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Theme Session on causes of observed Variations in fish growth (Theme session P)

DOES THE OTOLITH STRUCTURE REFLECT THE NUTRITIONAL CONDITION OF A FISH LARVA?
- A COMPARISON OF OTOLITH STRUCTURE AND BIOCHEMICAL INDEX (RNA/DNA RATIO) DETERMINED ON COD LARVAE -

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ABSTRACT

Cod larvae from laboratory rearing experiments aged from 1 to 12 days after hatching fed and deprived of food were analysed. The number of increments on the otolith and the width of these increments were determined together with the RNA/DNA ratios on the same larva. Alizarin marking of the otoliths was performed to validate the formation of daily increments. Cod larvae reared at 6°C formed the first ring right after hatching and deposited increments on a daily basis. A comparison of the measurements between the right and the left lapillus showed that the measurements between the right and the left otolith can differ, if the radius is taken. Specially the otolith-core showed high individual variability. Whereas the sum of the increments didn't differ between both otoliths. Until day 10 after hatching, while the larvae were still feeding on their yolk, the external food situation didn't effect the increment width of the otoliths or the RNA/DNA ratios. In larvae older than 10 days the widths of the daily increments was dependend on the nutritional situation and RNA/DNA ratios decreased in starving larvae in comparison to feeding larvae. RNA/DNA ratios and increment widths were correlated.

INTRODUCTION

Fluctuations in the size of fish populations may occur as a consequence of changes in the annual influx of young or recruiting fish. Recruitment variations are compounded by the effects of human exploitation and often attributed to the effects of environmental variations on the survival of egg and larval stages. The success or failure of annual recruitment can have a number of different abiotic and biotic causes. High mortality rate during early stages is considered one of the major factors causing stock fluctuations. The lack of food or a mismatch in larval fish and food organisms distribution are a principal cause for poor year class strength (Hewitt et al. 1985, Hjort 1914, Hunter 1976, Lasker 1978).

The relationship between RNA and DNA is an index of the cell's metabolic intensity and has been used to measure recent growth in fish (Bulow 1987, Buckley 1984, Buckley & Lough 1987, Hovenkamp 1990, Hovenkamp & Witte 1991) and has proven a useful indicator of the nutritional condition as shown in several larval fish studies (Buckley 1980, 1984, Buckley & Lough 1987; Clemmesen 1987, 1994, Fukuda et al. 1986; Martin et al. 1985, Raae et al. 1988, Robinson & Ware 1988).

All condition indices determine the nutritional condition at the time of catch reflecting the situation of the last hours, days or weeks before catching depending on the methods used. It is sometimes difficult to predict if the larvae are on the way of improving or deteriorating their condition. RNA/DNA ratios are commonly used to back- and forecast the growth and survival potential of larvae in order to help predicting recruitment. The validity of the RNA/DNA ratio can be improved by incorporating otolith increment structure studies (daily increment studies) on some subsamples to have the growth history of the larvae as well as the condition at catch.
Otolith structures reflecting daily patterns were described by Pannella (1971, 1974) and experimentally proven by Stuhsaker & Uchyama (1976). Brothers et al. (1976) used otolith increment structure to determine the age of larvae and juvenile fish.
The environment experienced by a larva will influence it's otolith structure as could be shown in several laboratory experiments (Panella 1980, Radtke & Dean 1982, Neilson & Geen 1982, Dale 1984, Berghahn & Karakiri 1990, Zhang & Runham 1992). Studies on Norwegian spring spawning herring and North Sea autumn spawning herring have shown the potential of using otolith microstructure in recruitment research (Fossum & Moksness 1993).
Daily ring formation was to be validated in this study on laboratory reared cod larvae by using Alizarin marking (Blom et al. 1994). The aim of this study was to combine otolith

microstructure analysis and RNA/DNA ratio determination on the same individual larva to compare the effect of food deprivation on the width of the daily increment and the RNA/DNA ratio and evaluate the potential use of this combination for recruitment research.

MATERIALS AND METHODS

Cod larvae were reared in the Havsfiskelaboratoriet Lysekil, Sweden from 03.05.94 - 27.05.94. Adult cod caught in November 1993 in the Bornholm Basin were transported to Lysekil, adapted to the higher salinity (30 ppm) and kept in 10000 liter tanks at 6-7°C as brood stock for the experiments. Cod spawned naturally, the fertilized eggs were transfered into 100 l tanks using a 300µm plankton net. The eggs float on the surface and are moved by a gentle water flow (0.5 l/min.). Temperature in the incubation tank was 6-7°C, larvae hatched after 13 days and were transfered to 100 l rearing tanks. Larvae were fed starting at day 4 after hatching with the rotifer *Brachionus plicatilis* reared on the flagellate *Isochrysis galbana*. Temperature throughout the experiment varied from 6°C to 7.5°C and salinity was between 33 and 34ppm. Tanks were illuminated by natural day light giving a 16hour day/ 8 hour night cycle. Larvae were fed three times a day at 8:00, 12:00 and 16:00 at a density of 0.65 *Brachionus*/ml. The daily food ration accumulated to 2 *Brachionus*/ml.

For marking of the otoliths 100 larvae were transfered to a 5 liter containing Alizarin (50mg/l, Tsukomoto 1988, Blom et al. 1994) on day 4 after hatching and kept there for 16h to set a fluorescent mark on the increment formed that day. After that the larvae were carefully transfered to clean sea water. Samples of the larvae were taken daily starting on day 4 at 11:00 after the larvae had been fed at 8:00. Five larvae per sample were taken, transfered into Eppendorf vials and stored in liquid nitrogen until the end of the experiment. After that the samples were put into a -70°C freezer and left there until analysis.

Prior to the determination of RNA- and DNA- contents the standard length of the larvae was measured and the otolith dissected. Sagittae and lapilli were identified using a polarisation-filter attached to a binokular and were fixed on a glass slide marking left and right side.The larva without the otolith was put into an Eppendorf vial kept on ice. After 5 larvae were dissected (necessary time: 30min.) the RNA/DNA analysis was performed. The analytical procedure was performed according to the method by Clemmesen (1993) using a specific nucleic acid fluorescent dye - Ethidium Bromid (EB)- for DNA and RNA. In order to measure

the DNA-content of a sample, RNA was enzymatically digested with RNase and the remaining DNA was determined with EB.

Measurements on the otoliths were performed using a digitalized computer aided video system with a CCD camera (Panasonic WVCL 700) connected to a fluorescence mikroskop (Zeiss Axioplan) using 1000-times magnification. Of the three otoliths the lapilli were chosen, since they are the largest at time of hatching up to age 25 in cod larvae (Bergstad 1984). Both lapilli were used for the analysis and the radii, number of increments and width were measured four times in four different directions on the otolith.

Results

To determine the accuracy of the age determination using the number of increments the results of the readings on the lapilli were compared with the known age of the laboratory reared cod larvae. Results are shown in Fig. 1a. 147 out of 150 analysed otoliths showed the deposite of a daily increment starting on the day of hatch. Only 2% of the analysed lapilli showed one ring less than expected.

16hrs treatment with Alizarin resulted in an orange coloured increment deposited on the day of marking. It could later be identified under the fluorescence- mikroskop. The number of increments formed after the Alizarin-marked increment were counted and set into comparison with the known number of days after the marking was performed. Results are given in Fig. 1b and show that the rings after the marking are deposited on a daily basis and give further validation of the formation of the increments in the cod otolith starting on the day of hatching.

A comparison of the measurements performed on the left and on the right otoliths showed that the radius or diameter of the otolith isn't only dependent on the growth of the daily increments but very much affected by the size of the otolith's core. The size distribution of the otolith cores between the left and the right side showed that the core size varied between 4.5µm and 13µm, but didn't show a statistically different distribution between left and right side (t-test, $p < 0.05$). The comparison of the otolith core of the left and the right otolith measured on the same individual larva revealed that there was a diffences in the size of the core when left and right otolith were analysed (Fig.2a).

A similar picture is seen when the radius of the left and the right otolith on the same larva are compared. The radius measurement between both sides was different (Fig.2b).

In comparison the sum of the increments deposited on the left and the right otolith did not differ (Fig.2c). The variability between the size of the left and right otolith is mainly caused by the difference in the size of the core.

To evaluate the effect of food availability or food withdrawal on the width of the daily increments two individual larvae fed or starved starting on day 4 were compared. Up to day 6 the larvae seem to deposite heterogenous increments depending on their individual yolk content. At day 7 the size of the increments seems to be determined by the food situation (Fig. 3a). With increasing age (ring number) the width of the increments increased in fed larva up to a width of 1.3µm whereas the starved larvae showed a constant decrease in the width of the increment and reached a value of 0.4µm (Fig. 3a). Fig. 3b gives a comparison of the size of the increments of all analysed fed and starved larvae showing mean values and standard deviations. The variability in the size of the daily increments is high in both groups but a comparison of the mean values shows a trend of increasing increment width in fed larvae starting on day 7 and decreasing width of the increments in the starved group.

DNA-content of fed larvae was stable between day 4 and 5. After that larvae started to grow, DNA-content increased and reached a value of 0.6µg/larvae (Fig. 4a). It is striking that the DNA-content of the starved larvae is higher between day 6 and day 10. Larvae from the starving group must have been larger at the start of the experiment. There is no significant difference in the DNA-content between the starved and fed larvae between day 10 and 13 (Fig. 4a). A comparison of the RNA-content of fed and starved cod larvae is given in Fig. 4b. The RNA-concentration decreased from day day 4 to day 5 and then slightly increased to reach a value of approximately 1-1.2µg/larva in the feeding group.Except for the value at day 9 the RNA-content of the starved larvae was lower than in the fed larvae. Although a strong overlap of the values was seen a trend to higher RNA-content in the fed group compared to the starved group (Fig. 4b) was shown. The RNA/DNA ratios during the course of the experiment are given in Fig. 5. From day 4 to day 7 the RNA/DNA ratio decreased from a value of 7 to below 2, this seems to be due to yolk-absorption causing a decrease in condition. Between day 7 and day 12 the RNA/DNA ratio in the starved group decreased further, on the contrary the fed group showed a slight increase. The effect of food availability starts to be visable on day 11 (Fig. 5). Fig. 6 shows the relation between mean RNA/DNA ratios and mean relative otolith growth of all analysed fed and starved larvae. The relative otolith growth was calculated by setting the width of the increment 4 to a value of 1 and calculating the relative growth of the following increments in relation to that value. Every data point represents the mean of 4-5 larvae. The relative growth of the otolith is reduced compared to the fed group. The RNA/DNA ratios of fed larvae doubled between day 6 and day 12, whereas the value in the starved group was reduce to half of the ratio. It can be seen that the feeding larvae have higher

RNA/DNA ratios and a higher growth of the daily increments showing that both the RNA/DNA ratio and the increment growth are coupled.

DISCUSSION

The study showed that cod larvae form then first increment on the day of hatching. This could be shown by the age determination based on increment numbers in comparison to known laboratory age as well as by the Alizarin- marking results which validated the deposition of daily increments. These results are in agreement with Dale (1984) who determined the increment formation in cod larvae using Electron- Transmission- Mikroscopy. By using a 12 hour day/ 12hour night cycle laboratory reared cod larvae produced an increment every 24 hours.

Neilsen & Geen (1982) showed differences in the size of the otoliths taken from the left or right labyrinth in salmonids (*Oncorchynchus tshawytscha*). Therefore both left and right otoliths were measured and compared in this study. The results clearly show that care has to be taken when measuring the total size (radius), since differences between left and right otoliths occurred. It could be shown that these differences were not due to the growth of the daily increments, but dependend on the size of the otolith core at the time of hatch. The individual egg-development might effect the size of the core as well as environmental factors. Influences on the formation of the otolith core should be further analysed.

It is known that environmental factors like constant darkness (Dale 1984)or too high temperatures (Mosegaard et al. 1988) can effect the increment structure. Based on the assumption that somatic growth is reflected in the growth of the otolith a well growing larva should deposit a wider daily increment than a starving larvae with slower growth. Maillet & Checkley (1990) and Zhang & Runham (1992) determined changes in the otolith structure in starving laboratory reared Atlantic menhaden and *Oreochronis nilotilus*.

The individual observations on the width of the otoliths in this study showed that the growth of the increments was effected by the availability of external food sources and showed differences in the growth of the increments between feeding and starving larvae starting on day seven.

DNA can be used as an indicator of cell number, reflecting the growth of the larva. During yolk absorbtion the DNA- content was stable but increased with age starting on day 7. The DNA-content wasn't affected by the feeding situation. RNA- content started to increase at day

7 in the feeding group getting more pronounced on day 11, whereas the RNA- content in the starved group decreases. The same picture was shown for the RNA/DNA ratio. Starting on day 11 the RNA/DNA ratio and the RNA-content reflect the different nutritional situations. Results shown here are in agreement with studies on cod larvae by Buckley (1979) and results on herring larvae by Clemmesen (1987, 1994). The minimum RNA/DNA ratio of 1 suggested by Clemmesen (1994) neccessary for the larva to survive is also found in this study. Alizarin-marking didn't effect the RNA- and DNA measurements, since the concentrations of nucleic acids determined on marked and unmarked cod larvae didn't differ (Doan pers. comm.). Unfortunately sampling of the larvae could not be performed after day 12, it is postulated that the trends shown in this study would have been much more significant if samples from older larvae had been available. Future studies must increase the sampling protocol up to an age of 21 days.

An evaluation of nutritional condition can not be performed in larvae before first feeding neither by analyzing the increment structure nor by measuring biochemical composition. Due to the process of internal yolk absorption and learning to capture food items the condition of fed and starved larvae in the first days after hatching is not significantly different. Therefore that life stage shows a general problem in condition analysis independent of the technique used (see Clemmesen 1994).

First observations showing the relationship between growth rates over the last five days (as determined from the width of otolith daily increments) and protein growth rates based on nucleic acid determinations are available for North Sea plaice (Hovenkamp 1990) but have not been performed on the same larva. To our knowlegde this study is on of the first to determine otolith microstructure and RNA/DNA ratio analysis on the same larva. During the yolksac phase no clear differences between feeding and starving larvae could be found. After yolk absorption the effect of an external food supply could be measured. The trend starts to be visable on day 7 and increases on day 11. High RNA/DNA ratios are coupled with greater increment width in fed larvae. Lower RNA/DNA ratios and smaller increment widths are found in starved larvae.

Biochemical indicators (RNA/DNA ratios) have also been used to demonstrate starvation mortality in the field and correlations between food availability and condition of the larvae have been found (Canino et al. 1991, Frank & McRuer 1989, Setzler-Hamilton et al. 1987). A

positive relationship of RNA/DNA ratio and prey abundance has been shown for stripped bass larvae (Martin et al. 1985) and larvae of the Atlantic cod and haddock (Buckley & Lough 1987). By coupling RNA/DNA ratio determinations and otolith increment structure analysis on the same larva the decision whether the larva is improving or deteriorating it's condition should be possible. The validity of these studies for the recruitment problem could be further improved.

Whether the findings in this study determined on laboratory reared larvae can also be found in field caught larvae has to be further evaluated. Results by (Clemmesen in prep.) on field caught anchovy larvae (*Engraulis anchoita*) revealed that the sum of the last increments was correlated with the RNA/DNA ratio, meaning that the effect of lack of food in the wild should be possible to determine by the combination of biochemical and otolith studies.

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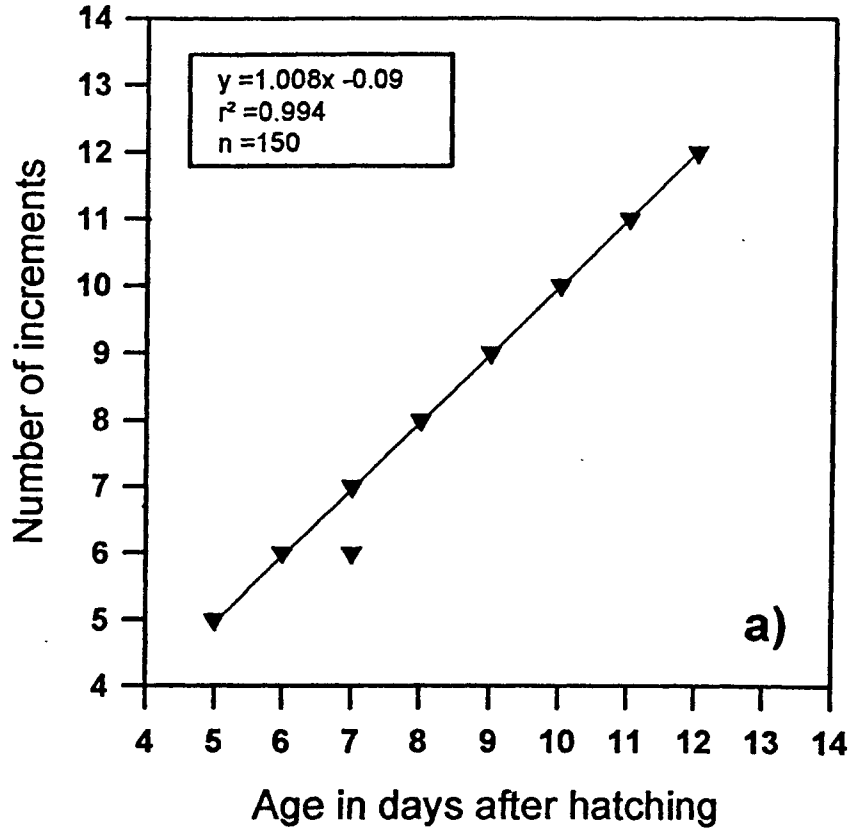

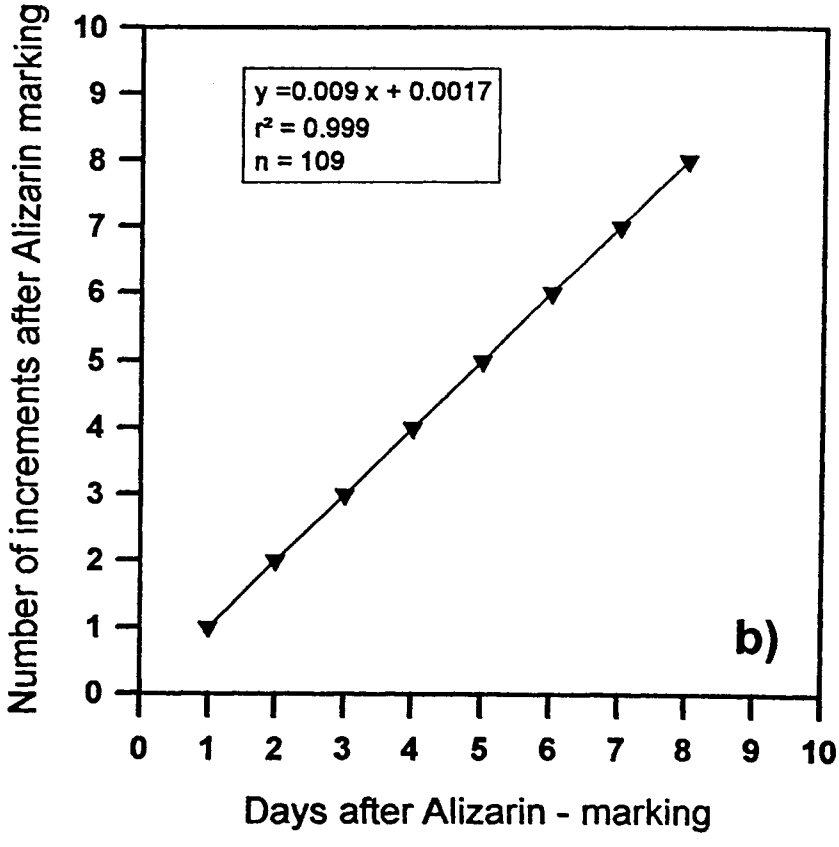

Fig. 1: Relation between number of increments and known age of laboratory reared cod larvae (Fig. 1a). Validation of the daily otolith deposition by marking the otolith with Alizarin (Fig. 1b).

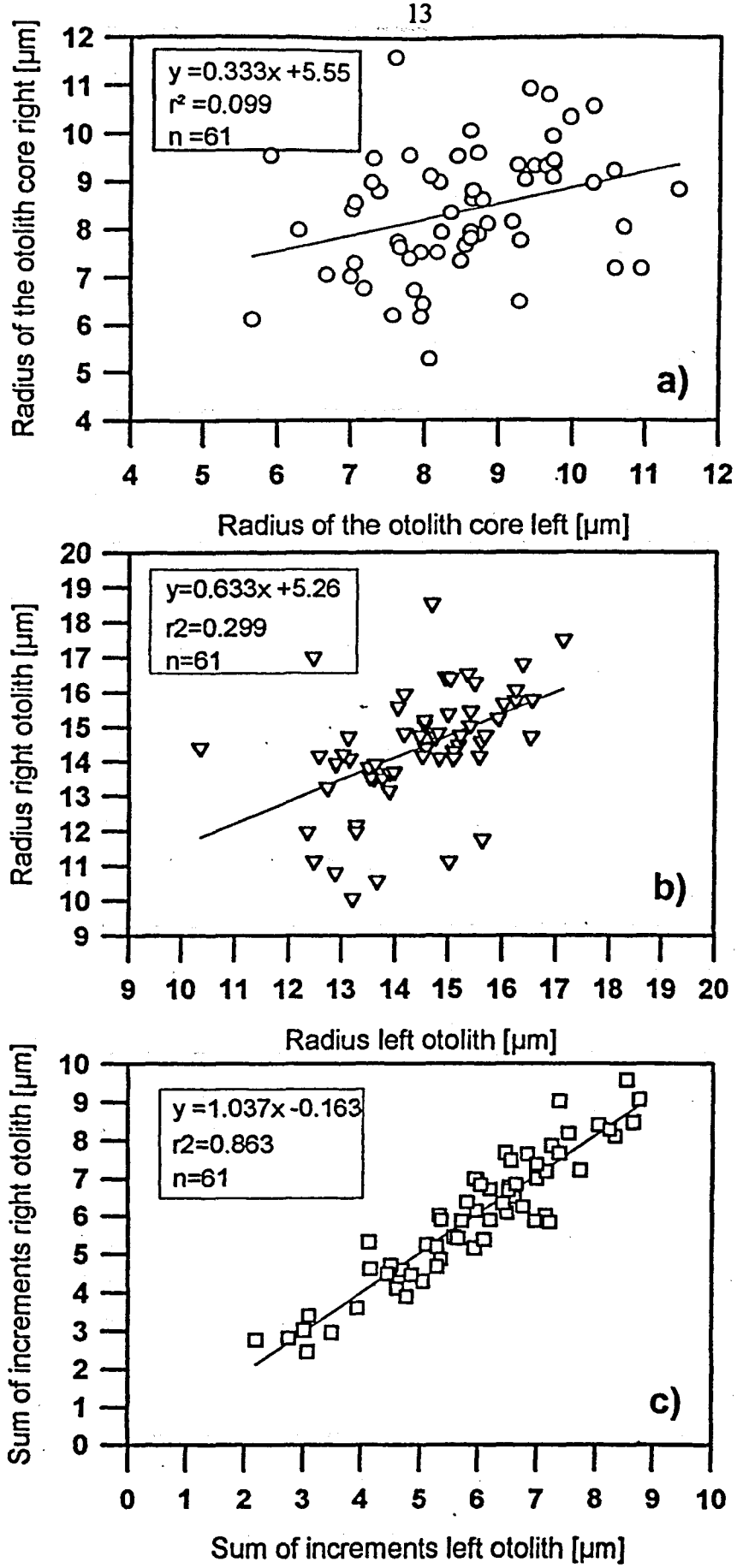

Fig. 2: Correlations between the radii of the core (Fig. 2a), the radii of the total lapillus (Fig. 2b) and the sum of the increments (Fig. 2c) of the left and the right lapilli based on measurements on 4-12 day old cod larvae.
Values are means calculated from 4 measurements on the otolith in four different directions. Linear regression model was fitted to the data.

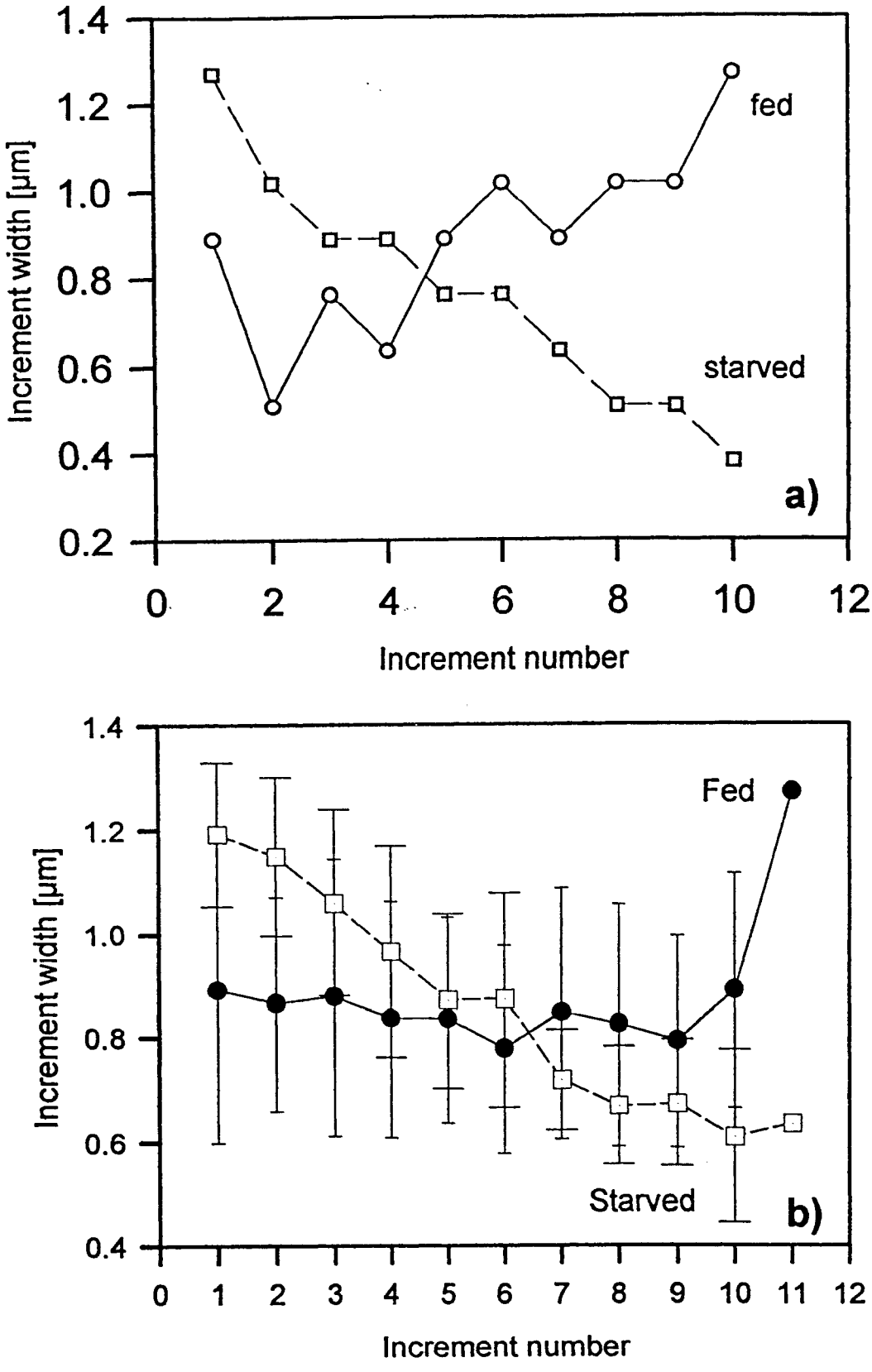

Fig. 3: daily otolith growth (increment width) of two individual cod larvae (Fig. 3a). Mean increment width of 59 fed cod larvae compared to 27 starved cod larvae (Fig. 3b). Error bars give the standard deviation.

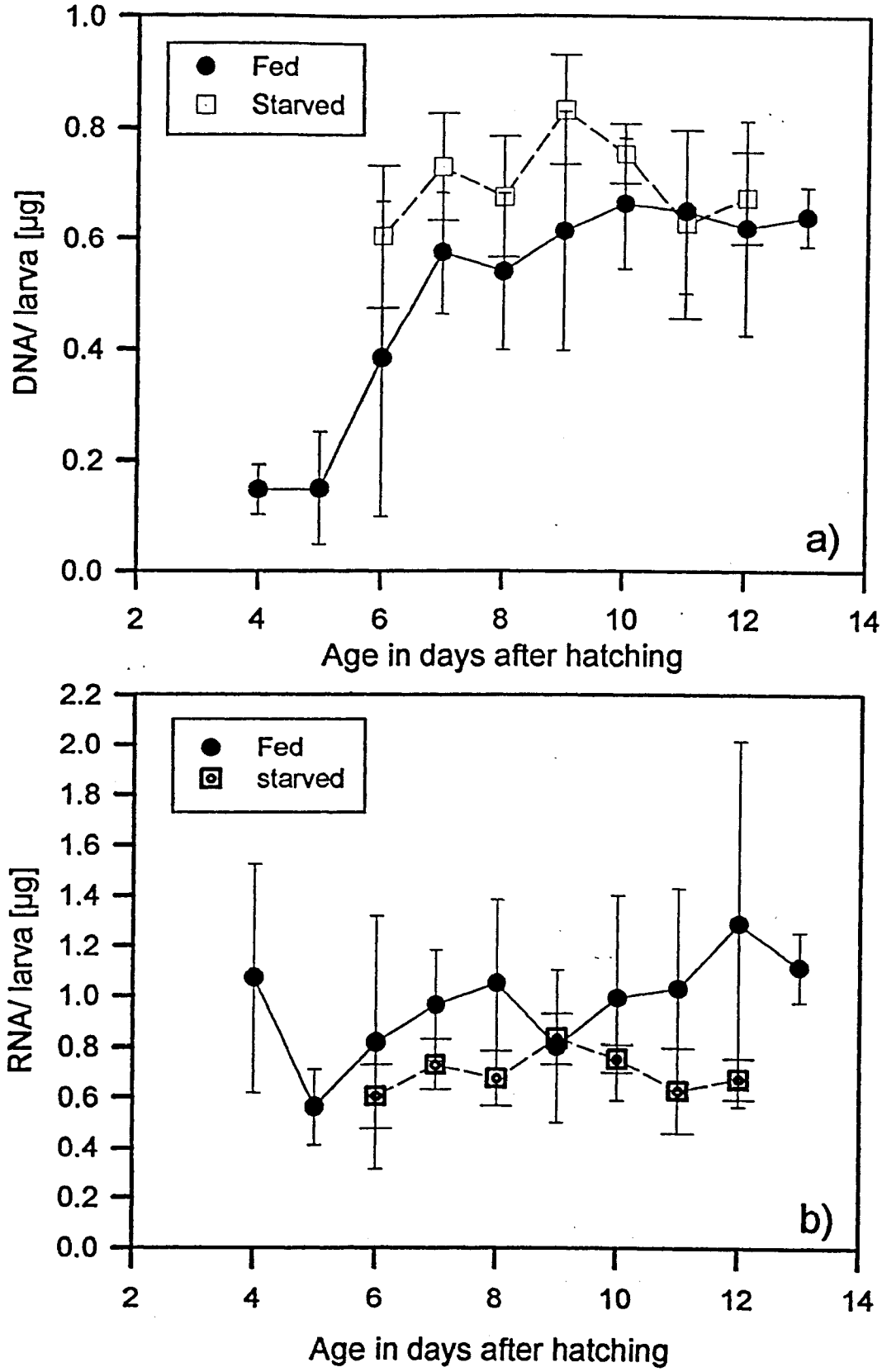

Fig. 4: DNA- content (Fig. 4a) and RNA- content (Fig. 4b) of cod larvae in relation to age and feeding situation. Starving larvae (n= 45) were deprived of food starting on day 4 and day 7 and compared to fed larvae (n= 86). Error bars give the standard deviation.

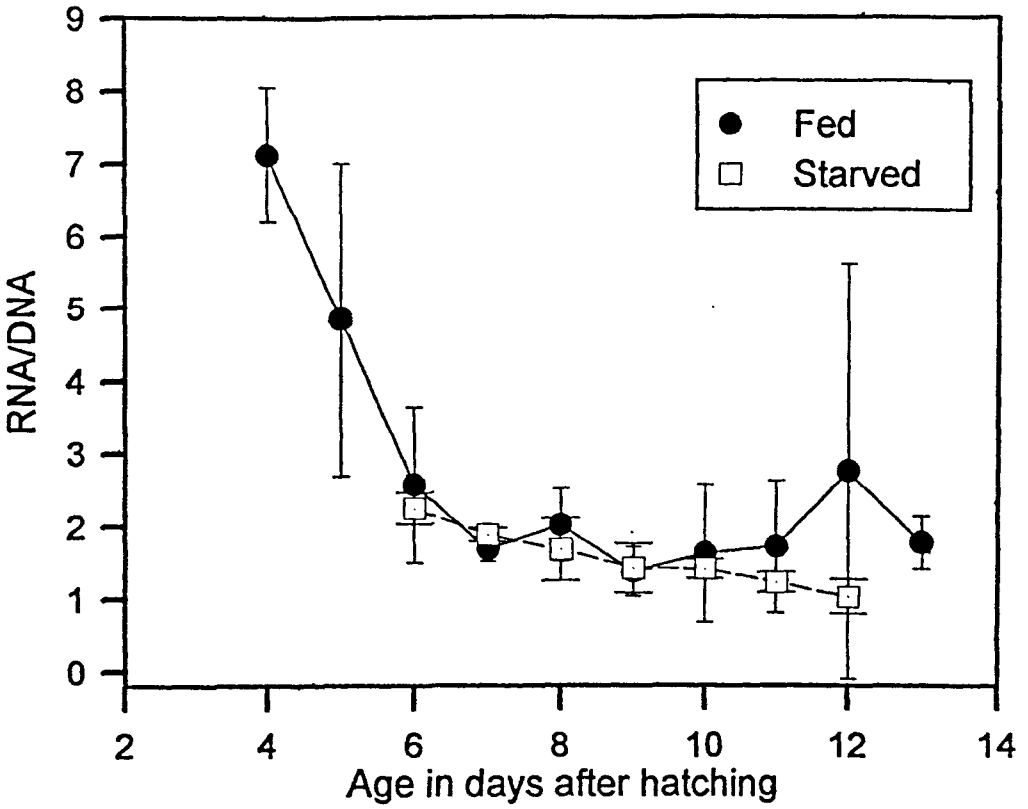

Fig. 5: RNA/DNA ratios of cod larvae in relation to age and feeding situation. Starving larvae (n=45) were deprived of food starting on day 4 and day 7 and compared to fed larvae (n= 86). Error bars give the standard deviation.

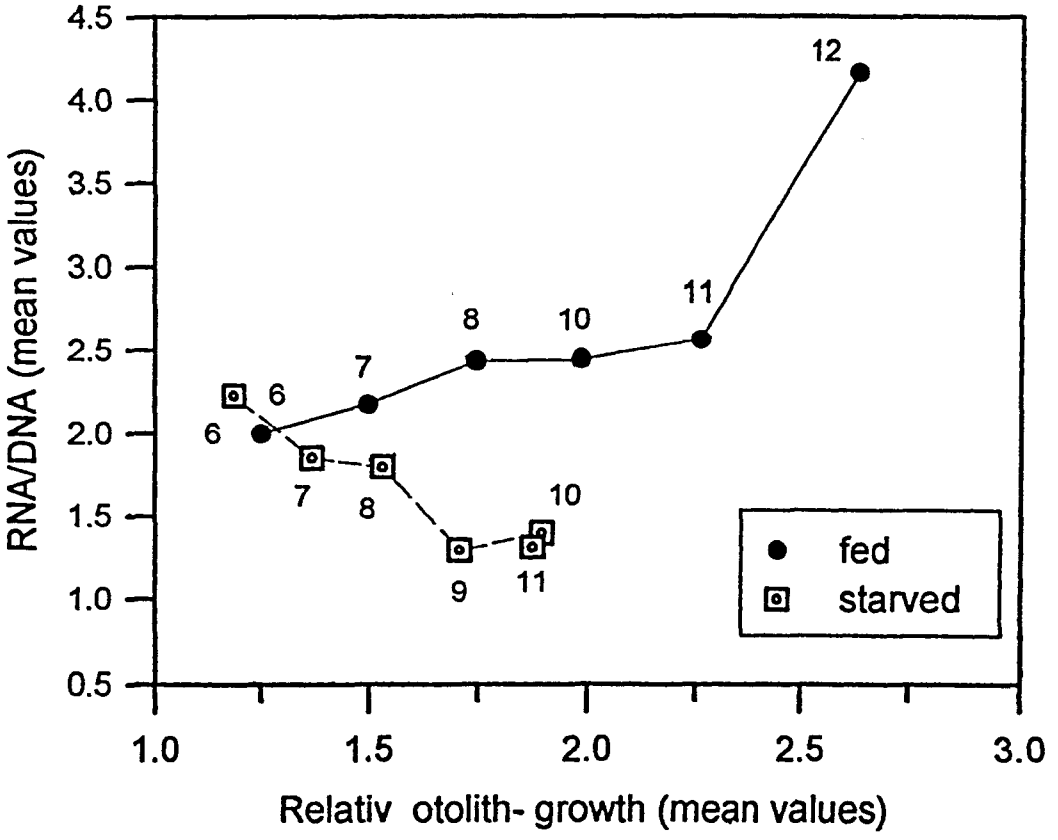

Fig. 6: Relation between relative otolith growth of fed and starved cod larvae and RNA/DNA ratios. For calculation of relative growth the width of ring number 4 was set to one. The numbers give the age in days.