The life-cycle of the flatfish nematode *Cucullanus heterochrous*

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Abstract

Mature specimens of *Cucullanus heterochrous* Rudolphi, 1802 (Nematoda: Cucullanidae) were obtained from the intestine of the flounder, *Platichthys flesus*, from Danish waters. Eggs embryonate in seawater but do not hatch. Fully developed larvae pressed out of eggs are 430 μm long with amphids and dereids and enclosed within the cuticle of a previous larval stage. Infective larvae are believed to be in their third stage. Experimental studies showed that the polychaetes, *Nereis* spp., *Scoloplos armiger*, *Brada villosa* and *Capitella* sp., may act as intermediate hosts. In *N. diversicolor* the larvae increase their length to 1 mm within four weeks (15°C) without moulting. Experimental infections showed that larvated eggs are not infective to fish, whereas >550 μm long larvae from polychaetes survived in 4–24 cm long flounders and plaice, *Pleuronectes platessa*. Third-stage larvae 550 μm to 1.1 mm long were found in the submucosa of the intestine one week post infection. At a length of about 800 μm to 1.4 mm they moult to fourth-stage larvae. Fourth-stage larvae, immature and mature worms occur in the intestine and rectum. Fourth-stage larvae and adults survived experimental transfer from one flounder to another. Similar developmental stages survived for two weeks in the intestine of experimentally infected cod, *Gadus morhua*.

Introduction

Members of the genus *Cucullanus* O.F. Müller, 1777 are common parasites of both marine and freshwater fish. Only a few cucullanid life-cycles are known (Anderson, 1992; Moravec, 1994). The type species *C. cirratus* O.F. Müller, 1777 with gadoid fish as final hosts may have both a direct and an indirect life-cycle involving transport hosts and fish intermediate hosts (Køie, 2000). Until now it was not known whether the life-cycle of *C. heterochrous* Rudolphi, 1802 is direct or indirect with intermediate hosts. *Cucullanus heterochrous* has been recorded in flatfish, mainly the flounder, *Platichthys flesus* (see Gibson, 1972; Køie, 1993; Moravec, 1994). The adult *C. heterochrous* has been described by Törnquist (1931), Berland (1961, 1970), MacKenzie & Gibson (1970), Gibson (1972) and Fagerholm (1982). According to Gibson (1972) the larvae from hatched eggs are 400–460 μm long, while the smallest specimens in flounders are 660 μm long. The question is: where do the larvae occur while they increase their length from 460 μm to 660 μm, and how are they transmitted to the final hosts, pleuronectid fish?

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Material and methods

Eggs and larvae

Eggs were obtained from gravid female *C. heterochrous* removed from the intestine of naturally infected flounders captured by trawling in the Øresund, Denmark. Naturally released eggs and eggs from dissected worms were placed in seawater (30‰ S) at 5,15 and 20°C to obtain larvae for experimental infections.

Experimental infections of invertebrates

The following potential food items of flounder were used for experimental infections: 50 specimens of the laboratory-reared calanoid copepod *Acartia tonsa*, harpacticoid copepods including *Tisbe furcata*, laboratory-reared *Capitella* sp. (mature, 1–2 cm long) (Polychaeta: Capitellidae), ten specimens of each of the following genera/species collected in the Øresund: *Balanus crenatus*, *B.*

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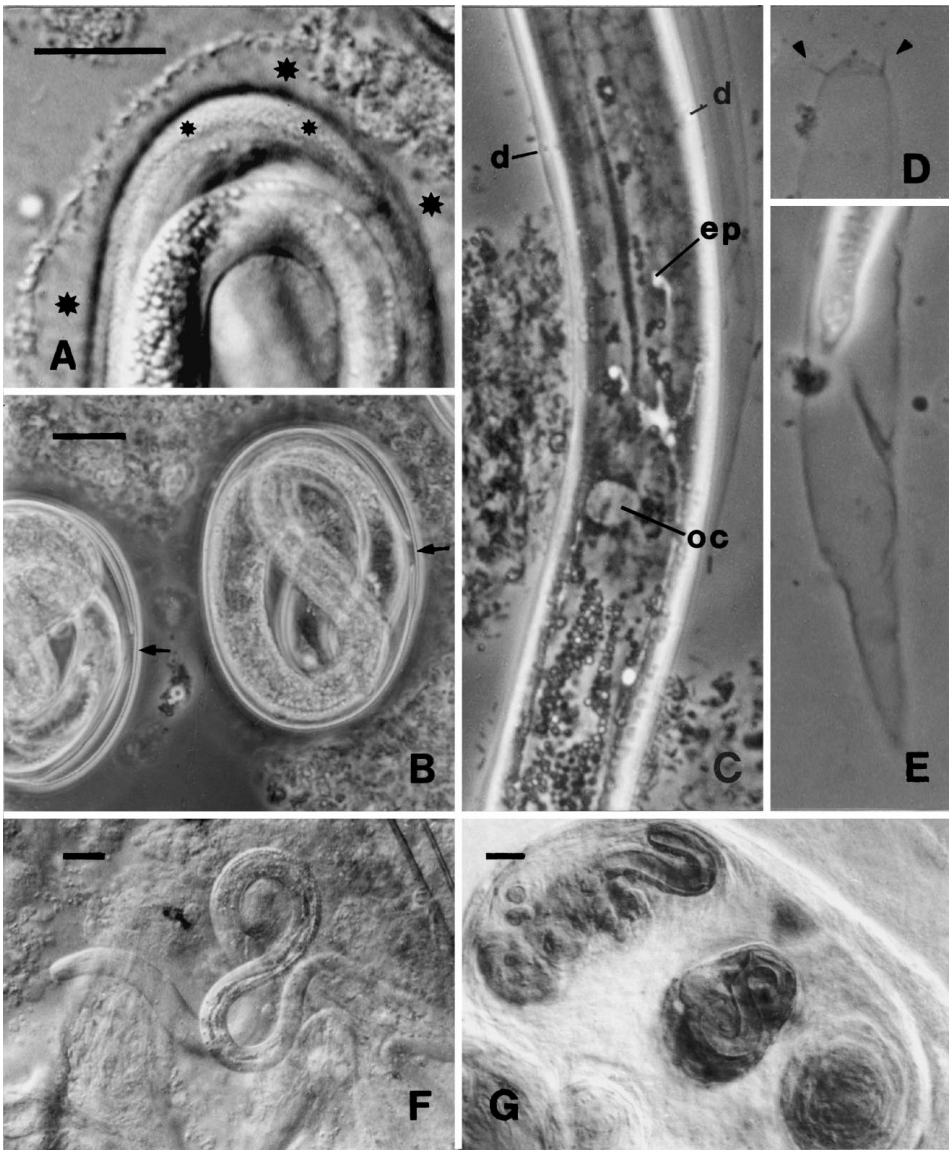

Fig. 1. Live eggs and larvae of *Cucullanus heterochrous*. A, F and G, interference contrast, B–E, phase contrast, A and C–E to same scale. (A) Surface structure of embryonated egg showing granular surface and thick mucous envelope (small and large asterisks, respectively). (B) Embryonated eggs with aperture (arrows) in internal egg shell. (C) Third-stage larva artificially forced out of egg showing the dereids (d) associated with the lateral alae, the excretory pore (ep) and the excretory system, large oesophageal cell (oc) and droplets posterior to the oesophagus. The larva is surrounded by the cuticle of the previous larval stage. (D) Anterior end of shed cuticle of larva artificially forced out of egg showing the everted amphids (arrowheads). (E) Posterior end of third-stage larva surrounded by the cuticle of the second-stage larva. (F) Third-stage larva in *Nereis diversicolor* one week p.i. (G) Encapsulated third-stage larvae in *N. diversicolor* three months p.i. Scale bars = 20 μm.

improvisus (Cirripedia), *Gammarus locusta*, *Corophium volutator*, *C. bonelli* (Amphipoda), *Idotea* sp., *Jaera* sp. (Isopoda), *Diastylis rathkei* (Cumacea), *Crangon crangon* (Decapoda), various species of mysidaceans including *Praunus flexuosus*, the polychaetes, *Nereis* (*Hediste*) *diversicolor* (1–6 cm long), *Nereis* (*Neanthes*) *virens* (3–7 cm long) (Nereidae), *Scoloplos armiger* (2–4 cm long) (Orbiniidae) and *Brada villosa* (1–2 cm long) (Flabelligeridae), various species of oligochaetes and the bivalves, *Mytilus edulis*, *Macoma baltica*, *Mya arenaria*, *Cerastoderma edule* and *C. lamarcki*. Twice the number of each genera/species were used as controls. The potential invertebrate hosts were repeatedly exposed over a period of one week to hundreds of infective eggs of *C. heterochrous* in glass containers up to

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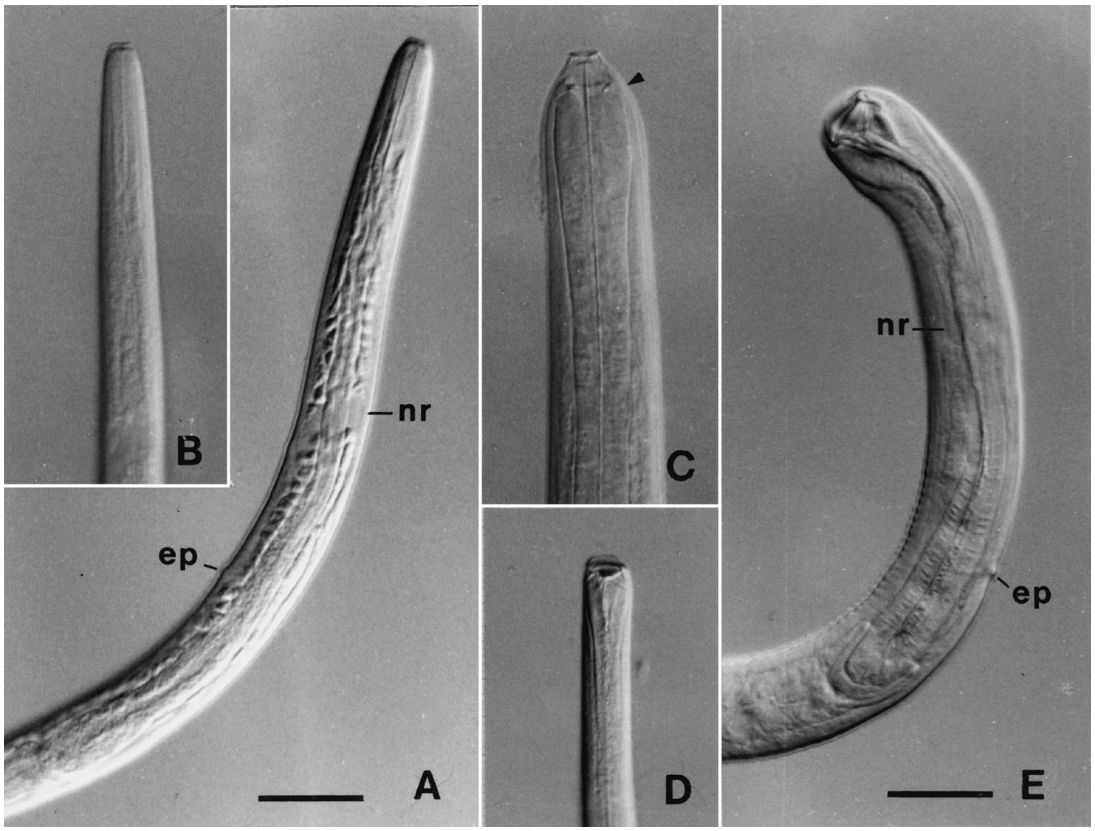

Fig. 2. The anterior end of *Cucullanus heterochrous* from *Nereis diversicolor* (A) and flounder (B–E). Interference contrast of fixed specimens. A and C to same scale (scale bar = 25 μm), B, D and E to same scale (scale bar = 50 μm). (A, B) Third-stage larvae. (C) Third-stage larva shortly before moulting, showing buccal structure of fourth-stage larva (arrowhead). (D) Fourth-stage larva shortly after moulting from the third stage. (E) Immature adult. ep, excretory pore; nr, nerve ring.

10 cm in diameter together with laboratory-reared algae (calanoid copepods) or chopped, thawed mysidaceans and boiled mussels (benthic invertebrates) (all at 15°C). A few specimens of each genus/species were examined shortly after feeding to confirm that eggs were consumed or the containers were examined to observe whether eggs remained uneaten. Specimens of *N. diversicolor* used as the intermediate host were exposed to hundreds of larvated eggs within a period of one week and then removed to a substratum without eggs.

Experimental infections of flatfish

Four 4–5 cm long 0-group flounders and four similar 0-group plaice, *Pleuronectes platessa*, captured in the Øresund were exposed four months post-capture to hundreds of larvated eggs mixed with chopped, thawed mysidaceans within a one week period (at 15°C). One or two of each flatfish species were examined shortly after the exposure to confirm that eggs had been consumed. The remaining 0-group flatfish were examined four weeks later.

Flounders and plaice (15–24 cm long) were used as experimental final hosts. They were isolated for more than six months and during this period fed on frozen mussels and prawns only. Large flounders were experimentally infected using gelatin capsules containing the following: (i) larvated eggs; (ii) small fourth-stage larvae to mature specimens of *C. heterochrous* carefully removed from naturally infected flounders; and (iii) pieces of experimentally infected *N. diversicolor* with larvae of different ages. The gelatin capsules were placed in thawed boiled mussels or thawed shrimps and fed to flounders three times within a one week period. Other flounders and plaice were fed live experimentally infected *N. diversicolor* with larvae of different ages. The experimentally infected flatfish were kept at 10°C (30‰) and examined one and two weeks and two and four months after initial exposure.

Experimental infections of cod

Small fourth-stage larvae to mature specimens of *C. heterochrous* recently removed from naturally infected flounders were fed, in gelatin capsules in pieces of thawed herring fillet, to four specimens of cod (30–40 cm long) previously isolated for six months. This was done three times within a one week period. Each cod ingested about one hundred specimens of *C. heterochrous*. The cod were examined three days, one week and two weeks post-infection (p.i.).

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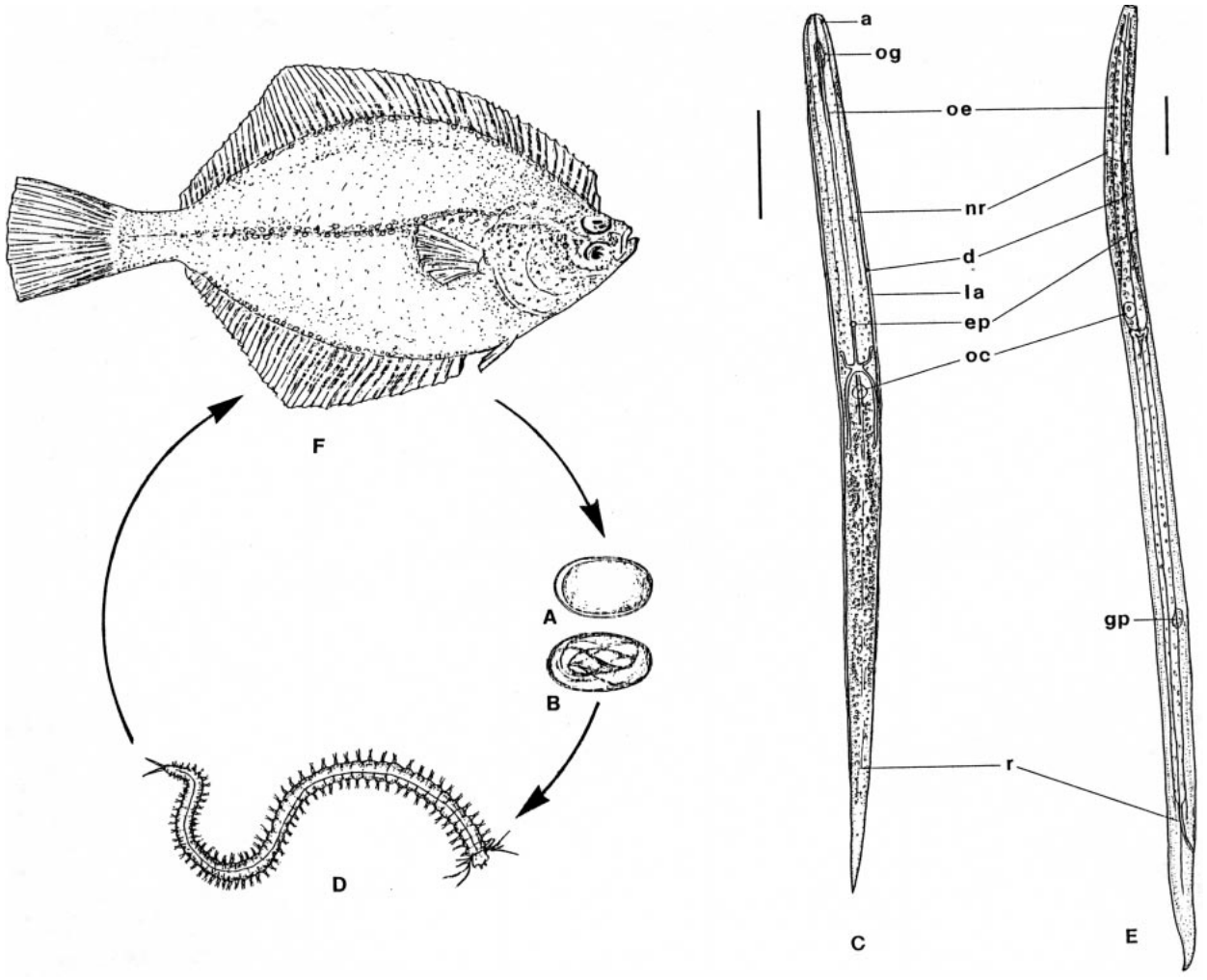

Fig. 3. The life-cycle of *Cucullanus heterochrous*. (A) Fertilized newly shed egg. (B) Embryonated, infective egg. (C) Infective third-stage larvae (430 μm) from hatched egg. Ventral view. (D) Intermediate host *Nereis diversicolor*. (E) Third-stage larva (800 μm) from intermediate host. Lateral view. (F) Final host *Platichthys flesus*. a, amphid; d, deirid; ep, excretory pore; gp, genital primordium; la, lateral ala; nr, nerve ring; oc, large oesophageal cell; oe, oesophagus; og, oesophageal gland; r, rectum. Scale bars = 50 μm.

Parasitic stages

Live eggs were placed in seawater and larvae of different developmental stages were released by coverglass pressure. Third-stage larvae from polychaete worms and flounders and older developmental stages from flounders were studied both live and after fixation in Berland's fluid (glacial acetic acid: 40% formalin, 19:1), cleared in lactid acid, and mounted in glycerol jelly. Measurements, of live third-stage larvae, and of older developmental stages of unflattened, fixed worms, are given in micrometres. Measurements are based on ten specimens unless otherwise stated.

Results

Eggs and free-living third-stage larvae

Infective eggs (fig. 1A and B) measure 80–100 × 45–60. The egg has a granular surface structure and a 5–10 thick mucous envelope (fig. 1A). Part of the egg shell is double with an aperture through the internal layer (fig. 1B). Larvae forced out of eggs by coverglass pressure do not emerge through this aperture. Eggs embryonate in seawater within seven days (20°C), two weeks (15°C) and two months (5°C). About 1% of naturally released eggs hatch. Larvae from naturally hatched eggs are not surrounded by a cuticle. Larvae forced out of eggs shortly before infectivity are partly surrounded by the cuticle of the previous larval stage. This cuticle is anteriorly provided with amphids (fig. 1D) and posteriorly with the lining of the rectum (fig. 1E).

Larvae forced out of infective eggs or naturally emerged larvae are 400–450 (430) in length (fig. 3C). Fixed larvae were 350 long. The posterior edge of the indistinct nerve ring and the posterior edge of the oesophagus are 95 and 180 respectively from the anterior

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extremity. There is a dorsal oesophageal gland anteriorly. The excretory system, including two anterior and two posterior lateral canals, is visible. The two deirids associated with the lateral alae and the excretory pore are 120 and 145 respectively from the anterior extremity. The large dorsal oesophageal cell is evident. Refractive droplets occur in the posterior part of the larvae. The anus is 50 from posterior extremity.

Free-living larvae from naturally hatched eggs only survive for a few days, whereas unhatched embryonated eggs remain infective for at least two and four months at 15°C and 5°C, respectively.

Experimental infections of invertebrates

Although the eggs were ingested by all the invertebrates studied, only the polychaetes became infected. All control invertebrates were negative. Nine of ten specimens of *Nereis diversicolor* became infected, three of ten *N. virens*, two of ten *Scoloplos armiger*, four of ten *Capitella* sp. and three of ten *Brada villosa* became infected with two to seven larvae. Both small and large specimens of *N. diversicolor* became infected, but the small larvae were difficult to detect in large specimens of the polychaete. *Nereis diversicolor* were used as experimental intermediate hosts. In this host some larvae of *C. heterochrous* grew to maximum length, 1.0 mm, within four weeks (15°C) others only grew to half this length within the same period. During this period the larvae did not moult. The larvae (fig. 1F) occurred free in the tissue throughout the polychaete body, most often in the parapodia, but also in the head and even in the palps. After about six weeks some larvae were partly surrounded by host cells. Three-month-old larvae were all encapsulated and non-infective (fig. 1G). The genital primordium is seen in infective larvae (550 μm to 1 mm long) in polychaetes (fig. 3E). A collar surrounds the mouth opening and there are no cuticular thickenings anteriorly (figs 2A and 3E).

Experimental infection of flatfish and cod

In experimentally infected flounders and plaice, third-stage larvae 550 μm to 1.1 mm long were found in the mucosa or submucosa of the intestine, rarely in the stomach one week after initial exposure. They occurred most often so superficially that they could be removed by scraping. In the mucosa and submucosa the third-stage larvae (fig. 2B) grow and moult to fourth-stage larvae (fig. 2C and D). The moult takes place at a size of 800 μm to 1.4 mm, or two weeks to two months p.i., apparently depending on the size of the third-stage larvae from the polychaete host. A few still remained as third-stage larvae four months p.i. Most moults apparently took place in the mucosa, and no remains of any capsules were found in the intestinal wall after the moult. Fourth-stage larvae and adults (fig. 2E) then migrate to the posterior part of the intestine and rectum, where they develop to the mature adult stage.

Fourth-stage larvae and later developmental stages survived the experimental transmission from flounder intestine to another flounder and to cod. In the experimentally infected flounders the worms survived and grew to maturity. In experimentally infected cod all specimens of *C. heterochrous* were lost within two weeks p.i. In cod about one hundred live worms were found throughout the intestine three days p.i. About ten worms were found in the intestine, most in the posterior part, one week p.i. The worms occurred in mucus and remains of food items in the lumen of the intestine. None was attached to the intestinal wall and none was found in the pyloric caeca. No dead worms were found. Two weeks p.i. no worms remained.

Natural infection of flatfish

In the Øresund, flounder, plaice and, rarely, common dab, *Limanda limanda*, and long rough dab, *Hippoglossoides platessoides*, harboured mature *C. heterochrous*. Off the Faroe Islands, mature specimens of *C. heterochrous* were found in plaice, long rough dab and lemon sole, *Microstomus kitt* (see Køie, 1993). Third-stage larvae, fourth-stage larvae, immature and mature adults were found in flounders from the Øresund throughout the year. The highest prevalence of mature worms was found in winter. Fourth-stage larvae and mature worms are often firmly attached to the flatfish intestine or rectum.

Life-cycle

The life-cycle of *C. heterochrous* is shown in fig. 3. The main final host, the flounder, acquires the infection by ingesting polychaetes, mainly *N. diversicolor* and *N. virens*. Transmission from one flounder to another *via* predation is hypothetical and is not believed to take place under normal conditions.

Discussion

The infective larvae in eggs of *C. heterochrous* are believed to be in their third stage. No moult was observed in the intermediate host, and infective larvae in polychaetes were identical with third-stage larvae in the fish. The larvae in the fish moulted to fourth-stage larvae. Larvae of *C. cirratus* forced out of eggs were surrounded by two putative cuticles of previous larval stages. Larvae from naturally hatched eggs of *C. cirratus* were directly infective to cod fry in which maturity was attained. It is likely that most or all larvae which emerge from eggs of *Cucullanus* spp. are third-stage larvae (Køie, 2000).

Even though third-stage larvae were found in the submucosa of the experimentally infected flatfish, no developmental stage of *C. heterochrous* has a true histotropic phase as recorded for other members of the Cucullanidae (see Anderson, 1992; Moravec, 1994). In this aspect it resembles *C. cirratus* which also occurs in the mucosa or in the lumen of the alimentary tract of the final host (cod, *Gadus morhua*) (Køie, 2000).

The larva of *C. heterochrous* has not previously been recorded from an invertebrate host. MacKenzie & Gibson (1970) did not find nereids (or various malacostracan crustaceans) infected with cucullanid larvae.

All the exposed species of polychaetes became infected with *C. heterochrous*. *Nereis diversicolor* is believed to be the most important intermediate host. Even though it has been used for physiological and morphological studies by numerous researchers, its role as intermediate host for *C. heterochrous* has until now been unknown.

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Nereis diversicolor is recorded from the Arctic, the North Pacific, the North Atlantic and adjacent seas to the Mediterranean Sea and the Black Sea. It is highly euryhaline and has been found in hypersaline conditions and in freshwater (Hartmann-Schröder, 1996). In brackish water with a constant low salinity the lowest limit for survival of adult worms is about 3‰. Its vertical distribution is from the supralittoral to a depth of about 40 m, and it does not have brackish water submergence (Hartmann-Schröder, 1996). *Nereis virens* has nearly the same distribution as *N. diversicolor*. *Scoloplos armiger* occurs from the eulittoral to a depth of 2000 m (Hartmann-Schröder, 1996). Neither the small specimens of *Capitella* sp. nor the mud-dwelling *Brada villosa* are likely to play a role as intermediate hosts under natural conditions.

Cucullanus heterochrous has been recorded in flatfish from the North Sea, the Russian Arctic, the Siberian coast, the Mediterranean Sea, the Atlantic and Pacific coasts of North America and the Far East (Gibson, 1972; Moravec, 1994) and from the Baltic (Fagerholm & Køie, 1994; Køie, 1999). The geographical distributions of *C. heterochrous* in flatfish and *N. diversicolor* are – apart from its absence from the southern part – nearly identical, indicating that *N. diversicolor* probably acts as the most important intermediate host. At the Faroes, the long rough dabs caught at 45 m were infected, whereas those caught at 342 m were uninfected with *C. heterochrous* (see Køie, 1993). In the Baltic Sea (from the Mecklenburg Bight to the Estonian coast), flounders were infected with *C. heterochrous* at all stations, with the highest prevalences and intensities in the western and southern Baltic Sea (Køie, 1999).

It appears that the shortest developmental time for *C. heterochrous* in flounders in the Øresund is about one year: eggs produced during the winter may embryonate and become infective during the spring and summer. Flounders may ingest infected polychaetes during the summer and autumn. In the flounder the larve may develop to maturity during the winter, the time of the year with the highest prevalence of mature specimens. Fagerholm (1982) found mature females throughout the year in flounders from the Finnish coast. As with the present findings Gibson (1972), Möller (1974) and Fagerholm (1982) found the highest prevalences of adult *C. heterochrous* in flounders in the winter. Gibson (1972) and the present study did not show a clear seasonal pattern of infection of different developmental stages in flounders. The lack of a seasonal pattern may be explained by the highly resistant eggs which may remain undeveloped at the sea bottom for months, and the retardation of the different developmental stages at low temperatures.

Cucullanus heterochrous has been recorded from cod, *Gadus morhua* (see Hemmingsen *et al.*, 1991). This record is unexpected since most species of *Cucullanus* are host-specific at least to family level. The present experiments indicate that specimens of *C. heterochrous* may survive for a short period of time in the intestine of cod, but that it is not a true gadid parasite.

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