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Induced deformities on larvae of the Atlantic halibut (*Hippoglossus hippoglossus* L.). A new experimental approach.

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ABSTRACT:

2 days before estimated hatching, halibut eggs from one egg batch were transferred to sterile polystyrene plates with 6 wells. Each well contained 10 ml of sterile seawater and one single egg. Chorion of the eggs was removed within 1 day after hatching and 5 ml of the seawater was renewed.
4 temperatures(range 0.9°C - 10°C), 4 light intensities(range 0.5 lux - 150 lux), 4 levels of O_2 concentrations (range approximately 1ppm - 8ppm), and 4 levels of H_2S concentrations (range 0.002-0.2mg/l Na_2S) were chosen as stressors. Each treatment was replicated with 60 or 48 eggs/larvae.
Overall survival in the control groups was somewhat lower (33% at 286 d° and 45% at 215 d°) than the rest of the egg batch reared in a "silo"(33% at 235 d°).
However, prevalence of healthy larvae was similar in control groups and silo(67 and 68 %, respectively). The percentage of healthy larvae were significantly lower ($p<0.05$) for low O2, H2S and light groups compared to control.
Low [O_2] caused higher prevalence of yolk sack oedema, abnormal jaw articulation and head deformities ($p<0.05$) than in control. Elevated temperature (10°C) did not increase prevalence of deformities compared to controls(7.5°C and 5.5°c; P=0.05).

INTRODUCTION.

The atlantic halibut is viewed as a promising species for cold water aquaculture, and have been subjected to extensive research efforts both in Norway and in U.K. the later years. As the biology, and especially the ontogeny of the halibut was little studied, a strategy of simultaneous basic and applied research have been chosen. It is known that several factors have effects on the developing fish larvae(Rosenthal and Alderdice 1976). We have the impression that answers to many of the basic problems concerning larval development have been sought in experimental units not suited to give these answers. We strongly felt the need to develop an experimental design were hypothesis could be tested.

MATERIALS AND METHODS.

Halibut eggs.

Approximately 20 females(weight range 15-120 kg) and 10 males(weight range 4-35 kg) were kept in a light shielded GRP-tank (volume 30 m^3) at Austevoll Aquaculture Research Station. Eggs were stripped according to Rabben (1987) and incubated according to Jelmert and Rabben (1987).

2 Days before hatching, approximately 2000 eggs from one single egg batch was transferred to a temperature controlled room (5.5°C) in 4 1l beakers. The rest of the batch(approximately 24000 eggs were transferred to a "silo" (Rabben *et al* 1987).

Experimental design.

After adjustment to ambient temperature in the temperature controlled room, the eggs were transferred to wells in multidish plates (Nunc a/s Kamstrupv.90, Roskilde, Denmark). The plates are made of transparent polystyrene and has a transparent lid which is positioned to give air exchange in the wells. Each plate has 6 cylindrical wells with a inner diameter of 3.5 cm With a height of 1.7 cm this gives an effective volume of ca. 15 ml. We use plates optionally presterilized by gamma-radiation.
In advance, the wells was filled with 10 ml autoclaved 70% seawater(SSW). The eggs were transferred with reversed sterile pasteur pipettes with rubber bulbs, one single egg to each well. Together with the eggs, approximately 1 ml of the incubation seawater was supplied, giving a total volume of 11 ml in each well.

Eggs were stored in darkness at 5.5°C until hatched. Within 1 day after hatching, 5 ml of the water, and the eggshell in each well was removed.
5 ml of SSW at ambient temperature was supplied. The newly hatched larvae was handled in dark-room illumination or in total darkness.
The multidish plates was split in groups of 10 at random, and transferred to the following environmental regimes.
Temperature:
1°C(range 0.9-1.6), 3°C(range 2.8-3-4), 5°C(range 5.4-5.8) 8°C(range7.5-8.0) and 10°(range 9.9-10.0). All plates were stored in darkness.
The different temperatures in the experiment was obtained by: a temperature controlled growth chamber(10°C), ambient temperature in the temperature controlled room(5.5°C), and a temperature controlled water bath. The bath was 100x100x10cm, and was continuously heated in one end, and cooled in the opposite end.
The multidish plates was placed floating in the water at fixed distances from the heating and cooling ends.

Light: 0, 0.6 lux, 6 lux, 33 lux and 115 lux.
A non-transparent tent (100x100x100cm) was placed in the temperature controlled room. The roof in the tent was covered with white paper and 4 desktop lights was mounted(Tungsram FD 11, 11W low-energy light tubes) to give reflected uniform light. Different light intensities was obtained when the plates was placed under lids of neutral density filters(Rosco cinegel 3403 and 3402). Temperature for the group was 7.5°(range 7.1-8.0). A small box of black plastic in the tent contained the control: 0(=8°C in temperature group).

O_2 concentration: Full (7.6 ppm), 6, 3 and 1 ppm O_2.
SSW was scrubbed with N_2 for 30 minutes to give a solution of approximately 1 ppm O_2.
Water in the wells was exchanged with different volumes of the scrubbed SSW to give theoretical concentrations of approximately 6, 3 and 1 ppm O_2. The renewal of water was made at 4 days intervals until day 32 were the treatment was terminated.
Once in the experiment, oxygen partial pressure was measured in the wells with a microelectrode (Revsbech and Jørgensen 1986) and recalculated to oxygen concentration.

H_2S concentration: In advance, oxygen-free SSW was made by supplying 1ml of a 0.3M solution of $Na_2S_2O_4$ to N_2-aerated (2h) SSW. A stock solution of Na_2S was made by dissolving 20 mg Na_2S in 1 l of oxygen-free SSW.
1,10,50 and 100 µl of the stock solution was added to the wells every second day to give instantaneous concentrations of 0.002, 0.02,0.1 and 0.2 mg/l. In the control, 100 µl of SSW was added. The addition of Na_2S was terminated at day 32.

The surviving larvae were harvested at different ages(d°). When harvested the larvae were immediately anaesthetized by addition of 100 µl of a 1 g/l stock solution of methomidate(Norsk Medisinaldepot) to each well. The dose was approximately 5 times stronger than suggested by Mattson and Riple (1989). Our procedure fixed the larvae with the vertebrae in an elongated position within 2 minutes at 22°C, and only hart musculature was active. The larvae was photographed in a Wild zoom binocular microscope at 6,4 times magnification. While being at the microscope stage, the following classes of deformities was monitored.
Yolk sack oedema(Y.S.O.(Rosenthal and Alderdice 1976)), Unarticulated jaws(U.J.(Pittman *et al* 1987), Head deformities, Disrupted mouth membrane((M.M)*op cit*), heart disorders(a: abnormal heart chambers, b:bacterial infections), gill infections and injuries of the vertebrae("tweezers dents").
The standard lengths and myotome heights were measured in a Wild M binocular microscope with a calibrated ocular graticule.
After the measurements the larvae were washed twice in tap water, thrice in distilled water and dried to constant weight at 60°C.
Dry weights were measured on a Mettler M3 µg electronic balance.

COMMENTS ON THE METHODS.

For reasons explained below we had to define the H_2S and O_2 concentrations in operational terms. It is not easy to maintain reduced sulphur-compounds in an environment the halibut larvae can tolerate. The chemistry of sulphur is in addition very complicated. The instantaneous "true" amounts of different sulphur compounds in the wells will remain unknown. However, by supplying different amounts of Na_2S at fixed intervals it should be possible to observe dose responses of H_2S. A similar problem was encountered for the experiment with lowered oxygen concentrations. We had to perform the experiment in normal atmosphere. An atmosphere with oxygen concentration similar to the actual experimental group would have been desirable. 24 hours after the supply of scrubbed SSW, we could measure a drift towards higher O_2 concentrations (table 1).
The concentration terms for H_2S an O_2 should therefore be treated with some caution.

Table 1. Oxygen concentration (recalculated as ppm from partial pressure).

Control	1ppm	3ppm	6ppm
7.56	2.6	4.7	6.2

From the experiences during the experiment it was evident that the two groups with 5 and 10 μl of Na_2S added lost its reduced sulphur content very rapidly(test by smell). These groups were therefore also included among the control groups. When later referring to H_2S-groups, we are referring to the two higher concentrations(50μl and 100μl Na_2S added.

It was difficult to obtain the different temperatures with acceptable accuracy in gradient water bath system. The wide temperature ranges implies that separate water baths or temperature controlled cabinets should be used.

RESULTS AND DISCUSSION.

Survival.

The cumulative mortality(=mortality at harvest) in the wells is given in figure 1. The following features should be noted: The cumulative mortality in control group at 286 d° was 77 % compared to 33% in the silo (at 235 d°). We believe that the difference was mainly due to bacterial growth and activity in

the wells.
In an infection experiment(Bergh *et al.*1990) carried out in similar systems, the cumulative mortality in uninfected group was 5% (198 d°). In a disinfection experiment(Bergh and Jelmert 1990), the control group had a cumulative mortality of 33% (198 d°) compared to 14 % in a group treated with an iodophor disinfectant.
A quite dramatic effect was observed in the group incubated at 1°C. At approximately 20 d°, more than 60% of the larvae had developed severe black necroses in the yolk sack. At 50 d°, 82% of the larvae was dead. Only 3 larvae survived through 200 d°.

The light exposed groups developed both eye and body pigmentation earlier than the dark groups. This phenomenon is treated in more detail in Helvik and Pittman (1990).

Dry weight.

The dry weights and time of harvest of the experimental groups are shown in figure 2.
A regression of dry weight as a function of age(d°) for the control groups was calculated. In the period of 170 to 300 d° the loss in dry weight was close to linear and can be expressed as.

1)	$y = -0.00251x+1.5386,\ r=0.9688$

Larvae from 1°C, 3°C and 6ppm O2 were significant lighter than corresponding controls ($P<0.05$; Comparison of percentages, Sokal and Rohlf, (1969)). The larvae from the silo were lighter than the larvae in the control groups in wells. We believe this can be attributed to swimming activity. In contrast to larvae in silos, larvae in wells will not be triggered by turbulence or tactile stimuli from other individuals .
Larvae exposed to the lowest O2-levels were heavier(not significant) than the controls. Together with the massive deformities(see below) this indicated a poorer utilization of energy reserves, probably coupled to oxygen consumption.
A very low dry weight of 0.436 mg was observed on the larvae at 1°C. As this number was only based on the 3 surviving larvae at 151 d° we will not stress its importance. However, together with the significant low dry weight for the 3°C larvae it indicates that age expressed as d° is not a proper measure for age at low temperatures (below 3°C). Pittman *et al.*(1989) reported differences in time to EYS(end of yolk sack) as a function of age in d°, when comparing groups grown at different temperatures.

Deformities.

The percentages of larvae without deformities are shown in figure 3. The results were pooled for each experimental group. The three experimental groups: light, low O_2 and H_2S had a significant lower percentage($P<0.05$; Comparison of percentages, Sokal and Rohlf, 1969) of healthy larvae than the control groups. The 3°C group had also significant lower percentage of healthy larvae($p<0.05$), but in contrast to earlier reports(Bolla and Holmefjord 1988, Pittman *et al.* 1989) the 10°C had not higher prevalence of deformities compared to control.

Several of the observed deformities was often coupled, mainly unarticulated jaws to heart deformities, unarticulated jaws to disrupted m.m. and gill infections to heart infections.
The prevalence of deformities (as % of surviving larvae) are shown in figure 4. Please observe that single larvae often had several deformities, so the percentages can add to more than 100%.
When looking at the individual classes of deformities, only the larvae subjected to lowered oxygen levels showed significant increase of yolk sack oedema, unarticulated jaws and head deformities compared to control groups ($P<0.05$; Comparison of percentages, Sokal and Rohlf, 1969).
All experimental groups had higher prevalence of head deformities than the control, but at a low significance level.($P<0.1$; Comparison of percentages, Sokal and Rohlf, 1969).
When using the term "low oxygen concentration" we can not rule out eventual effects of N_2 supersaturation for some of the deformities observed. In a pilot experiment however, we observed yolk sack oedema in wells with 2 or more larvae, indicating that oxygen deficiency at least is involved.
When the treatment with low O_2 water was ceased at day 32, a slow recovery of the yolk sack oedema was observed.

CONCLUSIONS.

All experimental groups(pooled data) except 10°C had significant higher prevalence of deformities than controls. The prevalence of deformities in control was 32 % compared to 31 % for the same egg batch incubated in a silo.

Prevalence of yolk sack oedema, unarticulated jaws and heart disorders was significant larger for groups exposed to low O_2 concentrations compared to control group.

The larvae that had developed a yolk sack oedema was able to partly resorb the oedema after the treatment with stressor was terminated.

The mortality in control groups at 204 d° was 43 % compared to 33% in silo at 235 d°.

The test system were found to be well suited to test hypothesis of the effects of several environmental parameters on halibut larvae. We believe the concept of single larvae in separate units have several advantages worth mentioning:

1) Death of larvae during experiment does not affect the rest of the experimental group. 2) The system allows a large number of replicates 3) Observations and measurements can be facilitated during experiment without disturbing the system. Thus the development of a single phenomenon can be monitored. 4) In the proposed or similar experimental systems, infection experiments can be safely run in the lab. Risk of infection to, or contamination of the environment can be minimized.

A better knowledge of antibacterial treatment procedures is expected to decrease cumulative mortality(Bergh and Jelmert 1990). A high mortality was a problem when incubating larvae longer than approximately 240 d°
We believe more results will come out from more comprehensive analysis of our data.

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LITERATURE.

Bergh, Ø. and Jelmert, A. 1990. Antibacterial treatment procedures of eggs of halibut(*Hippoglossus hippoglossus* L.)
I.C.E.S. C.M.1990/F:39

Berg, Ø, Hansen, G.H., and Jelmert, A. 1990. Bacterial diseases of eggs and yolk sac larvae of halibut(*Hippoglossus hippoglossus* L.): Characterization and experimental infection.
I.C.E.S. C.M.1990/F:38

Bolla, S. and Holmefjord, I. 1988. Effect of temperature and light on development of Atlantic halibut larvae.
Aquaculture 74:355-358 1988

Helvik, J.V. and Pittman, K. 1990. Light affects hatching, development and pigmentation of halibut(*Hippoglossus hippoglossus* L.)
I.C.E.S. C.M.1990/F:40

Jelmert, A. and Rabben, H. 1987. Upwelling incubators for eggs of the atlantic halibut(*Hippoglossus hippoglossus* L.)
I.C.E.S. C.M. 1987/F:20

Mattson, N. and Riple, T.H. 1989. Methomidate, a better Anesthetic for Cod (*Gadus morhua*) in Comparison with Benzocaine, MS-222, Chlorobutanol, and Phenoxyethanol. Aquaculture, **83**(1989) 89-94.

Pittman, K. Berg, L. and Naas, K.E. 1987. Morphological development of halibut *Hippoglossus hippoglossus* larvae with special reference to mouth development and metamorphosis.
I.C.E.S C.M. 1987/F:18

Pittman, K. Skiftesvik, A.B., and Harboe, T. 1989. Effect of temperature on growth rates and organogenesis in yolk sac larvae of halibut(*Hippoglossus hippoglossus* L.) Rapports et Proces-vebaux du Cocil international pourl'Exploration de la Mer 191:421-430

Rabben, H. 1987. A stripping method for atlantic halibut (*Hippoglossus hippoglossus* L.)
C.M.1987/F:40

Rabben, H. Jelmert, A., and Huse, I. 1987. Production experiment of halibut fry(*Hippoglossus hippoglossus* L.) in silos.
I.C.E.S. C.M 1987/F:42

Revsbech, N.P. and Jørgensen, B.B. 1986. Microelectrodes: Their use in Microbial Ecology. In: Advances in Microbial Ecology, vol 9. Ed. Marshall, K. C. Plenum Publishing Corporation, 1986.

Rosenthal, H. and Alderdice, D.F. 1976. Sublethal effects of environmental stressors, natural and pollutional, on marine fish eggs and larvae. J. Fish. Res. Board Can. **33**:2047-2065

Sokal, R.R. and Rohlf, F.J. 1969. Biometry.
W.H. Freeman and Company San Fransico U.S.A. 1969.

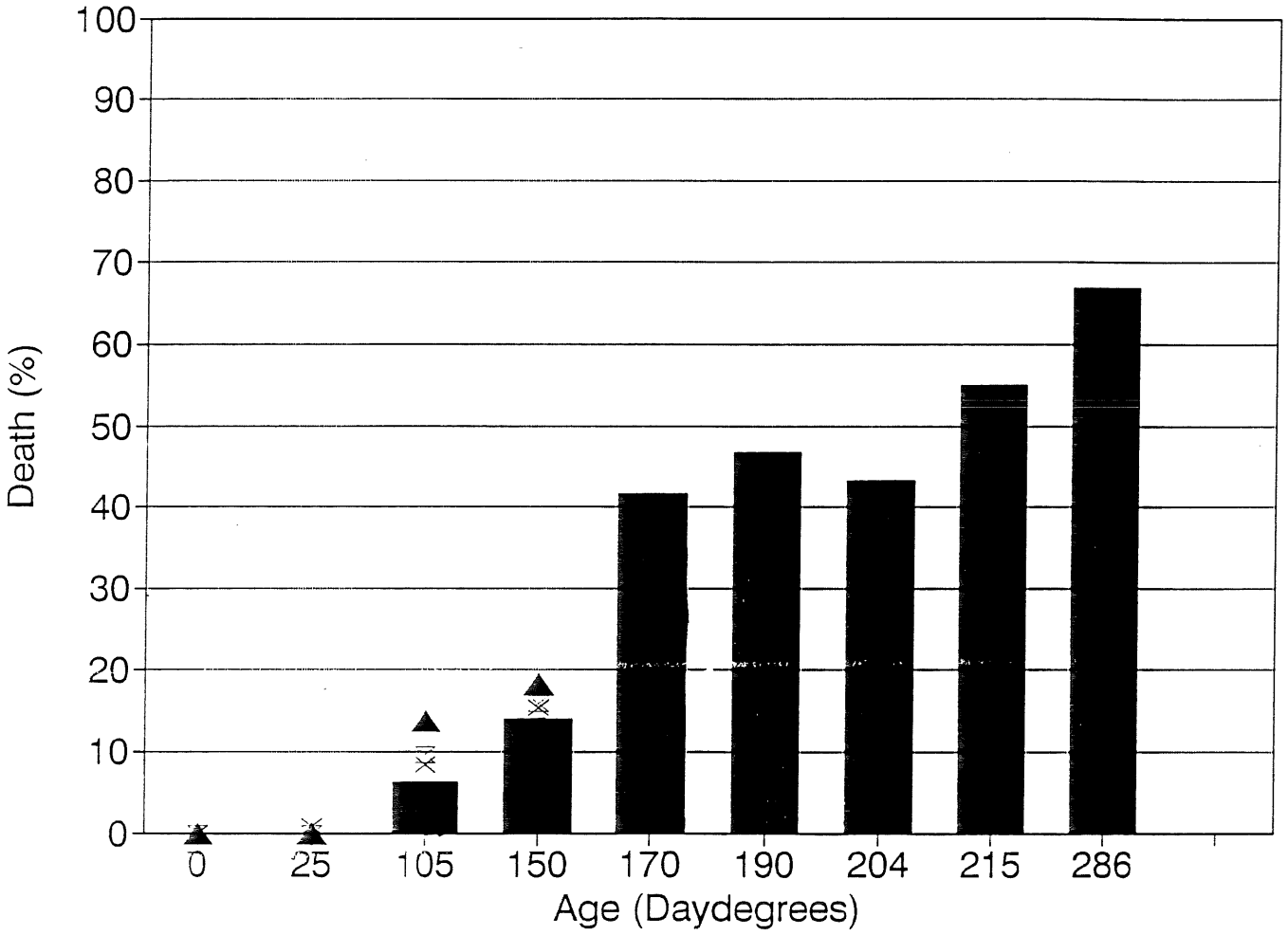


Figure 1. Cumulative(=total) mortality during experiment (below 150 d°), and at termiation of experimental groups. Black bars: Control groups. Filled triangles: H2S. Hourglass: Low O2. Crosses: Light.

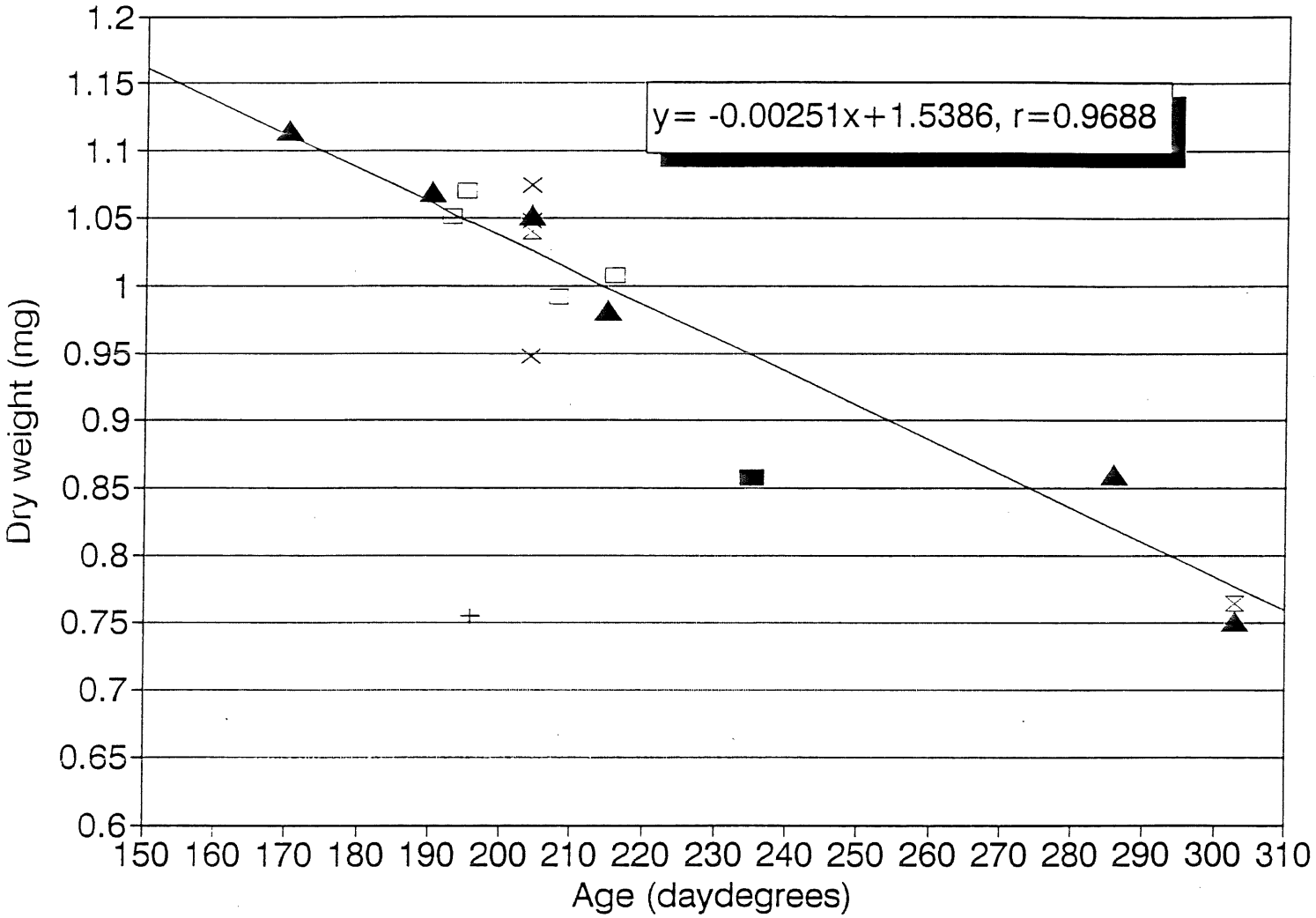
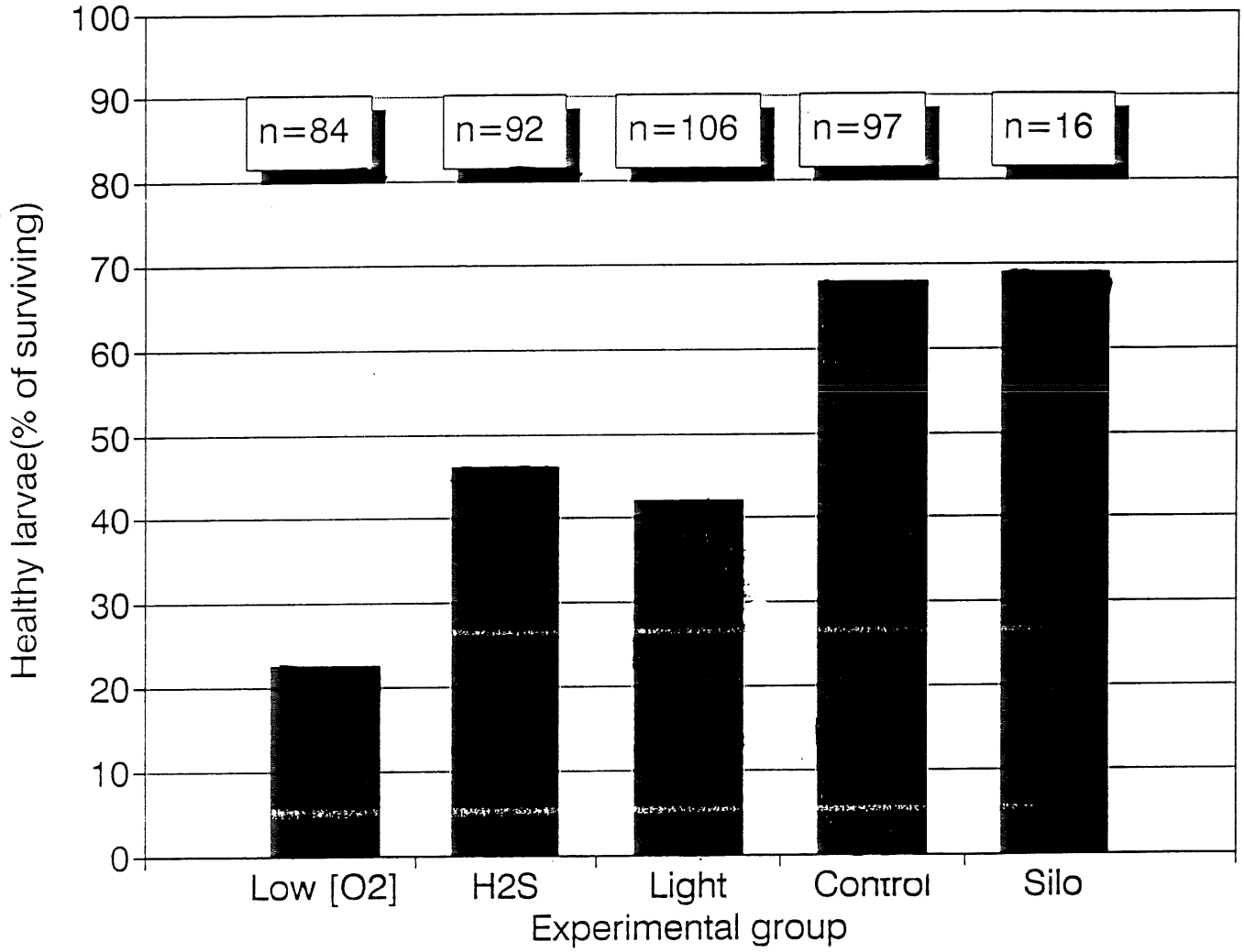


Figure 2. Dry weight as a function of age. Filled triangles: Control groups(Including 10°C at 170 d°). Open rectangles: light. Crosses: Low [O_2]. Hourglasses:H_2S. Plus: 3°C. Filled rectangle: Silo



Fgure 3. Prevalence of healthy larvae. Numbers in boxes are total number in pooled groups.

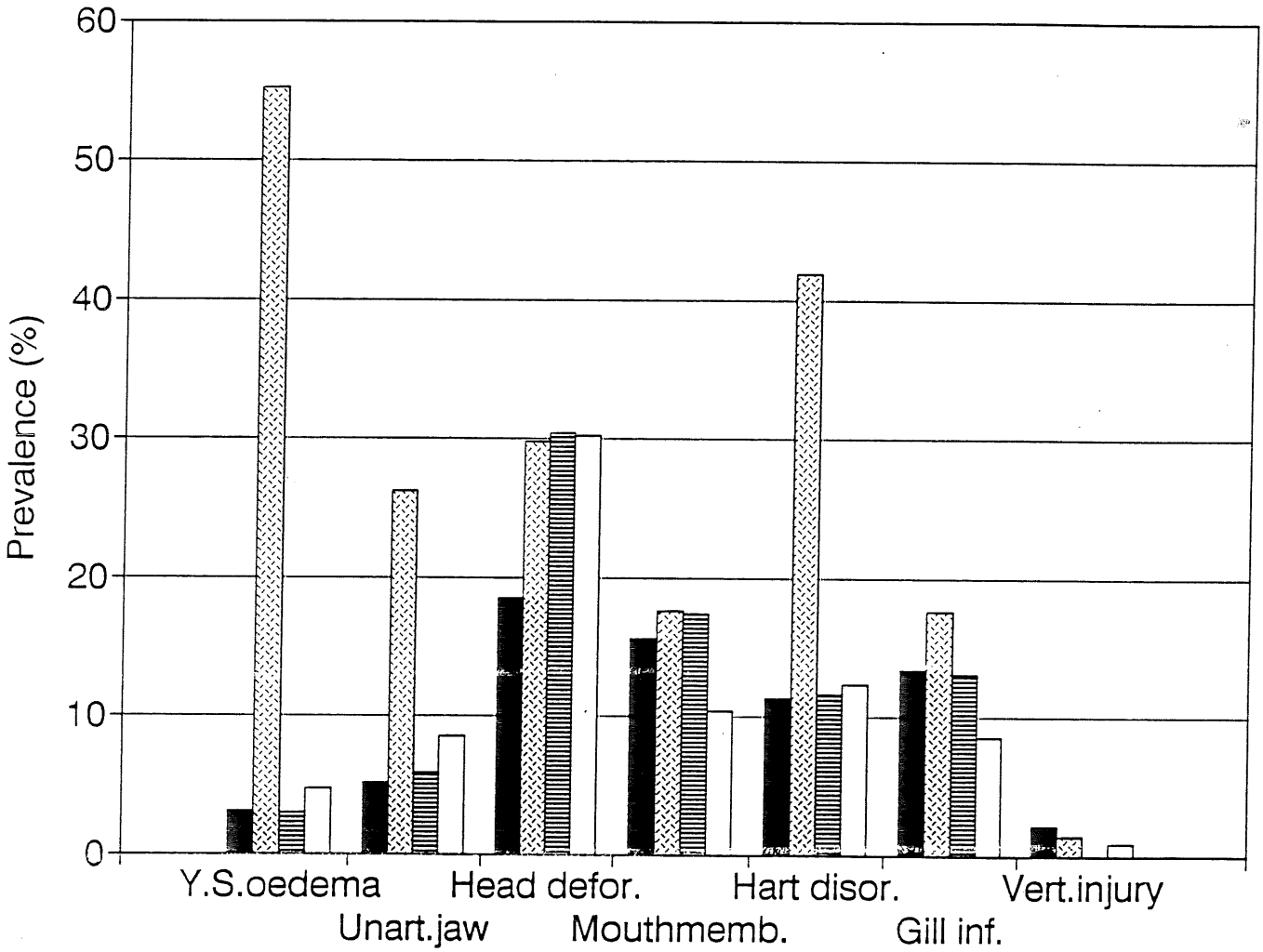


Figure 4. Types and distribution of deformities. Black bars: Control groups. Stitch: Low[O_2]. Horizontal stripes:H_2S. Open bars: Light.