Anisakis simplex (Nematoda: Anisakidae): Newly hatched third-stage larvae are not infective to adults of the euphausiid crustacean Euphausia pacifica

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Larvae of Anisakis simplex (Rudolphi, 1809) Dujardin, 1845 (Nematoda: Anisakidae) developed to the third-stage larvae enshathed by the two shed cuticles of the first- and second-stage larvae in the eggs. They hatched in seawater still enshathed by the shed cuticle of the second-stage larva. When experimentally exposed to adults of the euphausiid crustacean Euphausia pacifica, a natural host of A. simplex, newly hatched enshathed third-stage larvae were readily ingested intact by the euphausiids. Both enshathed and exshathed larvae were found in the digestive tract, but no larvae were obtained from the hemocoel. It is considered that hatched third-stage larvae are incapable of penetrating the digestive tract of adults of E. pacifica. Euphausia pacifica may become infected with third-stage larvae of A. simplex directly by swallowing hatched third-stage larvae in seawater while in earlier stages of euphausiids’ development, or indirectly by preying on small crustaceans such as copepods already harboring small developing third-stage larvae, or by both. It seems likely that in euphausiids (true intermediate hosts), larvae grow still in the third stage to become infective to fishes and squids (paratenic hosts) and possibly even to cetaceans (final hosts).

Key words: Anisakis simplex, Nematoda, hatched enshathed third-stage larve, Euphausia pacifica, infection experiments, life cycle

INTRODUCTION

The anisakid nematode Anisakis simplex (Rudolphi, 1809) Dujardin, 1845 (sensu lato) is parasitic in the stomach of marine mammals, especially cetaceans (see Oshima 1972, Smith and Wootten 1978). For the recent classification of the A. simplex complex, the reader is referred to Mattucci et al. (1997, 1998) and Pagli et al. (1998).

According to Oshima (1972), Shimazu (1974), Kagei (1979), and Smith and Wootten (1978), the life cycle of A. simplex is briefly as follows. This nematode uses cetaceans as final hosts, in the stomach of which third-stage larvae develop into adults after the third and fourth molts. Eggs in the feces of final hosts are passed into seawater. Second-stage larvae develop after the first molt in the eggs and hatch in seawater still enshathed by the shed cuticle of the first-stage larva. Hatched enshathed second-stage larvae infect marine crustaceans, especially euphausiids, or true intermediate hosts (see Shimazu 1974, Kagei 1979), in the hemocoel of which they develop into third-stage larvae after the second molt. Marine fishes, especially teleosts, and squids serve as paratenic hosts (see Shimazu 1974, Kagei 1979, Hays et al. 1998b), which acquire infection with third-stage larvae by preying on infected crustaceans or infected paratenic hosts and transmit them to final hosts. However, Keie et al. (1995) and Kikuchi (1997) have recently showed that larvae develop into third-stage ones after the first and second molts in the eggs. If they are right, hatched larvae will grow still in the third stage without molting in euphausiids.

Larvae of A. simplex have been obtained from the euphausiid Euphausia pacifica (Shimazu and Oshima 1972, Kagei 1974, our unpublished data 1996, 1998). Oshima et al. (1968) and Oshima (1972) reported that they were successful in experimentally infecting larvae of A. simplex to E. pacifica. The purposes of the present study were to confirm in which developmental stage larvae of A. simplex hatch, to observe the growth of the larvae in E. pacifica, and to discuss the development of larvae in euphausiid hosts.

MATERIALS AND METHODS

Gravid females of Anisakis simplex were obtained from the stomach of the truei-type Dall’s porpoise Phocoenoides dalli (Cetacea: Phocoenidae) caught off the Pacific coast of Tohoku, Japan, for local fishermen in March 1999 and 2000. Eggs were taken from a distal half of the uterus of them and incubated in 50-ml prefiltered seawater in a 100-ml Erlenmeyer flask at 15.5°C for the initial 3 days and at 20°C afterward in 1999 and at 15°C for the initial 8 days and at 20°C afterward in 2000. Their development was checked at irregular intervals.

In March 1999 and 2000, 180 and 70 almost sexually mature adults of Euphausia pacifica (Crustacea: Euphausiidae) (11.5–16.5 mm long in cephalothorax plus abdomen, disregarded sexes) collected off the Pacific coast of Tohoku by a local fisherman were used for infection experiments, respectively. Fifteen or 20 euphausiids each were exposed to newly hatched larvae of A. simplex (about 20 per 10-ml seawater) in 700-ml seawater in a 1000-ml beaker at 7°C for 24 hours. Only the euphausiids were then kept in new seawater at 6–11°C in 1999; but the euphausiids were used still in the same seawater with larvae at 7°C in 2000. The euphausiids exposed were examined either alive or after being preserved in 10% formalin for larvae under a stereoscopical microscope at irregular intervals. A few of the
RESULTS

Eggs were in the one-cell to morula stage when taken from the uterus of the female worms. In 1999, first-stage larvae molted to second stage 7 days after incubation. Second-stage larvae were found ensheathed by a thin shed cuticle of the first-stage larva (Fig. 1). On day 8, they molted to third stage. Third-stage larvae were found enclosed by two (outer thin and inner thick) shed cuticles of the first- and second-stage larvae, respectively (Fig. 2). On day 9, they started to hatch in seawater. Hatched third-stage larvae were enclosed by the inner thick shed cuticle but had left the outer thin shed cuticle in the eggs. In 2000, the first and second molts took place on days 11 and 12–13, respectively. On day 14, third-stage larvae started to hatch. Less than a half of the eggs incubated were fully embryonated in both years (Fig. 4).

Hatched ensheathed third-stage larvae 10 and 15 days after incubation, respectively, were used for one and three infection experiments in 1999 and 2000. Upon exposure, hatched larvae and unhatched eggs were readily swallowed by euphausiids (Fig. 5). Tables 1 and 2 give the results of the four infection experiments. In 1999, 157 of the 180 euphausiids used were examined after being preserved in formalin. A total of 37 larvae were obtained from 14 of 41 euphausiids examined 12 hours after exposure (Table 1). Neither the location of infection nor the presence or absence of the sheath of each of the larvae was determined. No larvae were found in the euphausiids examined 1, 5, 10, and 15 days after exposure (Table 1). In 2000, a total of 55 of the 70 euphausiids used were examined. Both ensheathed and exsheathed larvae were found in the digestive tract (Fig. 6, Table 2); but no larvae were found in the hemocoel, body muscles, or internal organs other than the digestive tract (Table 2). Some fully-embryonated eggs and empty eggshells were also seen in the digestive tract in both years.

DISCUSSION

The present results support Kое et al. (1995) and Kikuchi (1997). Larvae indeed molted twice to form third-stage ones ensheathed by a double sheath, or the outer thin and inner thick shed cuticles of the first- and second-stage larvae, respectively, in the eggs. They hatched still enclosed by the inner thick sheath, having left the outer thin one in the eggs. Shimazu (1974) described hatched ensheathed larvae (about 0.2–0.3 mm in body length) as second-stage ones at that time.

Larvae of *A. simplex* have been obtained from naturally-infected euphausiids of several species (Uspekshaya 1963, Smith 1971, 1983a, b, Oshima et al. 1969, Shimazu and Oshima 1972, Sluiter 1973, Kagei 1974, 1979, Lindley 1977, Shimazu 1982, Hurst 1984, Hays et al. 1998a), a caprellid amphipod (Uspekshaya 1963), caridean shrimps of two species (Shiraki et al. 1976), and a brachyuran crab (Uspekshaya 1963). Many of the larvae found in the euphausiids, except for a questionable larva of Sluiter, were morphologically similar to third-stage ones found in fishes and squids and accordingly they were considered third stage. Several authors (see, for example, Oshima 1972, Shimazu 1974, Kagei 1979, Smith and Wootten 1978) previously believed that second-stage larvae grow and undergo the second molt to the third stage in euphausiids. However, since hatched larvae are already in the third stage, they must grow still in the third stage without molting in euphausiids. Third-stage larvae from fishes and squids are 12.6–37.8 mm (see Shimazu 1974). When third-stage larvae reach about 12 mm in body length in euphausiids, they would become infective to fishes and squids and possibly even to final hosts (see also Shimazu 1974). Smith (1983b) postulated that three (4.2–5.9 mm in body length) of his larvae from euphausiids were second-stage larvae in the process of molting to the third stage. This is untenable. On the other hand, Hays et al. (1998a) obtained 100 larvae (10.00–39.30 mm long) from euphausiids artificially digested by an artificial gastric juice at 18–21°C for 24–72 hours and fixed them in hot glycero-alcohol. They observed that 56 (27.19 mm in mean body length) of the 100 larvae were morphologically and morphometrically identical with third-stage larvae from fishes but that the remaining 43 (26.54 mm) possessed two cuticles, each of which bore a mucron at the tail tip, excluding a questionable larva 10.00 mm in body length. They interpreted the latter as second-stage larvae in the process of molting to the third stage. This interpretation needs confirmation for the following reasons. Larvae with similar features are sometimes seen among third-stage larvae from fishes and squids after they are fixed in hot fixatives (70% ethanol, glycero-alcohol, water, etc.). It seems as if they were enclosed by a shed cuticle just like those of Hays et al. Third-stage larvae 12.6–37.8 mm long do not molt in fishes or squids and fourth-stage larvae have no mucron (see Shimazu 1974). Apparently, the cuticles of the above larvae have been split into two layers owing to the killing of them in hot fixatives (Shimazu’s unpublished data). It is almost certain that hatched third-stage larvae of *Pseudoterranova decipiens* (Krabe, 1878) Gibson, 1983 (formerly in *Phocanema* or *Terranova*) and *Contracaecum osculatum* (Rudolfi, 1802) Baylis, 1920 (*Anisakidae*) also grow still in the third stage in crustacean hosts (see, for example, McClelland 1982, 1990, McClelland and Ronald 1974a, b, Kое et al. 1995, Kое and Fagerholm 1993).

The present repeated experimental attempts to infect newly hatched ensheathed third-stage larvae of *A. simplex* to adults of *E. pacifica* failed. Larvae were totally absent in the euphausiids examined 1, 5, 10, and 15 days after exposure in 1999; and in the hemocoel, body muscles, or internal organs other than the digestive tract of the euphausiids examined in 2000. This suggests that all the larvae obtained in 1999 were present in the food basket or the digestive tract or both; and that ingested larvae were expelled from the digestive tract within 1 day. Accordingly, even an earliest growth of third-stage larvae of *A. simplex* in *E. pacifica* could not be observed in the present study. Oshima et al. (1968) and Oshima (1972) briefly reported that hatched larvae (regarded as second stage then) exsheathed but did not grow at all nor molt in *E. pacifica* and *E. similis* in 8 days after infection. Kagei (in Oshima et al. 1968, p. 586) added that the larvae were recovered mostly from the hemocoel of
Figs. 1–6. *Anisakis simplex*: larval development in eggs and infection experiments of newly hatched ensheathed third-stage larvae to adults of *Euphausia pacifica*.

Fig. 1. Second-stage larva (L2) enclosed by the shed cuticle (C1) of the first-stage larva, pressed out of the egg, phase contrast.

Fig. 2. Third-stage larva (L3) enclosed by the shed cuticles (C1 and C2) of the first- and second-stage larvae, respectively, pressed out of the egg, phase contrast.

Fig. 3. Hatched third-stage larva (L3) enclosed by the shed cuticle (C2) of the second-stage larva, phase contrast.

Fig. 4. Fully-embryonated eggs (arrows), just before hatching.

Fig. 5. Ensheathed third-stage larvae (arrows), a possible second-stage larva (asterisk), and an unhatched fully-embryonated egg (arrowhead) in the stomach of a euphausiid 12 hours after exposure in March 2000.

Fig. 6. Ensheathed (arrows) and an exsheathed (arrowhead) larvac in the intestine of a euphausiid 2 days after exposure in March 2000, interference contrast. (Scale bars: 0.1 mm in Figs. 1–5; 0.2 mm in Fig. 6.)
E. similis examined alive. Brattey and Clark (1992) also experimentally recovered exsheathed larvae (regarded as second stage then) with no evidence that they had molten or grown significantly from the hemocoel of harpacticoid copepods of Tisbe sp. and an unidentified species of the family Thaistriidae at 5 days after infection.

In spite of the fact that natural infection of larvae of A. simplex in E. pacifica occurs (Shimazu and Oshima 1972, Kagel 1974, our unpublished data 1996, 1998), hatched third-stage larvae of A. simplex did not infect adults of E. pacifica in the present experiments. They were certainly ingested intact by the adult euphausiids and both exsheathed and exsheathed larvae were found in the digestive tract. It is considered that ingested third-stage larvae are incapable of penetrating the digestive tract of adult euphausiids even after exsheathing. Oshima et al. (1968) and Oshima (1972) did not specify the developmental stage of E. pacifica used in their successful experiments. Hurst (1984) was unsuccessful in experimentally infecting hatched larvae (regarded as second stage then) of A. simplex to the euphausiid Nycitiphanes australis, a natural host of A. simplex in New Zealand waters, and to the galatheid crab Munida gregaria. Hurst did not specify the developmental stage of the euphausiids used, either. Hatched third-stage larvae of A. simplex may be infective to E. pacifica not in the adult stage but in earlier stages of development. In P. decipiens, larval gammaridean amphipods of Gammarus lawrenceanus and Unciola irrata, natural hosts of P. decipiens in Canadian waters, were most susceptible to infection with newly hatched ensheathed larvae (third stage; see Koie et al. 1995); but late larval instar and adult amphipods were much less susceptible (McClelland 1990). Besides, it cannot be denied the possibility that small crustaceans such as copepods swallow hatched larvae of A. simplex in seawater to store them in the hemocoel and then to transport small developing larvae almost as large as hatched larvae into E. pacifica when eaten by it (see also Shimazu 1974, Smith 1983b). Copepods constitute an important component of the diet of euphausiids of several species including E. pacifica (Mauchline and Fisher 1969). Because these euphausiids do not swallow whole copepods but break them into pieces with the mouthparts and stomach while preying on them (Mauchline and Fisher 1969), large larvae in copepods ought to be damaged or destroyed during this procedure. Brattey and Clark (1992) actually succeeded in experimentally infecting hatched larvae of A. simplex to copepods (see above). In P. decipiens, copepods transmit larvae to larval instar and adult amphipods; and copepods and amphipods transmit larvae to larger crustaceans, which were unable to attain direct infection with hatched larvae (McClelland 1982, 1990). Similarly, a transmission of third-stage larvae of A. simplex could also take place from euphausiids or copepods or both to a caprellid amphipod, caridean shrimp, and a brachyuran crab (as mentioned already), which then might act as paratenic hosts.

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**Table 1. Results of an experimental infection of newly hatched ensheathed third-stage larvae of Anisakis simplex to adults of Euphausia pacifica at 6–11°C in March 1999.**

<table>
<thead>
<tr>
<th>Time after exposure</th>
<th>No. of euphausiids*</th>
<th>No. of larvae recovered per euphausiid (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Examined</td>
<td>Infected</td>
</tr>
<tr>
<td>12 hours</td>
<td>41</td>
<td>14</td>
</tr>
<tr>
<td>1 day</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>5 days</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>10 days</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>15 days</td>
<td>22</td>
<td>0</td>
</tr>
</tbody>
</table>

* Euphausiids were examined for nematode larvae after having been preserved in formalin (see the text).

* Neither the location of infection nor the presence or absence of the sheath of each of the larvae recovered was not determined.

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**Table 2. Results of three experimental infections of newly hatched ensheathed third-stage larvae of Anisakis simplex to adults of Euphausia pacifica at 7°C in March 2000.**

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Time after exposure</th>
<th>No. of euphausiids* examined</th>
<th>Location of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 day</td>
<td>17</td>
<td>Stomach: 8: 1–6 (3.2), 26 (19+7), 3: 1, 3 (1+2), 2: 1–3 (2.0), 4 (4+0), 4: 1–10 (3.5), 14 (2+12)</td>
</tr>
<tr>
<td></td>
<td>2 days</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1 day</td>
<td>15</td>
<td>Stomach: 3 (1+2), 2: 1–3 (2.0), 4 (4+0)</td>
</tr>
<tr>
<td>3</td>
<td>2 days</td>
<td>15</td>
<td>Stomach: 4: 1–10 (3.5), 14 (2+12)</td>
</tr>
</tbody>
</table>

* Euphausiids were examined alive (see the text).

† No. of euphausiids infected: no. of larvae recovered per euphausiid (mean).

‡ Total no. of larvae recovered (no. of ensheathed larvae + no. of exsheathed larvae).
LITERATURE CITED


アニサキス線虫 *Anisakis simplex* (Nematoda: Anisakidae) の孵化第3期幼虫は、ツノナシオキアミ *Euphausia pacifica* の成体には感染しない

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アニサキス線虫 *Anisakis simplex* (Rudolphi, 1809) Dujardin, 1845 (*sensu lato*) (Nematoda: Anisakidae) の卵を培養したところ、卵殻内で、幼虫は第3期まで発育し、第1期幼虫と第2期幼虫の脱皮殻に包まれていた。この幼虫は、第1期幼虫の脱皮殻を卵殻内に残し、第2期幼虫の脱皮殻に包まれたまま海水中で孵化した。孵化した被雑性第3期幼虫をツノナシオキアミ *Euphausia pacifica* の成体に実際に接触させたところ、オキアミに容易に呑み込まれ、孵化した幼虫と脱皮した幼虫が消化管内に見られたが、血体腔からは検出された幼虫はいなかった。これは、幼虫が消化管を貫通できないために、オキアミ成体には感染できないことを示唆する。ツノナシオキアミには自然感染の幼虫が寄生しているが、幼虫はこのオキアミへつきの2つの経路のうちのどれか、あるいは両方で感染するのである。1) 孵化第3期幼虫はより未熟なオキアミへ感染する。2) 孵化第3期幼虫は槐脚類のような小形甲殻類にまず感染し、オキアミがその甲殻類を捕食したときに、小形幼虫がオキアミへ移行する。感染後、幼虫はその体内で第3期幼虫のままで発育し、待機宿主とおそらく終宿主への感染幼虫になると考えられる。

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