ACTA ICHTHYOLOGICA ET PISCATORIA
Vol. XII Fasc. 1 Szczecin 1982

*Jadwiga GRABDA*

Parasitology

# STUDIES ON SURVIVAL AND DEVELOPMENT IN VITRO OF *ANISAKIS SIMPLEX* STAGE 3 LARVAE IN TIME*

# BADANIA NAD PRZEŻYWALNOŚCIĄ LARW *ANISAKIS SIMPLEX* III st. I ICH ZDOLNOŚCIĄ DO ROZWOJU IN VITRO W ZALEŻNOŚCI OD CZASU

Institute of Ichthyology,
Academy of Agriculture,
Szczecin

The survival of *Anisakis simplex* larvaee isolated from herring and kept in a physiological NaCl solution at about: +5°C was studied. During the spawning of herring, the larval mortality rate was found reach 80%. The spawn herring leaving the Pomeranian Bay is infested to a minor degree only.

The development of larwal *Anisakis simplex* kept for 7 months in an artifical medium was followed.

## INTRODUCTION

The *Anisakis simplex* stage 3 larvae belong to parasites widely distributed all over the world. They occur in many fish species and are very resistant to various physico-chemical factors, which makes them one of dominants in the marine fish parasitic fauna.

* The project was financed in part by the Maria Skłodowska-Curie Fund under a Cooperation Agreement P-05-652-F between the Academy of Agriculture, Szczecin and the US Food and Drug Administration.

Studies on their biology were carried out by numerous authors, the purpose being the protection of man from infestation.

However, there are still gaps in our understanding of the species biology, larval survival in fish and infestivity in particular.

The follow-up of developmental cycle of the larvae kept *in vitro* (Banning, 1971; J. Grabda, 1976 b) allowed to broaden the scope of research on *Anisakis;* it is assumed that the ability of the larvae to develop in an artificial medium is an evidence of their infestivity and of a possibility that humans can become infested by incidentally consumed larvae.

Previous attempts to culture the larvae were usually limited to those larvae freshly (during the first few days after capture) isolated from fish under an assumption that such larvae only were wiable enough to develop both *in vivo* and *in vitro.* There was a lack of long-term investigations on larval survival under conditions similar to those created by a fish organism; no attempt to rear the larvae in vitro after a prolonged period of keeping them outside a fish was undertaken either.

Van Thiel et al. (1960) have already observed the larvae to survive for a few weeks in sea water half-diluted with fresh water, a medium of the osmotic pressure almost the same as that of the fish blood. Other workers (Young and Lowe, 1969; Asami and Inoshita, 1976) were keeping the larvae in the physiological solution for a shorter or longer period of time without making any systematic observations on larval viability.

The present studies are aimed at determining the temporal resistance of the *A. simplex* larvae kept in the physiological solution (0.65% NaCl) at about +5°C, i.e., under conditions simulating those naturally existing in a fish organism where the larvae stay for a long time in anabiosis.

## MATERIALS AND METHODS

The *A. simplex* larvae examined were isolated from the western herring entering the Southern Baltic to spawn here (J. Grabda, 1974).

The larvae isolated from fish were placed in a normal physiological solution (0.65% NaCl) and kept in a refrigerator at about +5°C. Every week the number of dead larvae was checked and the solution changed. In this way 300 larvae kept in glass crystalizers, 100 individuals in each, were being examined until the death of the last one. The larval mortality rate is presented in a graph (Figs. 1 and 2).

Another problem tackled was to check if the larval *Anisakis* kept under conditions as above would be capable of a further development in vitro so as described by J. Grabda (1976 b). A batch of the larvae, isolated from herring caught in November 1979 was kept in the physiological solution as above; every month some larvae were taken out to be reared in vitro. Culture 1, established immediately after catching the fish in November, was serving as a control. From January 1980, a new culture was established each month until July 1981 (Cultures 2–8). The results are presented in Table 1.

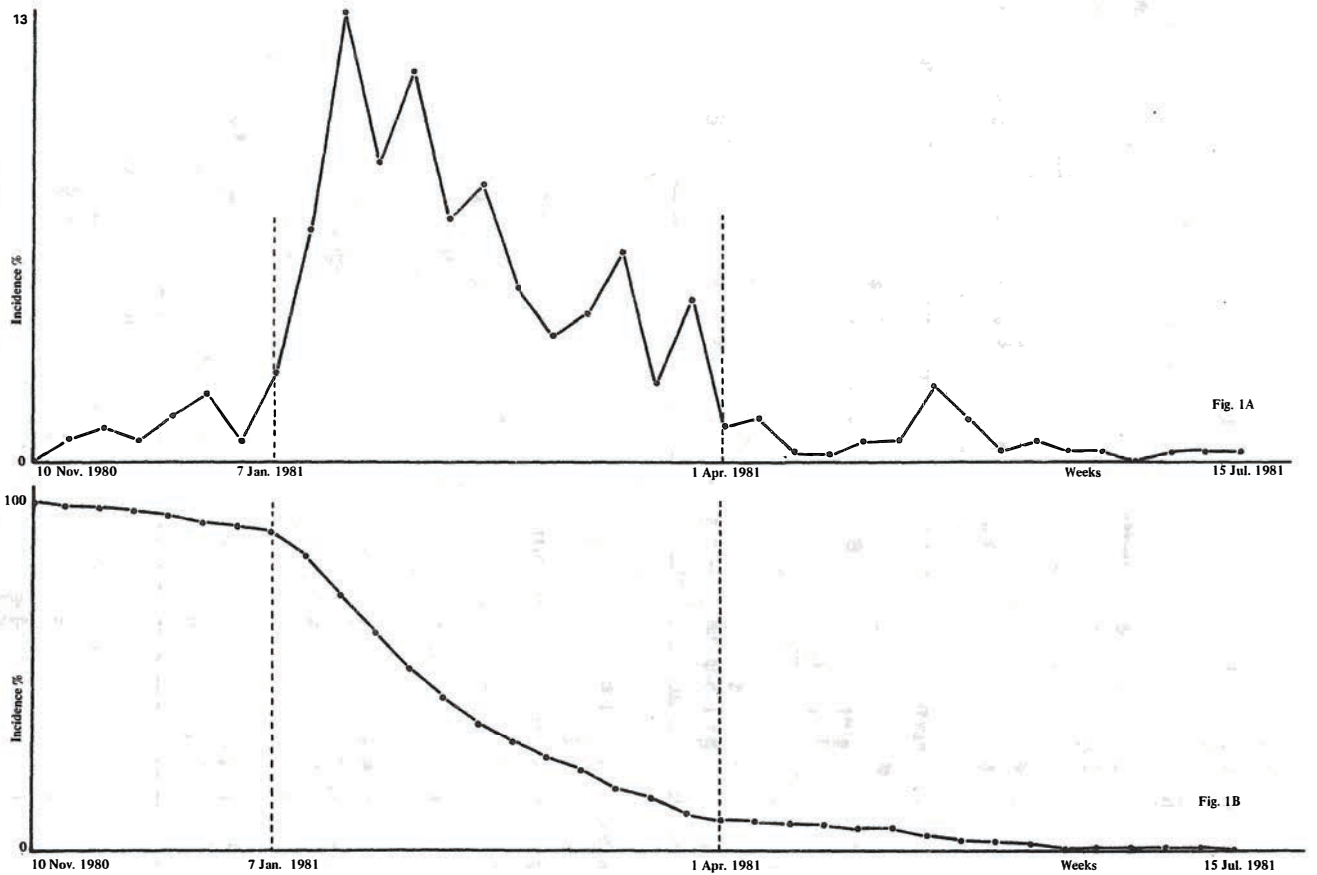


Fig. 1. Viability of stage III *Anisakis simplex* larvae kept in 0.65% NaCl for 8.5 months. A – mortality, B – survival

Table 1

In vitro cultures of *Anisakis simplex* larvae kept in physiological NaCl solution over varying periods of time

| | Culture 1 | Culture 2 | Culture 3 | Culture 4 | Culture 5 | Culture 6 | Culture 7 | Culture 8 | |
|---|---|---|---|---|---|---|---|---|---|
| Culture started | 16 Nov. 79 directly | 10 Jan. 80 after 1 mo. | 13 Feb. 80 after 2 mo. | 12 March. 80 after 3 mo. | 7 June 80 after 4 mo. | 7 July 80 after 5 mo. | 3 Nov. 80 after 6 mo. | 6 Sep. 80 after 7 mo. | 15 Nov. 80 after 7 mo. |
| No. of larvae cultured | 21 | 20 | 20 | 20 | 28 | 20 | 20 | 9 | 5 |
| Larval mortality prior to 3 rd month | all larvae living | all larvae living | 1 larva dead 5% | 4 larvae dead 20% | 8 larvae dead 28.5% | 12 larvae dead 60% | 5 larvae dead 25% | all larvae dead 100% | 2 larvae dead 40% |
| 3rd moult | day 5 | day 5 | day 6 | day 6 | day 5 (for 3 days) | day 6 (for 5 days) | day 5 (for 4 days) | | day 5 (for 2 days) |
| Larval mortality prior to 4th moult | 9 larvae dead 42.8% | 9 larvae dead 45% | 9 larvae dead 45% | 14 larvae dead 70% | 16 larvae dead 57.1% | 7 larvae dead 35% | 13 larvae dead 65% | | 3 larvae dead 60% |
| 4th moult | day 19 | day 19 | day 21 | day 20 | not observed | day 14 | not observed | | not observed |
| Eggs | fertilised on day 29 | fertilised on day 33 | fertilised on day 38 | fertilised on day 35 | no eggs | no eggs | no eggs | | |
| Culture period (days) | 36 | 36 | 44 | 57 | 38 | 32 | 32 | 5 | 22 |
| Nematode survival | 5 females 2 males 33.3% | 1 females 5% | 1 females 5% | 2 females 10% | 1 immature individual 3.5% | 1 immature individual 5% | 2 mmature individual 10% | 0 | 1 larva stage 4 |

## RESULTS

The *Anisakis* mortality rate is presented by a curve in which three phases can be separated (Fig. 1).

Phase 1 (November 10, 1980 – January 7, 1981): low mortality (0.6–2.6%); a total of 9% of the larvae died over that time. It can be assumed that the larvae retain fully their viability during this phase. Doubtlessly, the herring must have become infested by the nemtodes before spawning.

Phase 2 (January 7 – April 1, 1981): a considerable increase in mortality rate, up to 13% in different weeks, 81% of the larvae died within 3 months. The period corresponds to the herring spawning season.

Phase 3 (April 1 – July 15, 1981): survival of the strongest larvae. Larval mortality rate was relatively low (maximum 2.3% of the larvae dying during a week). 10.3% of the larvae died during this phase, the herring having terminated their spawning.

The larvae kept in the physiological solution survived over 248 days.

Graph 2 presents the larval survival based on the same data. An identical regularity is very clearly seen here.

Another problem deserving a consideration is whether the larvae are capable of development throughout their entire life span, in other words whether they retain their infestivity. The results of the in vitro culture are prsented in Table 1.

In every culture numerous larvae were lost so that only a low number of nematodes reached maturity.

As seen from Table 1 the highest number of mature and eggproducing nematodes was yielded by Culture 1 consisting of the larvae takne immediately from the caught herring. Larval die-off was relatively poor and the period can be assumed as one of the full viability of the larvae. In this case more than 33% of mature individuals producing fertilised eggs were obtained.

In the later cultures (Culture 2–7) more and more larvae were dying prior to the third moult (0–60%) which took place on the 5th or 6th day; usually in the early cultures all the larvae moulted within two days, whereas in the later ones the moult extended to 3–5 days.

The number of larvae dead before the 4th moult increased to 70%. A few individuals only (5–10%) survived until the termination of the culture; in Cultures 2–4 eggs were observed, no eggs being recrdedd in later months. Although the nematodes did moult for the fourth time, eggs were not produced, as seen in Cultures 5–7.

After 7 months (Culture 8), the development was greatly hampered: either the larvae died off on the fifth day, before the third moult, or some underwent a regular moult and died afterwards. Due to the fact that the batch of the larvae was exhausted, some individuals from other catches were used in this culture.

## DISCUSSION

The western herring yielding materials for the present study arrive in the Pomeranian Bay as early as in November and remain here until spring (J. Grabda, 1974). As opposed to the local Baltic stocks, they are strongly infested with the *A. simplex* larvae; the infestation has presumably taken place in the North Sea after their return from spawning grounds in the Southern Baltic. During their stay in the Pomeranian Bay, there is no possibility of an additional infestation as the first intermediate hosts (the *Euphausiacea*) are absent in the area and the feeding habits of herring (planktoneating) exclude any other path of infestation such as, for instance, that pertinent to predatory fish (cod) which become infested by eating already infested herring (J. Grabda, 1976 a). Moreover, the herring do not feed at all during spawning. Thus no supply of new parasites is available during the study period and the infestation was completed before the spawning migration.

The Southern Baltic is a natural experimental area not disturbed by any additional invasion of the *A. simplex* larvae in the spawning herring populations.

November is a month when the western herring commence their spawning migration. After spawning (February – April, depending on climatic conditions in a given year) the herring leave the Pomeranian Bay in May and migrate to their western grounds (J. Grabda, 1974). The duration of the culture corresponds almost exactly to the period of the western herring stay in the Baltic and it can be assumed that the processes taking place in the larvae kept in vitro are the same as those occurring under natural conditions in fish.

The period of an increased larval mortality is probably the end of viability of the larvae the herring become infested with the year before, after returning from spawning when an increased demand for food and increased feeding occur in order to compensate for losses incurred by spawning. The results obtained allow to conclude that the stage 3 *Anisakis* larvae live in anabiosis in fish for about a year.

Observations on the *Anisakis* invasion dynamics (J. Grabda, 1974) in the western herring arriving in the Pomeranian Bay, performed systematically over an annual cycle, confirm the larval die-off under natural conditions. In January – April 1973, 40–56% of the herring were infested, while in May that year they were almost completely free of parasites. The invasion incidence values on May 4 and 24 amounted to 12 and 3%, respectively. In June, the western herring disappeared from the Pomeranian Bay.

Those results then are almost identical with the present experimental data obtained from culturing the larvae outside hosts, in the physiological solution.

Under experimental conditions, a month-long acceleration of the larval mortality rate occurs, which may result from artificial conditions of the culture and also from differences in climatic conditions in various years.

As seen from Table 1, the highest viability and the best ability to develop was demonstrated by those larvae isolated from the fish immediately after capture. This is also a moment of the highest danger for man. Later on, the ability to develop in vitro and

thus the infestivity decreases gradually, a possibility of man being infested by chance still existing.

Considering the fact that the western herring leave the Pomeranian Bay in May, man is threatened by infestation over the entire period when the western herring remain in the Southern Baltic.

It is as late as after 7 months (Culture 8) that the larvae are weak enough to be presumably of no threat, in spite of some of them undergoing the third moult.

## CONCLUSIONS

1. The *A. simplex* larvae live in anabiosis in fishes for a year. Their mortality is most pronounced during the fish spawning. The spawn herring leaving the area are almost completely *Anisakis*- free.
2. The majority of the larvae reach maturity and are able to reproduce in vitro; they are thus invasive immediately after catching the fish.
3. The infestation potential becomes reduced with time; after 7 months the larvae are practically of no danger for human health.
4. In view of this, the *Anisakis* larvae in the western herring spawning population are invasive to man over the entire period of the herring stay in the Southern Baltic, i.e., from November untill May.

## REFERENCES

**Asami K. and Inoshita Y.**, 1967: Experimental anisakiasis in guinea-pigs; factors influencing infection of larvae in the host. – Jap. J. of Parasit. **16**, 6: 415–422.

**Banning P.van**, 1971: Some notes on a succesful rearing of the herring-worm, Anisakis marina L. (Nematoda: Heterochilidae). – J. Const. int. Explor. Mer, **34**, I: 84–88.

**Grabda J.**, 1974: The dynamics of the Namatode larvae Anisakis simplex (Rud.) invasion in the South-Western Baltic herring (Clupea harengus L.). – Acta ichthyol. et piscat., **IV**, 1: 3–21.

**Grabda J.**, 1976a: The occurrence of Anisakis Nematode larvae in Baltic cod (Gadus morhua callarias) and the dynamics of their invasion. – Acta ichthyol. et piscat., **VI**, 1: 3–22.

**Grabda J.**, 1976b: Studies on the life cycle and morphogenesis of Anisakis simplex (Rudolphi, 1809) (Nematoda: Anisakidae) cultured in vitro. Acta ichthyol. et piscat. **VI**, 1: 119–141.

**Thiel P.H. van, Kuipers F.C. and Roskam R.Th.**, 1960: A nematode parasitic to herring causing acute abdominal syndromes in man. – Trop. geogr. Med., **2**: 97–113.

**Young P.C. and Lowe D.**, 1969: Larval nematodes from fish of the subfamily Anisakinae and gastro-intestinal lesions on mammals. – J. of Compar. Pathol. **79**, 3: 301–313.

Translated: Dr Teresa Radziejewska

J. Grabda

## BADANIA NAD PRZEŻYWALNOŚCIĄ LARW, *ANISAKIS SIMPLEX* III st. I ICH ZDOLNOŚCIĄ DO ROZWOJU IN VITRO W ZALEŻNOŚCI OD CZASU

Streszczenie

Larwy *Anisakis simplex* III st. występujące w stanie anabiozy w śledziach zachodnich, przybywających na tarło do Południowego Bałtyku, po wyjściu z ryb przetrzymywano w roztworze fizjologicznym 0,65% NaCl w temperaturze około +5°C.

Do chwili śmierci ostatniej larwy przeżyły one w tych warunkach 248 dni.

Przeżywalność larw w hodowlach w roztworze fizjologicznym badano w okresie od 10 listopada 1980 roku do 15 lipca 1981 roku. Do badania użyto 300 larw hodowanych w 3 krystalizatorach po 100 sztuk w każdym. Co tydzień liczono martwe larwy, zmieniając im w tym czasie roztwór fizjologiczny.

Uzyskane wyniki przedstawiono na wykresach 1 i 2. Wskazują one, że okres tarła śledzi jest jednocześnie okresem wzmożonej śmiertelności larw, których zginęło w tym czasie 81%. Po tarle śledzie były prawie całkowicie uwolnione od larw *Anisakis*. W porównaniu do wyników uzyskanych w terenie w roku 1973 przez J. Grabdę (1974) istnieje tu duża zgodność.

Na podstawie tych badań można przyjąć, że w śledziach larwy przeżywają jeden rok.

Ponadto sprawdzano żywotność larw *Anisakis simplex* i ich zdolność do dalszego rozwoju w pożywce sztucznej co miesiąc używając larw przetrzymywanych jak wyżej. Badania prowadzono od 16 XI 1979 do 15 XI 1980 roku. (Tabela 1).

Stwierdzono, że największą żywotność wykazują larwy wyjęte ze śledzi bezpośrednio po ich przybyciu na tarło do Zatoki Pomorskiej. Przeżywalność larw wyniosła wtedy ponad 33%. W miarę dłuższego przetrzymywania larw śmiertelność larw w hodowlach znacznie się zwiększała. Pełny rozwój w hodowlach uzyskiwały tylko nieliczne nicienie jeszcze po ,6 miesiącach ale już nie produkowały jaj. Po 7 miesiącach rozwój ulegał znacznemu skróceniu a IV linka już się nie odbyła.

Я. Грабда

## ИССЛЕДОВАНИЯ ВЫЖИВАЕМОСТИ ЛИЧИНОК ANISAKIS SIMPLEX III St. И ИХ СПОСОБНОСТЬЮ К РАЗВИТИЮ IN VITRO В ЗАВИСИМОСТИ ОТ ВРЕМЕНИ

Р е з ю м е

Личинки Anisakis simplex III st. находящиеся в состояни анабиоза у западных сельдей, входящих на нерест в Южную Балтику, после изъятия, выдерживали в физиологическом растворе 0,65% NaCl при температуре +5 С.

До момента смерти последней личинки прожили они в этих условиях 248 дней.

Выживаемость личинок при выращивании в физиологическом растворе исследовали в период с 10.11.1980 г. до 15.07.1981 г.

Исследовали 300 личинок продерживаемых в 3 кристализаторах - по 100 штук в каждом. Еженедельно подсчитывали мертвые личинки меняя в это время физиологический раствор.

Полученные результаты представлены на рис. 1 и 2. Они показывают, что

период нереста сельдей является одновременно периодом усиленной смертности личинок, которых в это время погибло 81%. После нереста сельди были почти полностью лишены личинок Anisakis. По сравнению с результатами полученными в полевых условиях в 1973 г. автором (Грабда Я., 1974)имеется большая сходимость.

На основании этих исследований можно предположить, что в сельдях личинки живут 1 год.

Кроме этого проверяли выживаемость личинок Anisakis simplex и их способность к дальшему развитию на исскуственной питательной среде.Эти исследования проводили каждый месяц в период с 16.02.1979г. по 15.11.1980г. применяя личинки выдерживаемые в условиях определенных выше. Результаты содержит таб.1.

Обнаружили, что самую большую выживаемость имеют личинки взъятые из сельди непосредственно после их прибытия на нерест в район Поморского залива. Выживаемость личинок составила тогда 35%. По мере роста срока выдержки личинок их смертность значительно увеличилась. Полное развитие при исскуственном выдерживании имели только немногочисленные личинки еще после 6 месяцев, но не производили они уже яиц. После 7 месяцев развитие значительно сокращалось а 4 линка уже не происходила.

Перевод: dr Józef Domagała

Received: 10 IX 1981 r.

Address:

Dr Jadwiga Grabda-prof.dr Eugeniusz Grabda
Instytut Ichtiologii AR
71-550 Szczecin
ul. Kazimierza Królewicza 4
Polska - Poland