IMPACT OF TEMPERATURE, SALINITY AND LIGHT ON HATCHING OF EGGS OF *ANISAKIS SIMPLEX* (NEMATODA, ANISAKIDAE), ISOLATED BY A NEW METHOD, AND SOME REMARKS ON SURVIVAL OF LARVAE

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A new efficient method of isolating large number of eggs from anisakid worms is described. The method involves blending the worms in an electric mixer and isolating the eggs by centrifugation. Eggs were used to study factors affecting egg hatching and survival of emerged larvae of *Anisakis simplex*. The hatching time (range 3-21 days) varied inversely with temperature (5-21 °C), but did not differ significantly with salinity (0-28 psu). The final proportions of eggs which hatched, increased with salinity. This proportion was highest at 13 °C and lowest at 21 °C; larvae which emerged in fresh water died within a few hours irrespective of temperature. The mean survival time of larvae was in the range of 92-131 days. The survival time increased with salinity but decreased with temperature. Light exposure (at 13 °C) was found to reduce significantly the time needed for the hatching of eggs. The results support the hypothesis that *A. simplex* is adapted to off-shore pelagic marine environments.

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KEYWORDS: Anisakis; hatching; survival.

INTRODUCTION

The adults of the ascaridoid nematode *Anisakis simplex* (RUDOLPHI, 1809, det. KRABBE 1878), known as the 'herring worm' or the 'whale worm', live in the digestive tract of marine mammals, mainly whales (YOUNG 1972; HAUKSSON 1992a). Third-stage larvae have been found in euphausiaceans (SMITH 1983), cephalopods (KOBAYASHI & al. 1966; SMITH 1984a) and several species of marine teleosts (BERLAND 1961; HAUKSSON 1992b; KØIE 1993a). These larvae usually occur encapsulated on and in the viscera; but they may also be situated in the muscles, especially in the hypaxials or 'belly flaps' of fish (SMITH 1984b; MCCLELLAND & al. 1990). If consumed live, *A. simplex* larvae may cause 'anisakiasis' in humans (RUITENBERG 1970; OSHIMA 1972; SMITH & WOOTTEN 1978).

The life cycle comprises five stages separated by four moults. The third-stage larva is considered to be the infective stage; in the definitive host two moults take place, the fifth-stage becoming the adult worm (BAER 1971). The early phases of the life cycle are less well known and subject to controversy. When the egg is laid the parasite embryo develops into a larva surrounded by a thin sheath which has been assumed to be the cast cuticle of the first stage larva (see e.g. KOBAYASHI & al. 1968; VAN BANNING 1971; GRABDA 1976). Having studied early larval development and hatching in anisakid nematodes, KØIE (1993b), KØIE & FAGERHOLM (1993) and KØIE & al. (1995) found that the larva prior to hatching is surrounded by two cuticles and is therefore actually third-stage nematode; to date this has been seen in *Hysterothylacium aduncum*, *Contracaecum osculatum*, *Anisakis simplex*, and *Pseudoterranova decipiens*. However, MEASURES & HONG (1995) could not see this delicate cuticle in *Pseudoterranova decipiens* by electron microscopy.

The *Anisakis* larva, common in many fishes in the North Atlantic, was identified by PIPPY & VAN BANNING (1975) as *Anisakis simplex*. By enzyme electrophoresis *A. simplex* has subsequently been shown to consist of two sibling species: *A. simplex* A and *A. simplex* B, the former mainly distributed in the Mediterranean, the latter in the eastern North Atlantic (NASCETTI & al. 1986).

BRATTEY 1990 found an inverse relationship between temperature and egg hatching time, but differences in hatching times at the same temperature when comparing the eggs of the ascaridoid species *Pseudoterranova decipiens*, *Contracaecum osculatum* and *Phocascaris phocae*, all from seals in Canada. BURT & al. 1990 report the same clear effect of temperature on hatching time for *Pseudoterranova decipiens*, but salinity does not seem to have any effect, except on the survival of the larvae. A similar effect of temperature on hatching and an inverse effect of temperature on survival of *Anisakis simplex* from the white-beaked dolphin,

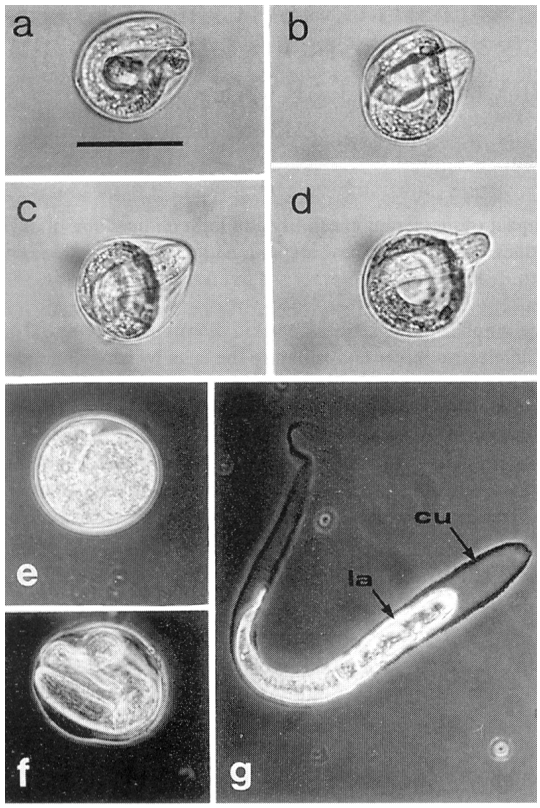

Fig. 1. Development and hatching of *Anisakis simplex* larva at different stages. A-C 'pushing'. D. 'break-through'. E-G (with phase contrast): from 'tadpole' (e) to newly hatched larva (g). cu: cuticle, second-stage larva. la: third-stage larva. scale bar = 40 µm.

Lagenorhynchus albirostris GRAY, were reported by BRATTEY & CLARK (1992).

The *Anisakis simplex* material (probably type B) analysed in this study was collected from the pilot whale, *Globicephala melas* (TRAILL, 1809), which is a known host for *A. simplex* (YOUNG 1972; RAGA & BALBUENA 1993; BLOCH & al. 1993). Experiments were carried out to analyse hatching at a range of temperatures, salinities and photoperiods, and the survival of larvae at different temperatures and salinities. The results are relevant to the understanding of the life cycle of *Anisakis simplex*. To speed up isolation of a large number of eggs, a new methodology has been worked out.

MATERIALS AND METHODS

Experiments were conducted using eggs of *Anisakis simplex* obtained from the fore- and main-stomachs of pilot whales killed at Bøur on 10 July 1992 and at Hvannasund 11 August 1993 in the Faroe Islands. The identification of the *Anisakis* material was based on DAVEY (1971) and HARTWICH (1974). The live nematodes were transferred in the field to physiological saline, 0.9 % at 10 °C, then stored at 5 °C. The nematodes were transported to the Marine Biological Laboratory in Helsingør, Denmark, in thermos flasks. Eggs were removed within 1-2 weeks from live or recently dead female worms. Dead *A. simplex* worms were used during the development of the blending method to isolate anisakid eggs.

Isolation of eggs

The dissection of female nematodes is usually very time-consuming and using a mechanical blender a faster method was developed to isolate eggs from a large number of females. All specimens (n = 373), including both male and female worms longer than approximately 4 cm, were used (sorting by sex was left out for time saving purposes). The worms were fragmented in a high speed Kenwood Electronic blender, model A518-519, at maximum speed for 4 minutes. The nematode 'soup' was centrifuged at 1000 rpm for 4 minutes. The supernatant was discarded and an equal volume of filtered (0.22 µm) seawater, salinity 28, added to the eggs and debris at the bottom of the centrifuge tubes (14 or 100 ml). Centrifugation and washing was done three times. After the first centrifugation the material was filtered through 65 µm filters. After the second and third centrifugation the contents of the centrifuge tubes were filtered through 30 µm filters, retaining the eggs, their size being 40 µm x 50 µm. The cleaned eggs were diluted to 1000 ml with filtered seawater, thoroughly mixed by hand and suspended in conical flasks, ready for use. The worms collected in 1992 were used for initial experiments and the development of the method described. The worms from 1993 were used for the hatching experiments and the analysis of the survival of the larvae.

Hatching experiments

After washing the eggs several times on the 30 µm filter with the appropriate saline solutions, they were transferred to 48 mm diameter petri dishes containing water of the salinities 0 (distilled water), 10, 19 and 28 (maximum salinity at Helsingør). The solutions were made from dilutions of the 0.22 µm filtered seawater, available at the laboratory (hereafter referred to as 'filtered seawater'). The original salinity was measured with an 'aerometer' and the 'The Tables of Knudsen'. Approximately 80 000-120 000 eggs were pipetted into each petri dish; two replicates were prepared for each combination of temperature and salinity. The replicates were placed in transparent polystyrene boxes wrapped with aluminium foil. Each box contained a standard thermometer for daily temperature control. The temperatures (± overall standard deviation, SD): 7 °C (6.7 ± 0.4), 11 °C (10.7 ± 1.2) and 13 °C (13.2 ± 1.1), were obtained in climatic rooms. The 5 °C (5.1 ± 1.5) in a refrigerator and 21 °C (20.7 ± 0.9) at room temperature. During the hatching experiments the liquid in the petri dishes was not changed, to eliminate the risk of reducing the number of eggs, as they might be suspended in the water. Oxygen, measured at the end of the experiments with micro-Oxygen-electrode MI-730 showed 92-98 % saturation in all cases. The pH-values of the original salinities 0, 10, 19 and 28 were measured with a PHM 80 Portable pH Meter and found to be 6.25, 7.04, 7.38 and 7.56 respectively.

To examine a possible effect of light exposure on hatching, one extra set of replicates with the same salinities and the same oxygen availability as above was kept at 13 °C in a box *without* aluminium foil wrapping. This was subjected to automatic light exposure daily for 16 h (6 a.m. to 10 p.m.). The light source was a battery of ten OSRAM 36W/20 fluorescent tubes at a distance of 1 m. Bacterial growth, ciliates (*Uronema* sp.) and flagellates (Bodonids and *Paraphysomonas* sp.), were seen in the petri dishes. Their effect on eggs or larvae was not estimated.

Counting of eggs and larvae

The success of hatching was determined by direct counting of the eggs and live larvae in the petri dishes, placed on transparent plastic film with 1 mm x 1 mm squares with a stereo microscope. Inspections of the petri dishes were carried out every day from the start of experiment. When the hatching could be observed repeated counts (3-9 times) were made once a day until no more increase in the hatching could be seen. Later on the countings were made every second to third day (Fig. 2). Each counting included four adjacent millimeter-squares selected at random. All samples were kept at 5 °C for 10-15 min at counting to minimize the activity and clustering of the larvae, which occurred if the temperature reached 15 °C during the counting procedure. The first hatching experiments (at 21 °C) were stopped 25 days after incubation. The last hatching experiments (at 5 °C) were stopped after 48 days.

Survival experiments

After the end of each hatching experiment, the sets of replicates were combined. Eggs and hatched larvae were separated by filtration, washed with the corresponding salinity solution, transferred to 40 ml tissue culture flasks and stored at the same temperature as before. Survival experiments and control for a delayed hatching were undertaken at the Marine Laboratory of Kaldbak, The Faroe Islands, 95 days after incubation of eggs in Denmark. The salinities used were 10, 19 and 28 , at (± SD): 2 °C (2.3 ± 0.7), 7 °C (6.6 ± 0.9), 10 °C (9.6 ± 0.8) and 13 °C (12.7 ± 1.0). The larvae, originally hatched at 5 °C, were exposed to 2 °C in the survival experiment, the larvae originally hatched at 7 °C were exposed to 7 °C, and so on (each survival experiment with an exposure temperature as close as possible to the prior hatching temperature). The samples were wrapped with aluminium foil and placed in refrigerators (2 and 7 °C) or rooms with a stable temperature (10 and 13 °C). The larvae were counted every 7 days for 7 weeks. Active larvae or larvae curled in an overlapping coil were registered as 'alive', while non-motile, slightly curved larvae were registered as 'dead'. In each flask 100 larvae were observed each time. As in the hatching experiments the water was not changed to minimize the risk of losing any larvae. The mean survival times were estimated as the difference in time between 50 % of the final proportion which hatched to the time when 50 % of the larvae were registered as 'dead.'

Data analysis

As the variances of the proportions which hatched were of the same magnitude, a two-factorial ANOVA was used; the data (proportions) were transformed with the arcsin-square root transformation (Bishop 1978). The data for the sigmoid hatching curves were assumed to fit the traditional logistic growth equation (as in Pielou 1969) and were transformed to a linear form with ln((b-y)/y), where b, the final proportions of hatching, was determined as the mean of the five last countings in each experiment. The requirements for residuals and normal probability in the multiple regression were fulfilled for the overall proportion of hatching analysis, but not completely when considered separately. Then the final proportions which hatched were analysed by a two-sample t-test, assuming equal variances. The results from the survival experiments were analysed by ANCOVA.

RESULTS

Several days before hatching the larvae were seen moving gently or twitching regularly inside the eggs. A few hours before hatching the movements became more swift and they could be seen actively pushing hard against the egg membrane (Fig. 1).

Prior to hatching cellular cleavage was observed. All developmental stages from tetrade to 'tadpole' could be seen, similar to those described by Grabda (1976) for

Table 1. Time until start of hatching (in days) of *Anisakis simplex* eggs, incubated at different temperatures and salinities. Light = 16 h light, 8 h dark; all others were kept in darkness, except during examination. Two replicates were used. One value means the same result for the two replicates, otherwise the two different values are shown.

	Temperature					
	5 °C	7 °C	11 °C	13 °C		21 °C
Salinity	Dark	Dark	Dark	*Light*	Dark	Dark
0	21-22	15	9	5	5-6	3
10	21-22	15	9	4	5	3
19	21	14-15	9	4	5	3
28	21	14	9	4	5	3

Table 2. Two-factorial ANOVA on hatching times of *Anisakis simplex* eggs at salinities 0, 10, 19, 28, temperatures 5, 7, 11, 13, 21 °C and *with* light (16h, 8h darkness) or *without* light exposure at temperature 13 °C (*: $p < 0.05$; **: $p < 0.01$; n.s.: not significant).

	df	F	Fcrit	P
salinity	3	3.3	3.1	0.04 *
temperature	4	4398	2.9	E-29 **
interaction	12	1.04	2.3	0.45 n.s.
with and *without* light				
salinity	3	9	4.1	0.006**
light	1	49	5.3	0.0001**
interaction	3	1	4.1	0.440 n.s.

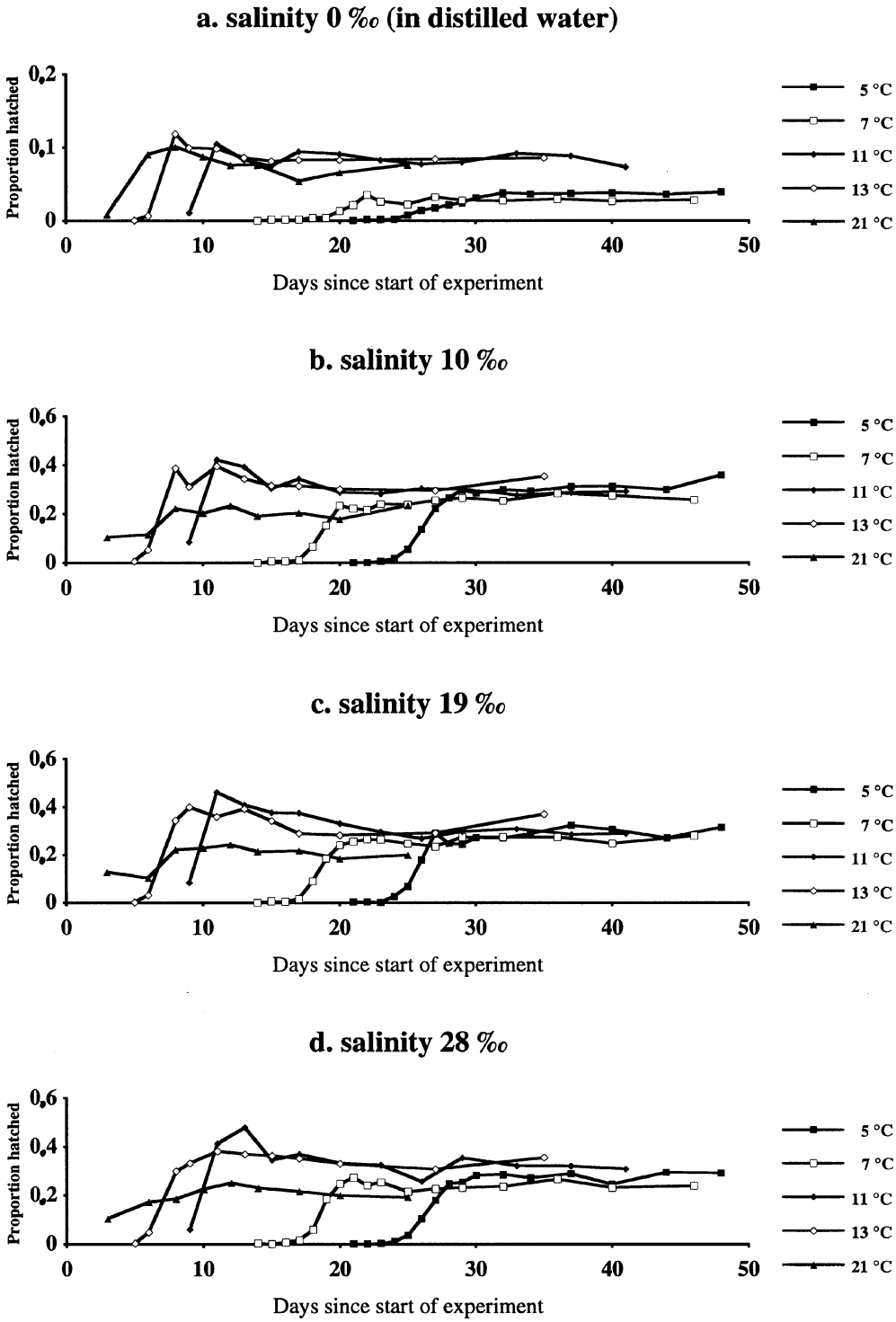

Fig. 2. Proportion hatched of *Anisakis simplex* eggs with time, at temperatures 5, 7, 11, 13, 21 °C and salinities 0 10, 19 and 28. The points represent means of two replicates.

A. simplex. It was found that the time until the hatching of eggs began, ranged from 3 days at 21 °C, to 21 days at 5 °C (Table 1). The differences in hatching times due to varying temperatures are significant (ANOVA, $p < 0.01$, Table 2).

The mean proportion which hatched reached a maximum of 0.50 (Fig. 2). The total egg counts were steadily falling with time (about 40 % during the first two weeks after start of hatching) and determining whether eggs were dead or immature was not feasible. Consequently, the proportions which hatched were calculated relative to the *initial* total egg counts. Furthermore, there was poor correlation between the number of empty eggs and the number of live larvae at the salinities 10, 19 and 28 . At salinity 0 (in distilled water) a positive correlation was found between empty egg shells and the number of larvae, but here all larvae died immediately at the higher temperatures and within few hours after hatching at the lower temperatures. Sometimes the larvae were observed to have died before hatching was complete. Estimates of eggs which did not hatch were approximately 50-70 % at salinities10-28 and 88-98 % at salinity 0.

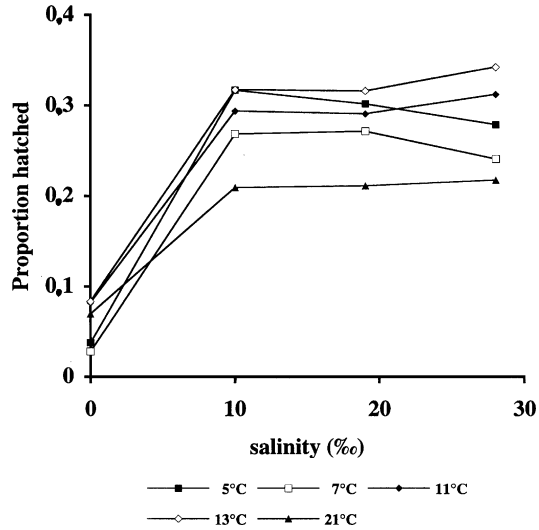

Fig. 3. Final proportions hatched of *Anisakis simplex* eggs at temperatures 5, 7, 11, 13, 21 °C and salinities 0, 10, 19 and 28. The term proportion is used for the relative amounts which hatched.

The hatching process

The eggs in the same replicates started to hatch almost simultaneously. Fig. 2 shows that the curve for the hatching is sigmoid at low temperatures and can be divided into three sub-periods: (1) 'The lag phase' (start of hatching), (2) 'The exponential phase' (rate of hatching increases rapidly with time) and (3) 'The stationary phase' (rate of hatching remains fairly constant). The duration of the lag phase, 2-6 days, and the exponential phase, 2-8 days, was inversely related to temperature. The stationary phase was monitored for 20-30 days, depending on temperature. Fig. 3 shows the final proportions of eggs which hatched at the end of the stationary phase.

The effect of temperature and salinity on the hatching proportions

Two-factorial ANOVA was applied to the hatching proportions for all experiments, from the start of hatching, and to salinity and temperature. A highly significant difference was seen in hatching proportions when data from salinity 0 were included in the test ($p < 0.01$). However, when the salinity 0 samples are excluded, the difference due to salinity is not significant ($p > 0.05$). This is an indication of osmotic stress on the eggs at salinity 0, resulting in very low hatching proportions. (0.001-0.134, Fig. 2A), compared to 0.002-0.500 for salinity 10-28 (Figs 2B-D).

The final proportions of eggs which hatched at salinity 0 are especially low, (0.03-0.08) compared with 0.21-0.34 for salinity 10-28 (Fig. 3). The statistical differences are generally significant; this could also be seen in a multiple regression analysis* and a two-sample t-test*. It was found (two-factorial ANOVA)* that when the data from salinity 0 were included, the effect of salinity on the overall hatching proportions was highly significant (ANOVA, $p < 0.01$). When the data from salinity 0 were excluded, the effect of salinity was not significant (ANOVA, $p > 0.05$). This effect of the salinity 0 was found at all temperatures and also at exposure to the 16 h light period.

* statistical tables are available upon request from the journal or the author

The effect of light exposure

Apparently light exposure affects the hatching time in the experiments carried out at 13 °C. Table 1 shows 4-5 days hatching time for samples exposed to light and 5-6 days for those not exposed to light. Table 2 shows these differences to be significant (ANOVA, $p < 0.01$), indicating a positive light-effect. The result is based on one set of experiments only with or without light exposure and therefore should be taken with some reservation.

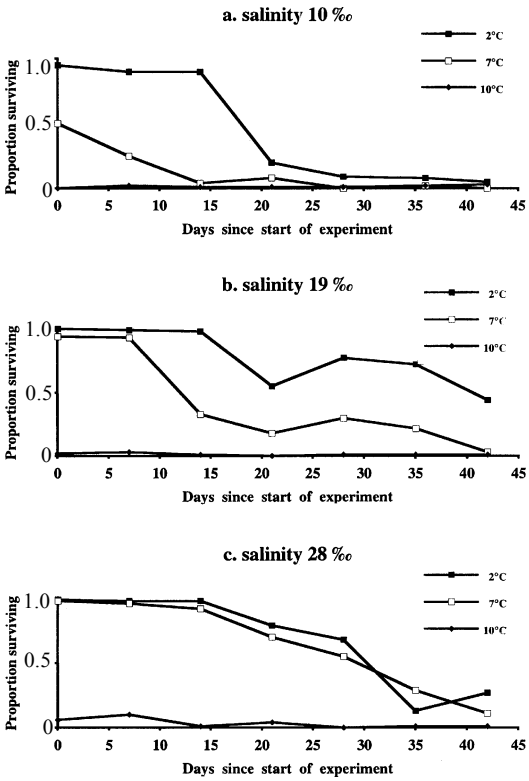

Fig. 4. Proportion of *Anisakis simplex* larvae, which survived at temperatures 2, 7 and 10 °C and at salinities 10, 19 and 28. The experiments started 95 days after incubation.

The survival of larvae

The mean survival times range from 92-131 days (Table 3). Survival was found to increase with salinity but decrease with temperature (ANCOVA, $p < 0.01$; Fig. 4). No surviving larvae were seen at 13 °C.

Delayed hatching and stickiness of eggs and larvae

No further hatching of eggs was observed in the egg flasks, indicating that the low hatching proportions were not due to delayed hatching of some eggs.

During the inspection of eggs and larvae in the survival experiments, 'stickiness' of both eggs and larvae was observed. About 50-100 eggs could be seen adhering to each other. Clusters containing 10-30 live and active larvae were common. Usually the posterior end of the larvae showed the stickiness observed.

DISCUSSION

This experimental study on *Anisakis simplex* eggs and larvae revealed varying effects of temperature and salinity on the hatching times, the hatching proportions and the survival time of larvae. A possible effect of light on the hatching time was also observed. How do these results apply in nature?

Encapsulated third-stage *A. simplex* larvae are mainly found in pelagic oceanic fishes. One could assume that *A. simplex* is better adapted to the offshore regions than to the coastal or the estuarine environment. Both the results from hatching experiments (Figs 2 & 3) and from the survival experiments (Fig. 4) support this hypothesis. The spreading of free-swimming larvae of *A. simplex* into estuaries or rivers is not plausible, due to a very short survival time in fresh water (salinity 0). By contrast, *Pseudoterranova decipiens* can tolerate lower salinities than *A. simplex*. Burt & al. (1990) found that in *P. decipiens* 'neither the time taken to hatch nor the hatching success rate was influenced by the salinity of the water in which the eggs were incubated.' Their conclusion is based on the experiments conducted at the temperatures of 4 to 22 °C, but their results at 11 and 12 °C show a delay of 2-3 days of hatching time in seawater (salinity 34), compared to salinities 17 and 0. Despite some overlaps in the distribution of some hosts, however, it seems that the main biotopes of *A. simplex* and *P. decipiens* are separated: an offshore marine one for *A. simplex* and a coastal marine one for *P. decipiens*.

The investigations of Brattey (1990), Burt & al. (1990) and Brattey & Clark (1992) on the hatching of ascaridoid eggs did not take into account any possible influence of light conditions. In the present study the light exposure seemed to reduce hatching time by one day at 13 °C (Table 1). The 0.22 µm filtered seawater used in the experiments should not originally contain any oxygen-producing algae, so the reason for the difference in hatching time due to light exposure remains to

Table 3. Mean survival times for *Anisakis simplex* larvae at different temperatures and salinities. Two replicates were used. One value means the same result for the two replicates, otherwise the two different values are shown.

SALINITY (psu)	TEMPERATURE (°C)	SURVIVAL TIME (days)
10	11	92
10	7	123
10	2	127
19	11	96
19	7	121
19	2	127
28	10	122
28	7	115-122
28	2	124-131

be explained and also requires more investigations at other temperatures. Photosensitive enzymes within the unhatched egg is a possibility, but no significant effect of light on the hatching proportions could be detected (analysed by two-factorial ANOVA). On the other hand, the results of Brattey & Clark (1992) on the hatching of *A. simplex* eggs (with no photoperiod-control) and the present study (principally without light exposure) are comparable to a degree which makes the light factor negligible.

Under natural circumstances both eggs and larvae would be situated in considerably larger water volumes. Because the experimental setup did not allow change of water both the data for hatching and survival presumably must be considered to represent minimal values. E.g. if not influenced by predation in the marine environment the larvae most likely would survive for a longer time than inside the flasks in the laboratory.

Apparently the blending procedure used in this study does not damage the eggs isolated, because the hatching data are comparable to those obtained by Brattey & Clark (1992) who dissected *A. simplex* females for their experiments. To some degree the blending procedure is comparable to the manual grinding method by Costello (1961), who was working with *Ascaris lumbricoides* var. *suum*. Even if Costello (1961) does not recommend motor-driven homogenization because of damage to *Ascaris* eggs, the blending method developed seems to facilitate isolation and purification of a high number of eggs from nematode species found in marine mammals, and those involving fish as intermediate hosts. Thus it could be used for experimental studies into different aspects of the early life history of other nematode species. Also it should make possible more extensive research on the importance of environmental parameters already examined such as temperature, salinity and lesser known parameters such as light, pH and oxygen.

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