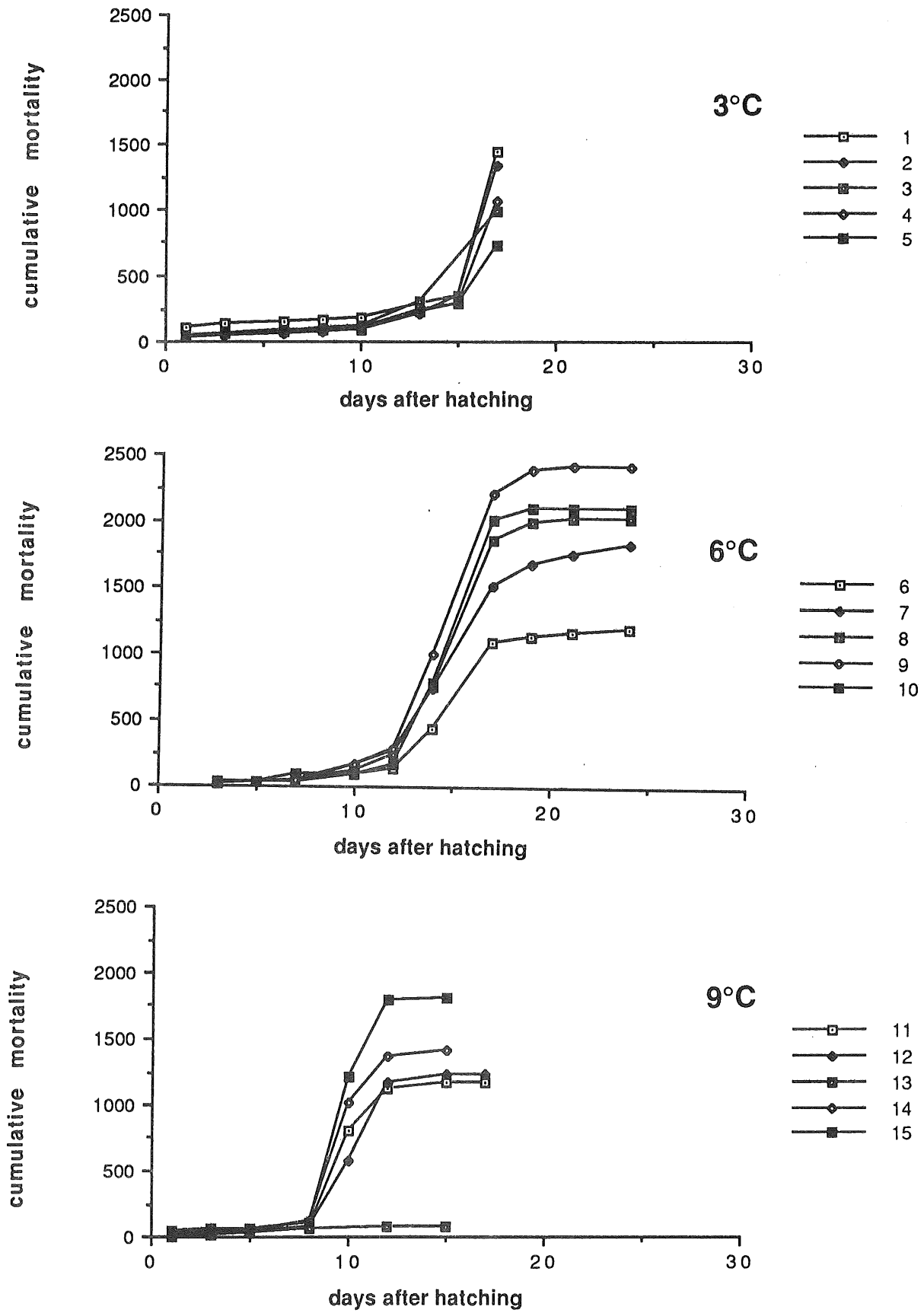


FIG. 15. Cumulative mortality of halibut larvae incubated at 3°, 6° and 9°C (top to bottom).

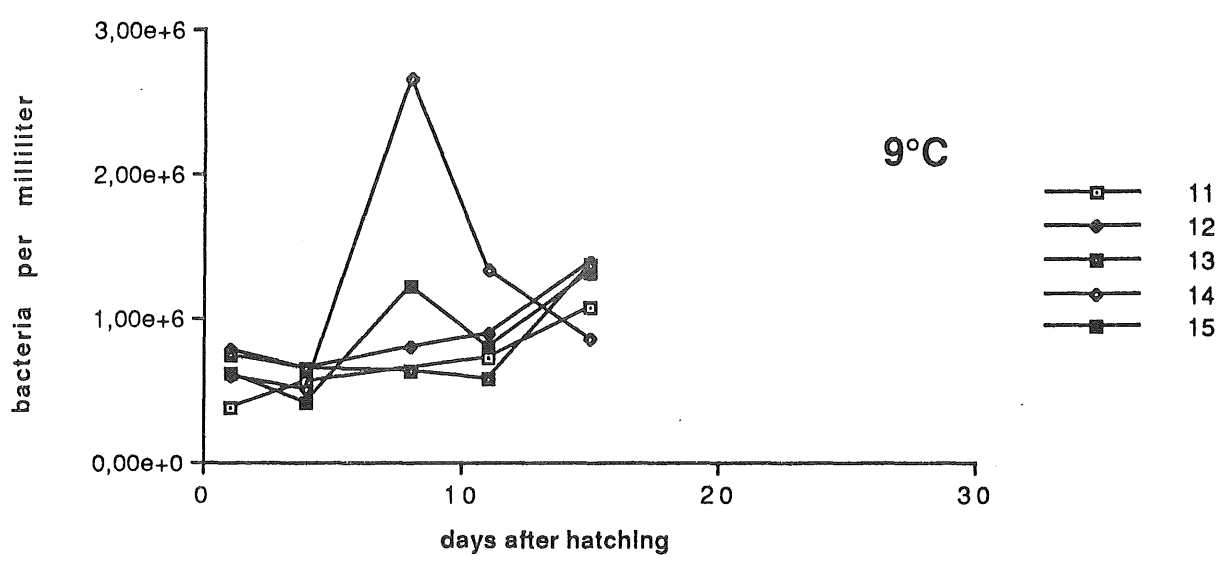
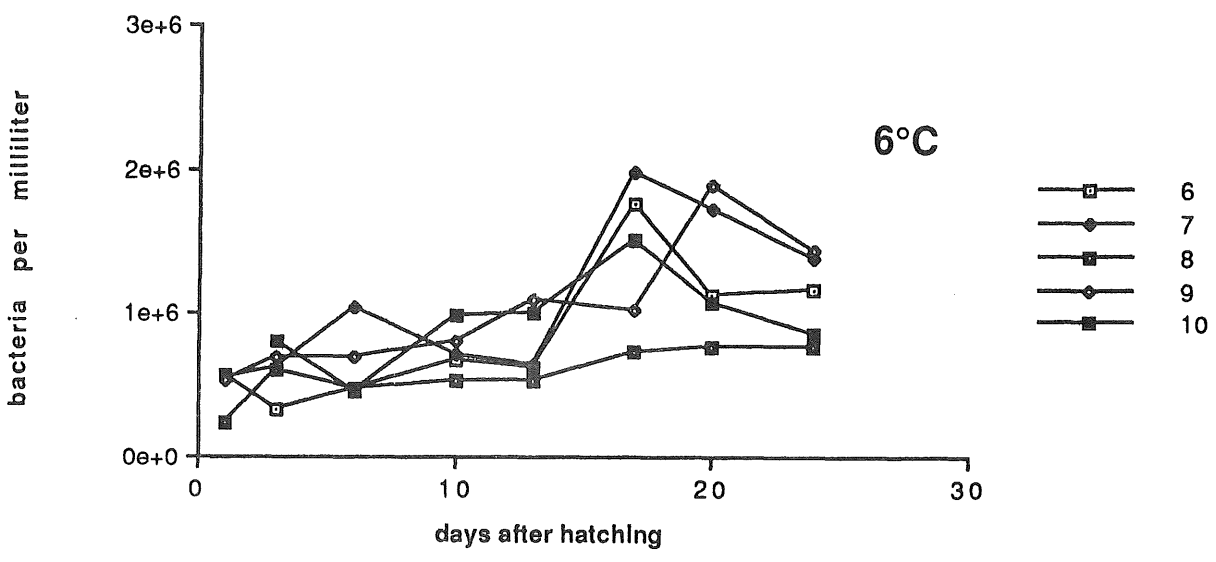
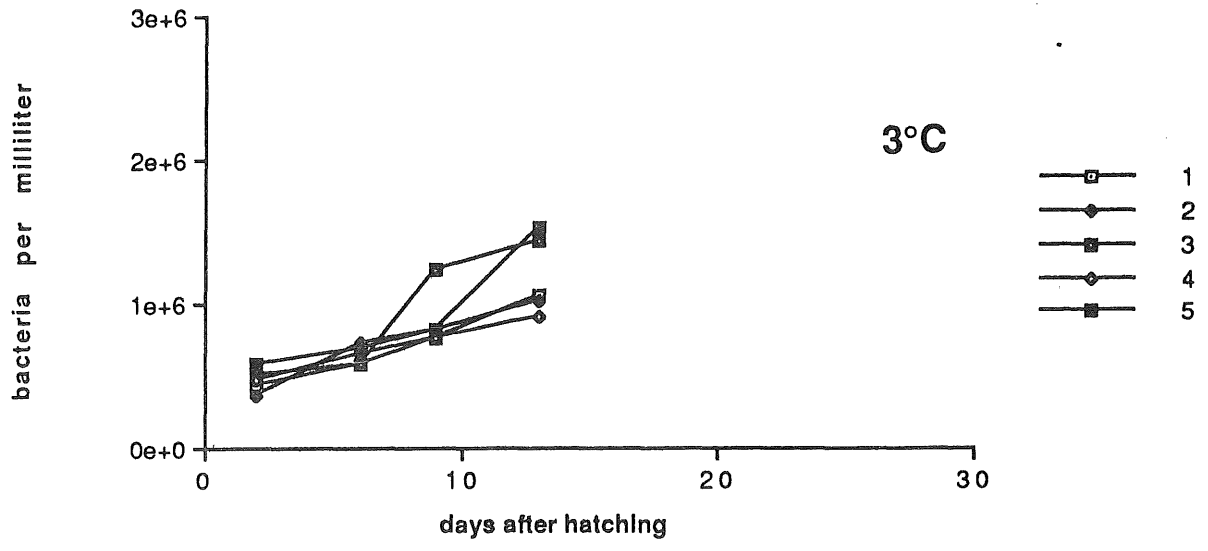


FIG. 16. Total counts of bacteria in larva incubators at 3°, 6° and 9°C (top to bottom).

DISCUSSION

Egg development

Development of the eggs showed a difference in rate already within the first cell divisions. Hatching took place after 600 hours at 3°C, 336 hours at 6°C and 211 hours at 9°C. This deviates from a time to hatching of 13 days (312 hours) found at about 5.7°C (Finn 1989), but may be due to small temperature variations. Some anomalies were observed, such as the appearance of Kupffer's vesicles and an incomplete tail development. The mean number of vesicles was highest in the 9°C group whereas many larvae in the 6°C group had no vesicles. This leads us to question whether these are in fact vesicles or artifacts due to (suboptimal) abiotic factors. It also seemed that the nature of the vesicles was different in each of the temperature groups and samples have been taken to examine their histology.

Larval growth and development

It seems that in the 6° and 9°C groups there was no increase in the number of cells (DNA) from hatching until about day 14, whereas the number of cells increased earlier in the 3°C group. Total protein synthesis (RNA) increased throughout the experiment and seemed to be best at 3°C. Since the dry weight data is incomplete, we cannot say whether these larvae also have a higher Specific Growth Rate. The DNA data indicate that the embryos raised at 9°C had developed furthest at hatching, a finding which is supported by the smaller yolk sacs and the larger space between the membranes. This would seem to indicate that although the timing of hatching was shortened by the warm temperature, the emergence was delayed relative to larval development. The other groups were not significantly different in standard length at hatching but did have larger yolk sacs. Further confirmation by histological examination and protein analysis is needed.

The RNA/DNA ratios seem to follow an approximate bell curve in all three groups, indicating growth without cell division during the first two weeks and thereafter an increased cell division. This is supported by morphological observations showing that organs arise and increase in complexity about the time of the downward trend in RNA/DNA. The ratios were also about twice as high as those for yolk sac larvae of salmon (Taranger, 1989) although the curve for the 9°C group started at about the same level as that of Atlantic salmon. The values for halibut larvae are lower than those reported for cod (Raae et al. 1988) but about the same level as those reported for winter flounder (Buckley 1981). The difference between temperature groups supports the hypothesis forwarded by Raae et al. (op. cit.) that differences in nucleic acid contents reflect environmental factors.

The RNA/DNA ratios decreased to the same level as at hatching about 25 days after emerging from the egg. In Buckley's study of winter flounder larvae, the lowest ratios were found in starving fish, and were independent of standard length. Cessation of feeding resulted in a decrease in the RNA/DNA ratio of winter flounder and cod within 2 and 4 days respectively, and Buckley proposed that the ratio analysis could be used to detect the early stages of starvation. Although not conclusive, the data presented here for halibut support the hypothesis that exogenous nutrients are needed already halfway through the yolk sac stage.

Larval growth showed a rapid increase in standard length in the 9°C group from the time of hatching, while the 3°C group showed the slowest increase in standard length. This repeats results from 1988 (Pittman et al.) where those larvae raised at 9°C were also longest until about day 25 when those from 6° and eventually 3°C overtook, the latter having the greatest length at end-of-yolk-sac.

Larval mortalities

Larval mortality peaked after 8 days at 9°C and 12 days at 6°C, when development had reached approximately the same stage. At this stage, the heart was still tubular, the eyes were partially pigmented, the head was lifted to mid-eye, there was a cellular aggregation for the liver and a groove at the area of the prospective mouth. This stage in development corresponds to the time at which the larvae naturally begin to sink (Pittman et al, submitted),

an event which was compensated for by increasing the flow rate to lift the larvae. Unpublished data (Opstad and Bergh) show a clear increase in mortality with increasing flow rates. Mortalities at 3°C closely followed daily temperature fluctuations and these larvae died earlier in development than those at warmer temperatures.

Deformities

Two deformities were observed, gaping (at 9° and 6°C) and incomplete caudal development (at 3°C). Gaping occurred during the larval stage. It may have been due to either mechanical stress caused by flow or by an effect of temperature on jaw development and repeats findings which associate gaping in halibut larvae with warm temperatures (Bolla & Holmefjord 1989 and Pittman et al. 1989). Caudal development was affected during embryonal development and may have been due to the incidence of very cold temperatures (less than 3°C) during the egg stage, or contamination by a disinfectant ("Algedreper" by Mastercare) which had a protein-fixing effect. Many of the dead in all groups had narrow tail fins which may be the result of flow rates, disinfectant and a subsequent colonisation by bacteria. Scanning electron micrographs of damaged tail fins revealed wounds colonized by large amounts of filamentous bacteria (Bergh and Pittman, unpub. data). Many of the abnormalities reported here, such as yolk, organ and mouth defects, have been described in other fish larvae as responses to sublethal environmental stressors (Rosenthal & Alderdice, 1976).

Bacteria

We were not able to find significant differences among the bacterial populations of different temperature groups. Both total count and viable count data indicate that factors other than temperature, such as flow rate, larval number and larval mortality, are more important in determining bacterial activity and numbers in the incubators. The lower total counts in the 3°C incubators were probably caused by the termination of these experiments at an earlier stage in larval development. There is also evidence (Holmefjord, pers. comm.) that larval density greatly influences bacterial counts but the latter is not correlated to larval mortality. Opstad and Bergh (unpubl. data) have shown differences in bacterial population composition and bacterial activity as a response to differences in incubator flow rate, and, probably, larval mortality.

The similarity in development, growth and mortality patterns in the incubators shows that the larvae experienced these as replicates, despite differences in flow rates and bacteria concentrations. The egg incubators which had the highest rates of mortality were those which were closest to other sources of influence (door, noise). Total ammonia levels and temperature were such that no more than 0.4% could be unionised (toxic) and the levels were below those recommended for chronic exposure (Wedemeyer et al., 1976) until high mortalities occurred.

CONCLUSIONS

Temperature during egg incubation affected the rates of development and growth, as well as the rates of protein synthesis and cell division at hatching. Development was slowest, but total and relative protein synthesis highest in eggs and larvae raised at 3°C. Yolk size at hatch was also largest at 3°C, although there was no significant difference in standard length. The halibut raised at 9°C appear to have come furthest in development at hatching although the time required to reach hatching was shortest. These larvae quickly developed abnormalities associated with sublethal stressors, such as small hearts and livers, and large peritoneal and pericardial spaces.

Relative protein synthesis (RNA/DNA) was highest at 3°C and lowest at 9°C throughout the experiment. The growth data point to the period between 25 - 30 days after hatching as the time at which an uptake of exogenous nutrients is needed, to continue a level of protein synthesis above that found at emergence. This is supported by behavioural and developmental data (Pittman et al. 1989 submitted, Pittman et al. 1987).

The primary cause of larval death is not clear. As larval death took place at the same stage in development at 6° and 9° C, before the increase in amount of free-living bacteria, bacteria were not the cause. There is a possibility that surface-bound bacterial infections of larva were involved, although viable counts indicate that numbers of free-living potential pathogens, such as *Vibrio sp.* and *Cytophagales* were low throughout the experiment. The filamentous bacteria involved in fin deformities could be of importance. An increase in total bacteria was in turn followed by a large increase in ammonium concentration, probably caused by bacteria metabolizing compounds from dead larvae, which are rich in proteins and amino acids, the surplus nitrogen being excreted. Thus a vicious circle is started, where larval death leads to increased bacteria numbers and increased ammonia levels which then weaken the remaining larvae, allowing further mortality and bacterial growth, and so on.

There is some evidence that high flow rate could cause increased larval mortality and increased bacterial activity, although the variable flow rates in this experiment make the calculation of bacterial growth rates extremely difficult. Brown spots were noted on the larvae early in development (Pittman and Strand, unpub. data). These had previously been shown under scanning electron microscopy to be areas where the tissue was worn down, in some cases to the cartilage, and the prevalence was higher in these incubators than it had been in previous years (Pittman, unpub. data). Overall water exchange rates were also higher in this experiment, and exceeded the level at which Opstad and Bergh obtained high mortalities (unpub. data). If the rate of flow is indeed a contributing factor, this may explain the coincidence of the periods of passive sinking, counteracting flow rates and an increase in mortality. It may also have contributed to the general wear on the fins and snout, allowing bacterial colonisation of the wounds.

Further investigation is continuing to add dry weight, specific growth rates, amino acids, protein content, histology, pathology, yolk conversion factors and characterization of bacterial isolates to the data already treated here.

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