In vitro development of the nematode *Contracaecum osculatum* Rudolphi 1802 (Nematoda: Anisakinae)\(^1\)

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Received December 31, 1973


Eggs of *Contracaecum osculatum* were dissected from the vagina and uterus of adult worms from seals and were incubated in seawater at 15°C. Freshly hatched larvae were cultivated to preadult in Eagle’s medium (MEM) with 20% foetal calf serum.

At 15°C, the exsheathed second-stage larvae molted and grew to the infective stage without further molts. Infective larvae of *C. osculatum* were 6.5 mm (5.9-9.5) in length after 32 weeks.

At 35°C, 58% of infective larvae >2 mm in length molted to subadults which possessed cuticular lips but lacked interlabia. Subadults were 2.9-13.8 mm in length and morphologically similar to larvae found attached to the gastric mucosa of seals. Four subadults subsequently molted to preadults 13.1-17.2 mm in length with interlabia. However, an attempt to infect a seal with cultivated infective larvae was unsuccessful.

**Introduction**

McClelland and Ronald (1974) successfully cultivated *Terranova decipiens* in vitro. Eggs used in that study were dissected from adult *T. decipiens* collected from stomachs of harbor seals (Phoca vitulina) and grey (Halichoerus grypus) seals from Cape Breton Island.

About 5-10% of the adult nematodes from the above-mentioned seals belonged to a second anisakine species, *Contracaecum osculatum*. Adult anisakines in samples from the stomachs of harp seals (Pagophilus groenlandicus) from the Gulf of St. Lawrence were exclusively this species. Davey (1969) pointed out that while much attention had been devoted to studies of the biology of *T. decipiens*, there were no parallel studies of *C. osculatum*.

**Materials and Methods**

Adult and subadult *C. osculatum* were obtained from the stomachs of grey and harbor seals from Fourchu, Nova Scotia, and from harp seals from the Gulf of St. Lawrence.

Eggs were dissected from the vagina and uterus of gravid worms. Incubation of eggs and subsequent cultivation of second-stage larvae were according to the methods of McClelland and Ronald (1974). In this case, however, second-stage larvae were stimulated to exsheath in 0.05% sodium hypochlorite in seawater (Davey 1969) before they were introduced to medium.

The medium was Eagle’s minimum essential medium (MEM) with 20% foetal calf serum and 50 U penicillin, 50 μg streptomycin, and 50 μg kanamycin per milliliter. Temperature was 15°C; pH, 7.2; gas phase, air or 5% CO₂ in air. Larvae were cultivated in 5 mm of medium in 150 x 16 mm culture tubes or 35 x 15 mm Petri dishes. The tubes were placed in a roller drum at 1 rpm. Initially, there were 200-300 larvae/ml of medium, but this was reduced to 40-60/ml and 20-30/ml at 10 and 22 weeks respectively. Medium was changed weekly.

The temperature was raised to 35°C for cultures of second-stage larvae >2 mm in length. Medium, pH, and gas phase were as above, but culture vessels were 100 x 15 mm Petri dishes or 200-ml culture flasks. Each contained 20 ml of medium. There were 10-20 larvae/ml and medium was changed daily.

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\(^1\)Migration Series No. 083.
Specimens from in vitro cultivation and from seals were fixed in hot glycerin alcohol and cleared in glycerin for morphological study.

Results

Cultivation of Second-stage Larvae

Eggs, multicellular when removed from the vagina and uterus, hatched after 19-25 days of incubation in seawater at 15°C. The exsheathed second-stage larvae (400 microns (μ) long) were free-swimming and extremely active. The outer layer of the sheath or first-stage cuticle was expanded into fin-like folds.

Larvae exsheathed after 5-10 min in 0.05% sodium hypochlorite in seawater. Larvae not exposed to exsheathing solution exsheathed 1-2 weeks after introduction to the medium. Nearly 100% of the larvae exsheathed in each case. Exposure to hypochlorite did not seem to affect subsequent growth and development in vitro.

In medium, exsheathed larvae were fully extended and motionless. They began to grow immediately, although the gut did not appear functional initially. Peristaltic activity of the muscular oesophagus and contractions of the rectum were first detected after 1-2 weeks, when larvae were 0.5-1.0 mm in length.

All larvae grew and there was little mortality. No molts occurred over 32 weeks of continuous cultivation at 15°C. Second-stage larvae were 6.0 mm (5.5-7.3) in length and 243 μ (217-279) in diameter after 22 weeks, but had only increased to 6.5 mm (5.9-9.5) in length after 32 weeks.

Morphology of Second-stage Larvae

At time of exsheathment, and throughout the development of second-stage larvae, there was a triangular mouth with a blunt cuticular tooth at its ventral border (Figs. 1 and 2). The tooth was apical in freshly exsheathed larvae but came to occupy a more ventral position as larvae grew. The excretory pore was a transverse slit at the base of the tooth. There were three rounded lip masses with the dorsal mass becoming particularly prominent in large larvae. The dorsal lip had two submedial papillae; and each subventral lip, one papillae and an amphid. The two cervical papillae were slightly posterior to the nerve ring (Figs. 3 and 4).

In recently exsheathed larvae, there was a rudimentary muscular oesophagus, ventriculus, intestine, and rectum. There was a large nucleus in the ventricular appendix (Fig. 3). The excretory gland, the most prominent internal structure, was flat, granular, tapered at the extremities, and extended along the left perienteric region from the nerve ring to 0.6 of the distance from the anterior extremity. The conspicuous nucleus of the gland lay near the posterior end of the ventricular appendix. The cuticle-lined excretory duct ran ventral to the oesophagus from the pore to the anterior end of the gland. The oval genital primordium (10-15 μ in length) appeared unicellular and lay in the left subventral part of the pseudocoel near the posterior end of the excretory gland.

In larvae >1 mm in length, the elongate muscular oesophagus appeared striated and multinucleate; the ventriculus, glandular and opaque (Fig. 4). The dorsal oesophageal gland was evident; its duct opened to the lumen of the oesophagus anterior to the nerve ring. The oesophageal-intestinal junction was on the ventral wall of the intestine. The resulting dorsal intestinal caecum was attached distally to the body wall by a ligament. Although there was a slight increase in size (17-25 μ in length in 6-mm larvae), the genital primordium did not appear to change in cell number or morphology. The rectum and associated muscles, ligaments, and glands were clearly defined (Fig. 5). There were three lobular rectal glands, one dorsal and two subventral.

From morphometric regressions (Fig. 6), it is apparent the oesophagus and appendix were about the same length throughout development of second-stage larvae. Covariant analysis with significance at the 5% probability level reveals no difference in regressions for the two structures. Caecal length, 8-23 μ in 0.8- to 1.0-mm larvae,
increased 10-fold in 1.0- to 2.0-mm larvae. In larvae >2 mm in length, the ratio of caecal length to appendix length was 0.51 (0.34-0.88). Growth was slightly greater between the genital primordium and the posterior extremity than in the rest of the larva. In larvae >5 mm in length, the genital primordium lay at 0.52 (0.43-0.58) of the body length from the anterior end.

Cultivation to Preadult

The temperature of incubation was raised from 15°C to 35°C in cultures containing a total of 15,000 second-stage larvae >2 mm in length. After 6 weeks, 58% had molted to subadults possessing three cuticular lips but lacking interlabia; 28% were viable second-stage larvae or subadults which retained the cuticle of the second-stage; 14% were moribund.

Before molting, second-stage larvae attached to the walls of the culture vessel by means of a cap which encased the head. Molting began at day 3 and continued throughout the 6-week
FIG. 7. Anterior end of cultivated subadult of *C. osculatum* 12 mm in length (*en face* view). Fig. 8. Anterior end of cultivated subadult of *C. osculatum* 10 mm in length (*lateral* view). Fig. 9. Anterior end of cultivated preadult of *C. osculatum* 17 mm in length (*en face* view). Fig. 10. Oesophagus, intestinal caecum, and ventricular appendix of cultivated subadult of *C. osculatum* 10 mm in length (*lateral* view).
period. Subsequent to molting, cephalic caps reformed and subadults attached to the walls of the vessel or to cast off cuticles.

Second-stage larvae and subadults were fed before and during attachment. Sampling before and after the 6-week period indicated larvae had increased in length by an average of 85%. Subadults were 2.9–13.8 mm long.

Four subadults had evidently undergone a second molt to preadults (13.1–17.2 mm in length) possessing interlabia. One of these remained partially ensheathed in the cuticle of the subadult.

**Morphology of Sub- and Pre-adults**

Cultivated sub- and pre-adults were morphologically similar to larvae found attached to the gastric mucosa of seals. Subadults possessed three cuticular lips with lateral projections (Figs. 7 and 8). The papillae on dorsal and sublateral lips were double and the characteristic collar of cuticular folds was evident. In preadults, interlabia were about the same length as the lips, and the lateral projections of the lips were more pronounced (Fig. 9).

In both sub- and pre-adults, the oesophagus was conspicuously striated; the ventriculus and appendix, homogeneous and translucent (Fig. 10). Intestinal folds formed a distinct herringbone pattern. There was a marked decrease in the relative growth of the appendix (Fig. 11). The caecum appendix length ratio was 0.93 (0.53–1.56), and 1.24 (0.65–1.88) in cultivated and field-collected subadults respectively.

The genital primordium of subadults was multicellular and sexually dimorphic. In small subadult females, a stout primordial vagina extended through the ventral hypodermis. The distal uterine-ovarian portion was bilobed and ran posteriad in the ventral pseudocoel. In large subadult females, there was a distinct vagina, a uterus, and ovaries, while the vulva appeared as a tiny pore through the cuticle of the preadults. In the latter case, the cuticle-lined aperture extended only to the proximal end of the vagina. The primordial vagina lay 0.46 (0.39–0.60) and 0.41 (0.34–0.45) of the body length from the anterior end in cultivated subadults and subadults from seals respectively. In 20 adult females (28.3–67.2 mm in length) from seals, the vulva lay 0.32 (0.28–0.37) of the body length from the anterior end.

In small, subadult males, the genital primordium was short and cylindrical, and lay in the left subventral region of the pseudocoel near the midbody. It became elongate and convoluted in large subadult males. The primordial vas deferens extended from the anterior end of the genital primordium to the ventral wall of the rectum. Spicule primordia were present but genital papillae lacking. There was no increase in the number of rectal glands. The tail, however, was significantly smaller in males than in females (Fig. 11).

Covariant analysis reveals that regression coefficients for the position of nerve ring, and lengths of oesophagus, appendix, and caecum were significantly lower in cultivated subadults than in subadults from seals (Fig. 11). Comparison of regressions for the position of primordial vagina seems to indicate that the above differences were attributable to more rapid growth in the posterior half of the smaller cultivated larvae. Cultivated and field-collected subadults of similar size did not differ significantly in respect to any of their major dimensions.

**Transmission to Seals**

Cultivated second-stage larvae of *C. osculatum*, 2–8 mm in length, were implanted in the body cavities of herring and fed to a 14-month-old worm-free harp seal. The seal received a total of 3000 larvae over a 2-week period beginning 6 weeks before its eventual necropsy. At necropsy, there were no nematodes in the gastrointestinal tract.

**Discussion**

According to Davey (1969), eggs of *C. osculatum* are in the blastula stage in seal faeces and hatch after 13 days in seawater at an optimal temperature of 16°C. In the present study, eggs dissected from the vagina and uterus of adult *C. osculatum* hatched after a longer incubation period (19–25 days) at a similar temperature (15°C). The appearance of the ensheathed second-stage larvae, and in vitro exsheathed larvae in hypochlorite solution were as described by Davey.

The life cycle of *C. osculatum* is not known. Possibly, it is similar to that described by Huizinga (1966, 1967) in his studies of *C. spiculigerum* and *C. multipapillatum* of piscivorous birds; that is, an invertebrate (copepod) may
serve as a precursor host for the second-stage larva, but development to infective stage occurs in the fish host. Davey (1969) successfully transmitted freshly hatched larvae of *C. osculatum* to harpacticoid copepods (*Tisbe furcata, Amphiascus similis*). The second-stage larvae exsheathed in the gut of the copepod and penetrated to the haemocoel. They apparently underwent little growth or development therein. Sudarikov and Ryzhikov (1951) identify nematodes from the viscera of yellowfin sculpin (*Cottocompeorus growingki*) as the infective stage larvae of *C.*

![Graph](image)

**Fig. 11.** Morphometric regressions for the lengths or positions of various structures in cultivated subadults of *C. osculatum* (*n* = 45) and subadults of *C. osculatum* from seal (*n* = 40). Distance from posterior extremity, *;* distance from anterior extremity, **.
osculatum. These larvae are similar in size (6.7–11.1 mm in length) and morphology to larvae cultivated from the egg at 15°C in the present study. Berland (1963) found what he suspected were the infective-stage larvae of *C. osculatum* (with cephalic tooth and 5–6 mm in length) in the stomach of harp seal.

According to field-collected evidence (Sudarikov and Ryzhikov 1951; Osche 1959; Berland 1963), *C. osculatum* undergoes two molts (presumably the third and fourth) in the seal stomach; the fourth-stage larvae possess three cuticular lips but interlabia do not appear until the fifth stage. The fourth-stage larvae collected from Baikal seals (*Pusa sibirica*) by Sudarikov and Ryzhikov, and those obtained from harp seals by Berland were 13.8–23.8 and 7.1–12.1 mm in length respectively. The above molts were demonstrated in vitro in this study when the temperature of cultivation was raised to 35°C. Cultivated subadults are similar to specimens from harp, grey, and harbor seals, and they also resemble the fourth- and fifth-stage larvae described by Sudarikov and Ryzhikov.

Evidently, *C. osculatum* (see Sudarikov and Ryzhikov 1951; Osche 1959; Berland 1963) and *Contracaecum* spp. of fish (Berland 1961) and piscivorous birds (Huizinga 1966, 1967) become infective to the final host as third-stage larvae. According to Huizinga, the molt to the third stage occurs in the tissues of the fish host. The cuticle of the second stage is retained as a tightly fitting sheath. However, the second-stage larvae of *C. osculatum* like those of *Terranova decipiens* (see McClelland and Ronald 1974) developed directly to the infective stage in vitro. In the latter study, McClelland and Ronald point out that during in vitro cultivation, larvae did not encounter physical or chemical stimuli which might normally induce them to molt in vivo. On the other hand, they suggest that possibly *T. decipiens* develops neotenically from the second stage to the infective stage under natural conditions. The second molt appears to be lacking during the in vitro development of *C. osculatum*, but this molt has not been detected in vivo.

After the molt of the larvae from infective (third) stage to subadult (fourth) stage herein, the ventricular appendix had changed in appearance and grew at a slower rate than the intestinal caecum. Ratios describing the relative lengths of caecum and appendix invariably appear in description of adults of *Contracaecum* spp. Such ratios are also used in separating *Contracaecum* spp. larvae into morphologically distinct groups (Kagei et al. 1970). Yet, according to data herein and in the literature, there is no direct relationship between the length of the caecum and appendix in *C. osculatum*. The appendix is present in freshly hatched second-stage larvae but the caecum is lacking (Davey 1969). The caecum–appendix length ratio changes from 0.54–0.81 in third-stage larvae to 1.18–1.25 in fourth-stage larvae (cf. Sudarikov and Ryzhikov 1951), and is 1.9–2.5 in adults (cf. Berland 1963).

The infective (third stage) larvae of some *Contracaecum* spp. possess well-developed genital primordia and are sexually dimorphic (Kagei et al. 1970). This appears to be the case in the infective larvae of *C. aduncum*, a species which uses predacious marine fish as a final host (Berland 1961), but Huizinga (1966, 1967) did not detect a genital primordium in the infective larvae of avian *Contracaecum* spp.

In the present study, the genital primordium began to differentiate in the subadults (fourth-stage larvae) of *C. osculatum*. Development of genitalia was similar to that in *T. decipiens* (McClelland and Ronald 1974), although, in the latter species, the genital primordium begins to differentiate before larvae reach the infective stage. As a result of allometric growth in *C. osculatum*, the genital primordium, which lay at 0.6 of the body length from the anterior end in freshly hatched second-stage larvae, came to lie anterior to the midbody in large infective-stage larvae and subadults. In large subadult females, the proximal end of the primordial vagina lay at a position nearly corresponding with that of the vulva in adults, that is, at about one-third of body length from the anterior end (cf. Berland 1963).

**Acknowledgments**

The authors are indebted to M. L. MacDonald and S. Dudka for collecting nematodes from seals. Special thanks to Mrs. U. Strelive for preparing the final drawings, and to Dr. R. C. Anderson who reviewed the manuscript.


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