Aquat. Living Resour., 1992, **5**, 307-318

Description of the larval stages of *Anguillicola crassus* (Nematoda, Dracunculoidea) using light and scanning electron microscopy

Guillaume Blanc [1], Stéphane Bonneau [2], Sylvie Biagianti [3] and Annie J. Petter [2]

[1] *Service d'Aquaculture et Pathologie Aquacole, École Nationale Vétérinaire de Nantes, C.P. 3013, 44087 Nantes Cedex 02, France.*
[2] *Laboratoire de Biologie parasitaire, Protistologie, Helminthologie, Muséum National d'Histoire Naturelle, 61, rue Buffon, 75231 Paris Cedex 05, France.*
[3] *Laboratoire de Biologie marine, Faculté des Sciences de Perpignan, avenue de Villeneuve, 66025 Perpignan Cedex, France.*

Received September 23, 1992; accepted October, 26, 1992.

Blanc G., S. Bonneau, S. Biagianti, A. J. Petter. *Aquat. Living Resour.*, 1992, **5**, 307-318.

Abstract

The larval stages of *Anguillicola crassus*, a parasite of the swimbladder of eels, are described, using light and scanning electron microscopy. Second-stage larvae in copepods are characterized by a globular cephalic end and third- and fourth-stage larvae by an apical sclerotized ornamentation consisting of two U-shaped median pieces. Third- and fourth-stage larvae are difficult to distinguish from each other using light microscopy because the third moult has never been observed and there are no obvious characters differentiating the two stages. Three characters, however, observed by using high magnification of light and scanning electron microscopy are considered as distinctive between the two stages, namely presence or absence of deirids and lateral alae and differences in the structure of the cuticle. These morphological characters will permit epidemiological studies at the larval level.

Keywords: *Anguillicola crassus*, Nematoda, Dracunculoidea, larval morphology.

Étude de la morphologie des stades larvaires de Anguillicola crassus *(Nématoda, Dracunculoidea) en microscopie optique et électronique à balayage.*

Résumé

La morphologie des stades larvaires de *Anguillicola crassus*, nématode parasite de la vessie gazeuse des anguilles, est étudiée en microscopie optique et électronique à balayage. Chez les hôtes intermédiaires (Cyclopidae) les larves du 2e stade sont caractérisées par une extrémité antérieure globuleuse; chez les Cyclopidae, les hôtes paraténiques et l'hôte définitif des larves des 3e et 4e stades ont une structure buccale et céphalique caractérisée par une ornementation sclérifiée constituée de pièces médianes en forme de U renversé ainsi que par 4 papilles sub-médianes et 2 amphides. La 3e mue n'ayant jamais été observée, la distinction entre le 3e et la 4e stade est délicate en l'absence de caractéristiques discriminantes d'observation aisée. Toutefois, trois caractères observés en utilisant le plus fort grossissement du microscope optique et le MEB sont considérés comme distinctifs: la présence ou l'absence de déirides, la présence ou l'absence d'ailes latérales ainsi que les différences dans la structure de la cuticule. Ces caractéristiques morphologiques permettront la poursuite d'études épidémiologiques au niveau des stades larvaires.

Mots-clés : *Anguillicola crassus*, Nematoda, Dracunculoidea, morphologie des stades larvaires.

INTRODUCTION

Anguillicola crassus Kuwahara *et al.*, 1974, a parasite of the swimbladder of the Japanese and European eels (*Anguilla japonica* Temminck and Schlegel and *Anguilla anguilla* L.), has become increasingly widespread within Europe in recent years. The parasite was first described by Kuwahara *et al.*, 1974 and the genus was recently revised by Moravec and Taraschewski (1988). Its biology has been studied by several authors (De Charleroy *et al.*, 1990; Haenen *et al.*, 1989; Petter *et al.*, 1989; Kennedy and Fitch, 1990, Petter *et al.*, 1990; Bonneau, Blanc and Petter, 1991): the life-history includes a crustacean intermediate host (mainly cyclopid copepods); small fish may act as paratenic hosts, in which the infective third-stage larvae remain alive and able to infest the definitive

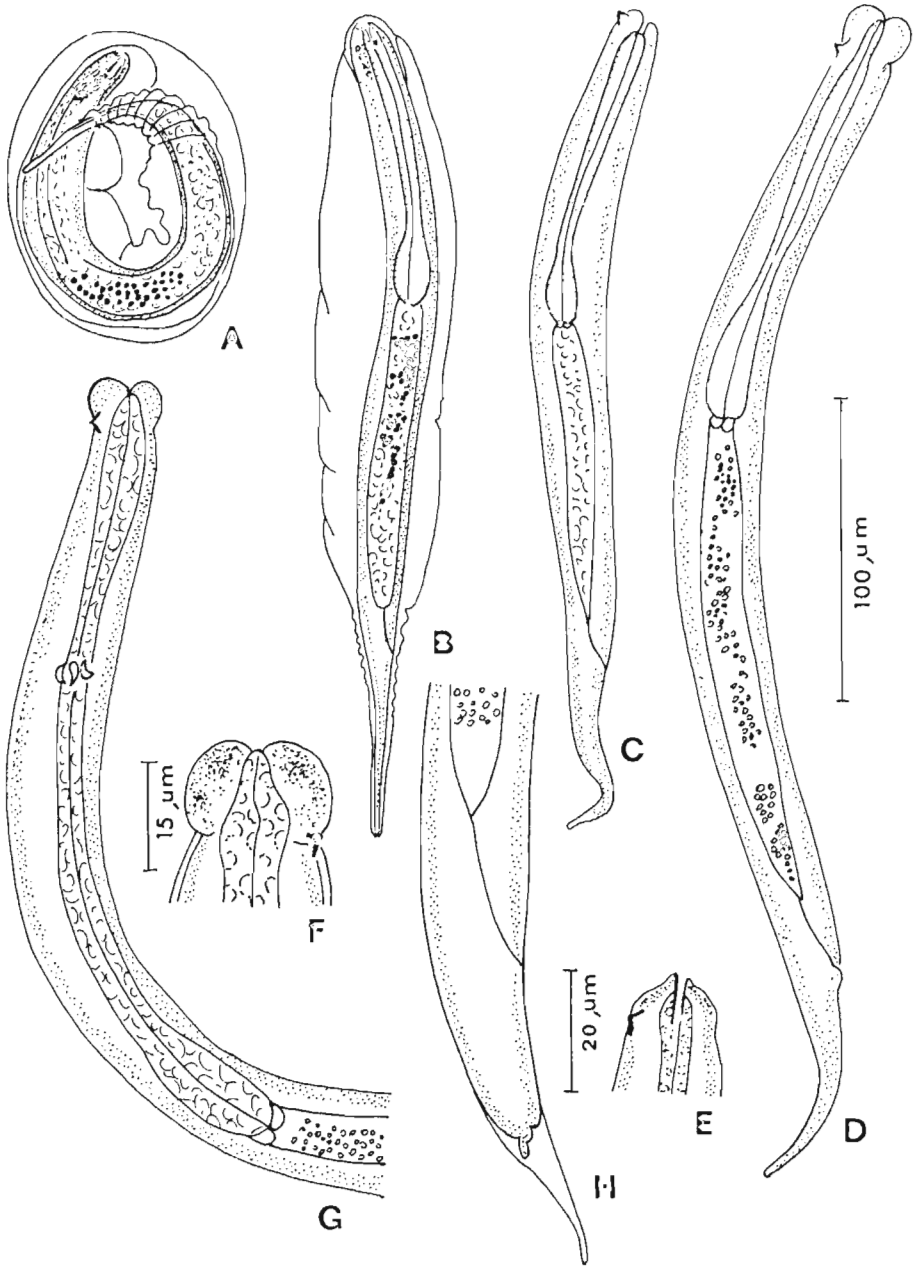

Figure 1. - *Anguillicola crassus*. Second-stage larvae. *A*: mature egg. *B*: free-living larva. *C* to *G*: larvae from the haemocoel of intermediate hosts. *C*, *D*,: entire larvae. *E*, *F*: anterior ends. *G*: anterior end of a larva showing three small crescent-shaped pieces. *H*: posterior end of a larva showing the second moult. Scale-bar: *A*, *B*, *C*, *D*, *G*, *H*, 100 µm; *E*, 20 µm, *F*, 15 µm. This figure includes data from Petter *et al.* (1989) with additional information.

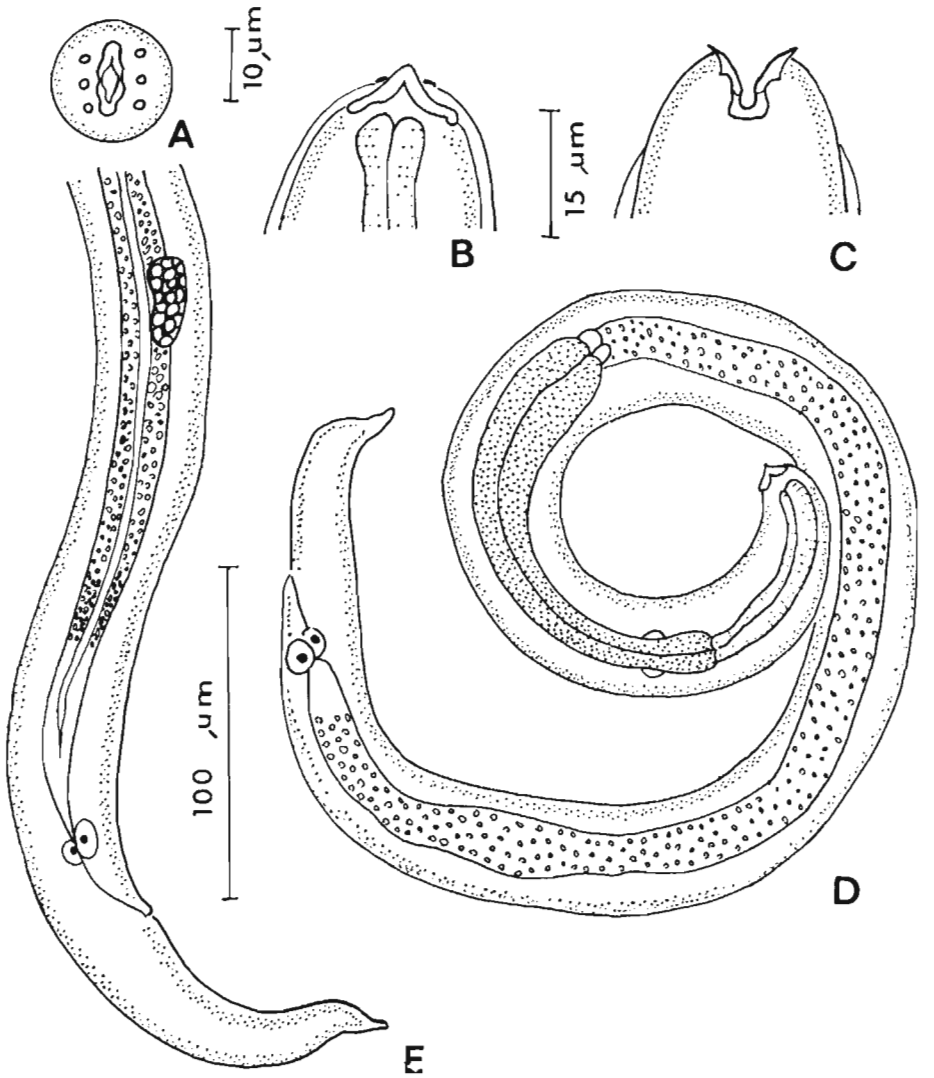

Figure 2. – *Anguillicola crassus*. Infective third-stage larvae. *A*: *en face* view. *B*: anterior end, lateral view. *C*: anterior end, median view. *D*: entire larva, lateral view. *E*: posterior part showing the genital primordium, lateral view. Scale-bar: *A*, 10 µm, *B*, *C*, 15 µm, *D*, *E*, 100 µm. This figure includes data from Petter *et al.* (1989) with additional information.

Table 1. – Growth of second-stage larvae of *Anguillicola crassus* in copepods (all measurements are given in µm).

No.	1	2	3	4
Length	290	410	730	740
Width	20	30	35	35
Oesophagus	105	140	220	250
Tail	65	78	110	90

host; third- and fourth-stage larvae develop in the swimbladder wall of eels, and the adults live in the swimbladder lumen. Petter *et al.* (1989) described the early larval stages in the intermediate and paratenic hosts. An extensive study of the larval stages of this nematode using scanning electron microscopy and light microscopy is presented in this study.

MATERIALS AND METHODS

During the course of a survey of this parasite in northern, southern and western France, eels were captured by means of both electrofishing and fykenetting. Infective third-stage larvae were obtained from various species of cyclopoid copepods and three species of fishes (*Phoxinus phoxinus* L., *Gasterosteus aculeatus* L. and *Pygosteus pungitius* L.) caught in Nantes or in the Paris area. Copepods were experimentally infested as outlined by Petter *et al.* (1989). Fishes were infested by consuming these copepods placed in their aquaria. The older third-stage and fourth-stage larvae came either from naturally- or experimentally-infested eels captured in the Loire-Atlantique district or collected from an eel brood

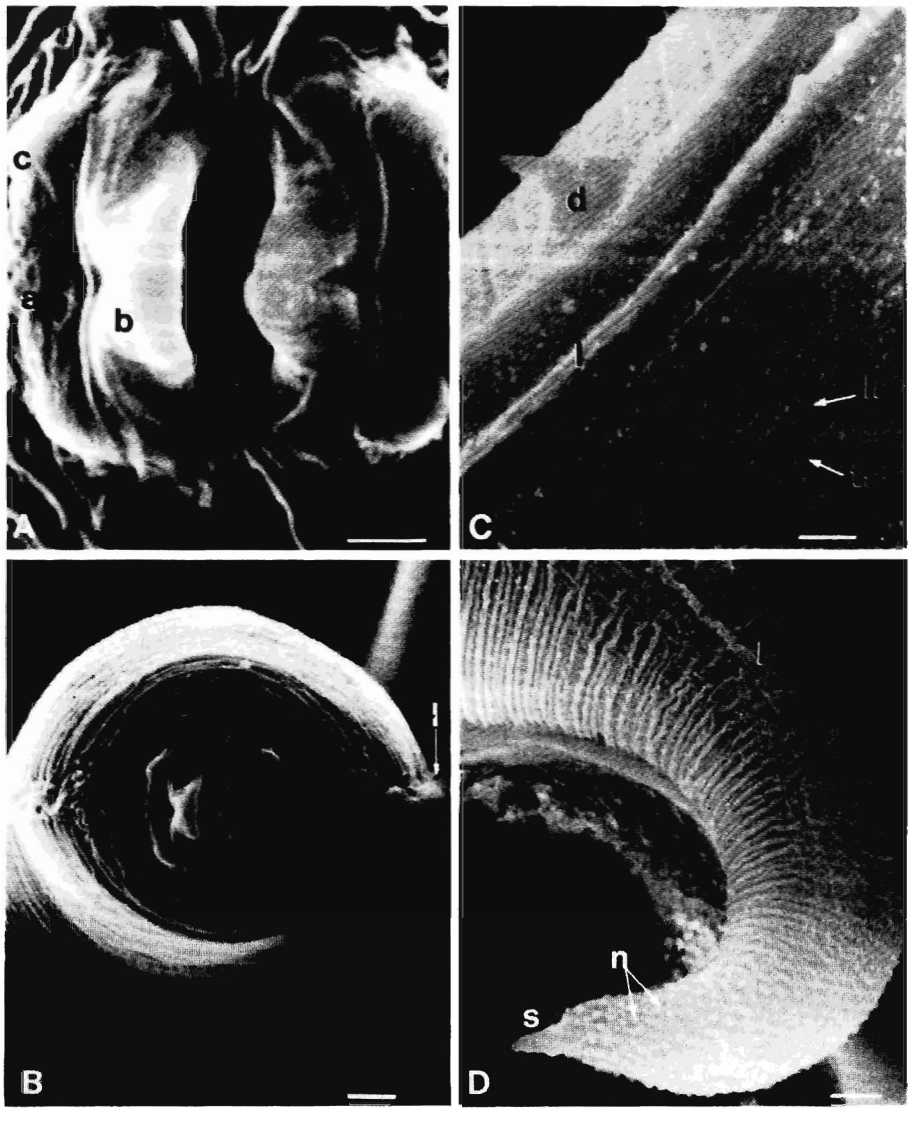

Figure 3. – *Anguillicola crassus*. Infective third-stage larvae; scanning electron micrographs. *A*: Apical view: *a*, amphid; *b*, buccal ornamentation; *c*, submedian papilla. *B*: Same, lower magnification: *l*, lateral ala. *C*: Portion of body at level of deirids: *d*, deirid, *l*, lateral ala; *lr*, longitudinal crests; *ts*, transverse striae. *D*: Caudal end: *l*, lateral ala; *n*, nodules; *s*, terminal spike. Scale-bar: *A*, *C*, 1 µm, *B*, *D* 2 µm.

Table 2. – Measurements of the infective third-stage larvae of *Anguillicola crassus* (all measurements are given in µm).

	Mean ± standard deviation	Range	Number of specimens studied
Length	738 ± 82.6	577-967	27
Width	35 ± 4.4	29-47	27
Oesophagus	224 ± 25.1	166-264	12
Nerve ring	92 ± 16	67-112	13
Length of genital primordium	26 ± 3.6	20-31	10
Tail	72 ± 6.9	59-84	14

stock; infested copepods were introduced into the oesophagus of eels.

Light microscopy

Light miscrocopy observations were carried out on live or fixed nematodes. The live nematodes were kept in saline (1% NaCl) at a temperature of 4°C prior to examinations. Worms were fixed in either 70% ethanol or 10% formaldehyde and, if necessary, cleared in lactophenol. *En face* views were mainly prepared according to Anderson's (1958) method. Drawings were made with the aid of the Leitz microscope drawing apparatus. Tissue sections of parasites were made with and LKB microtome after fixation, post-fixation, dehydration, infiltration and embedding in

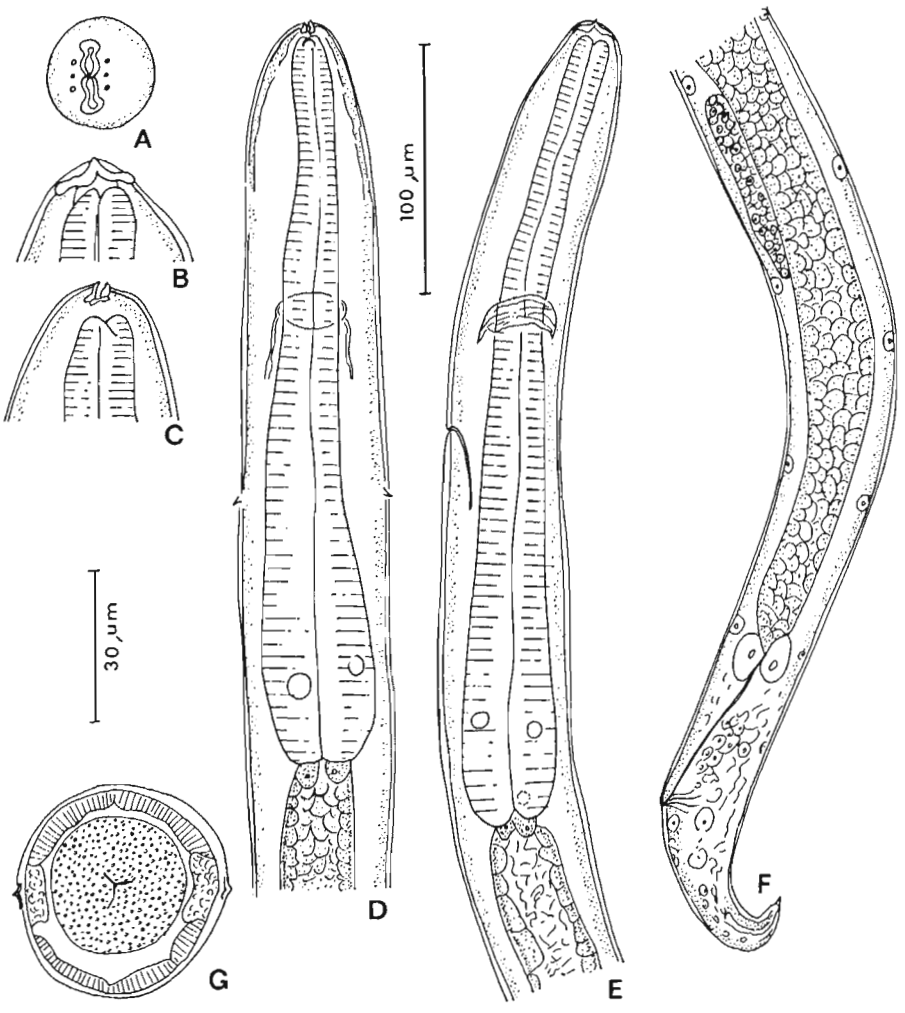

Figure 4. – *Anguillicola crassus*. Third-stage larvae from the definitive host. *A*: *en face* view. *B*: anterior end, lateral view. *C*: anterior end, median view. *D*: anterior part, median view. *E*: anterior part, lateral view. *F*: posterior part, lateral view. *G*: transverse section at the level of oesophagus. Scale-bar: *A*, *B*, *C*, *G*, 30 µm; *D*, *E*, *F*, 100 µm.

Table 3. – Growth of third-stage larvae of *Anguillicola crassus* in eels (all measurements are given in µm)

No.	1	2	3	4	5	6	7	8	9	10
Length	815	852	963	991	1027	1074	1120	1148	1200	1278
Width	32.6	34.7	37.9	40.8	41.6	38.9	39.6	70	60	69.4
Oesophagus	261	259	284	296	262	282	282	310	310	294
Nerve ring	114	83.9	109	102	97.9	125	137	126	120	135
Excretory pore	75	160	165	174	170	172	170	186	175	193
Deirids	177	163	168	202	183	172	199	202	195	226
Length of genital primordium	9.3	19	27	35	34	37	36	32	80	44
Tail	82	70	86.2	86	89	81	84	123	80	119

Spurr's low viscosity medium. The semithin sections (1 µm) were stained according to Richardson, Jarret and Finke (1960). Parasites were also fixed in Bouin's fluid, embedded in paraffin wax and sectioned at 5 µm thickness. These were stained with May-Grünwald-Giemsa, and examined for eel erythrocytes.

Scanning electron microscopy

Parasites were fixed in 2% glutaraldehyde in 0.2 M Sörensen buffer (pH = 7.2) for 20 min at about 20°C. The samples were rinsed twice in the buffer and then post-fixed in 1% buffered OsO_4 solution, dehydrated

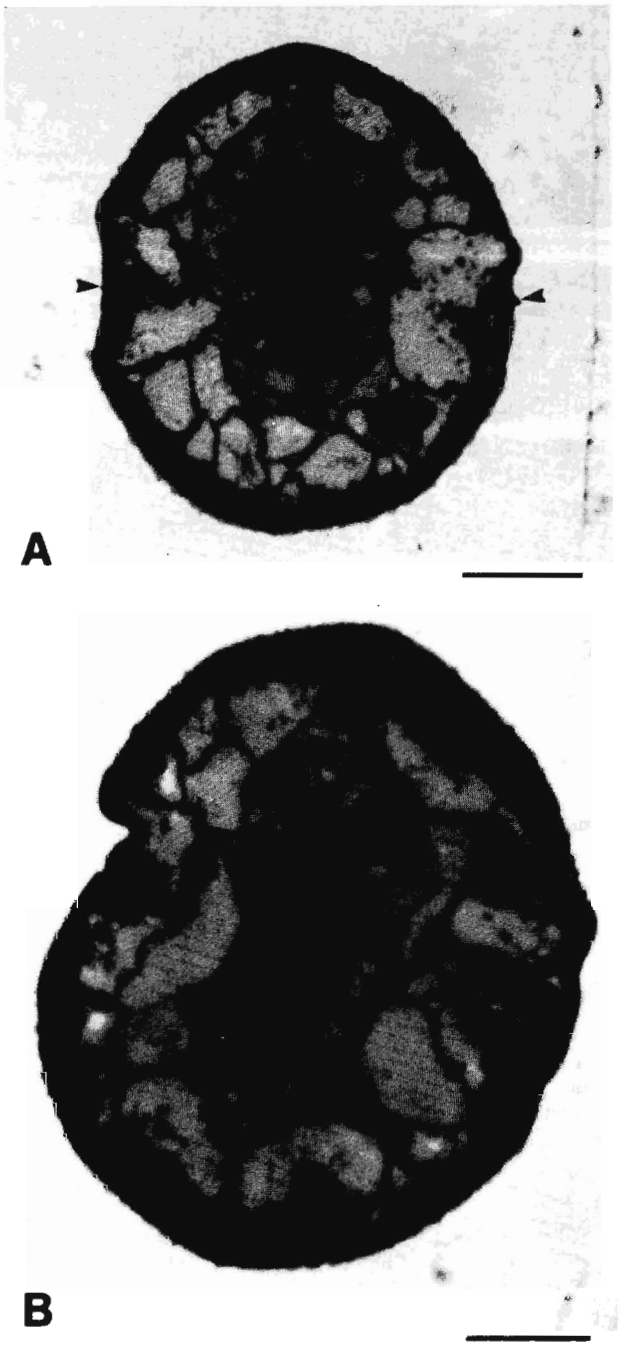

Figure 5. *Anguillicola crassus*. Transverse sections; light micrographs. *A*: third-stage larva in eel; V-shaped sclerotized support of the lateral alae (left and right arrows). *B*: fourth-stage larva. Scale bar: 10 µm.

Table 4. – Growth of fourth-stage larvae of *Anguillicola crassus* in eels (all measurements are given in micrometre)

No.	1	2	3	4	5	6	7	8	9	10	11	12
Length	1111	1204	1318	1333	1520	1676	1759	1815	2158	2175 male	2287 male	2656 female
Width	58.2	67	93	70	76	90	116	132	134	150	130	160
Oesophagus	273	340	285	338	343	365	302	454	393	425	491	445
Nerve ring	116	123	109	129	125	143	151	162	172	175	190	172
Excretory pore	168	172	186	194	204	264	261	267	275	300	338	335
Length of genital primordium	38	65	102	81	79	130	80	399	148	210	583	–
Tail	93	116	92	109	95.5	79	128	112	88	135	107	148

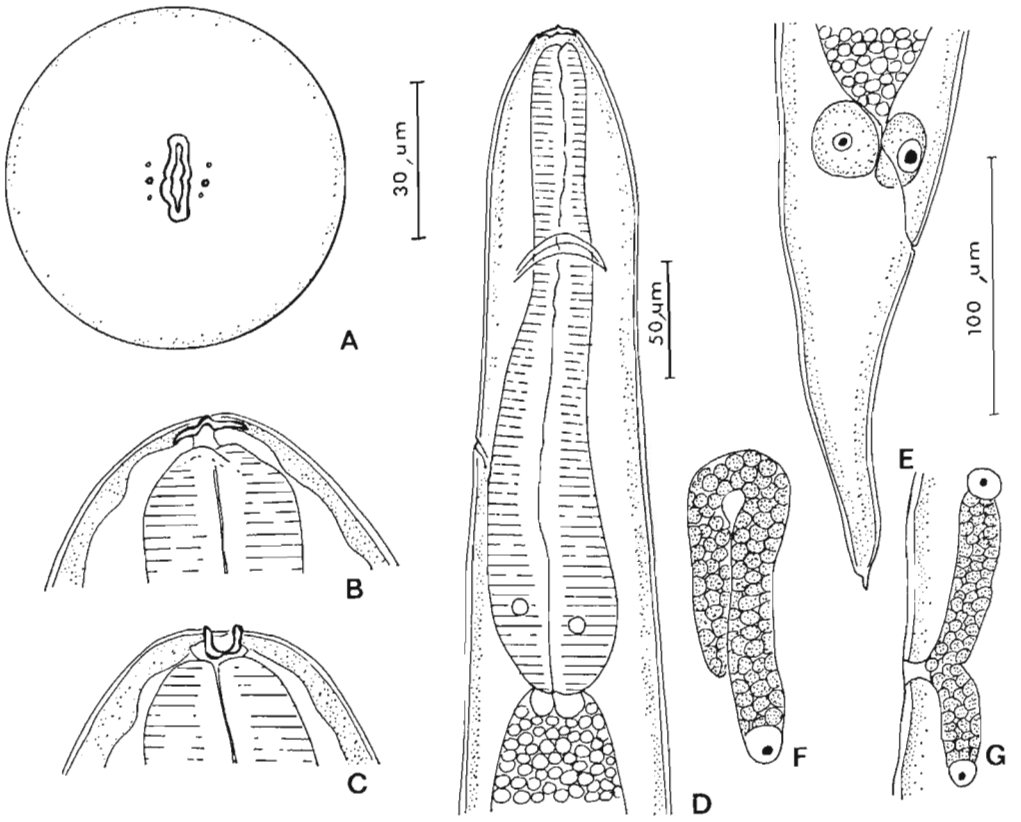

Figure 6 – *Anguillicola crassus*. Young fourth-stage larvae. *A*: *en face* view. *B*: anterior end, lateral view. *C*: anterior end, median view. *D*: anterior part, lateral view. *E*: posterior part, lateral view. *F*: male genital primordium. *G*: female genital primordium. Scale-bar: *A*, *B*, *C*, 30 µm; *D*, 50 µm; *E*, *F*, *G*, 100 µm.

through graded ethanol and critical-point dried using liquid CO_2. The material was examined under a JEOL electron microscope. All measurements are given in micrometre unless otherwise stated. The measurements given in *tables* 1, 2 and 3 were made on live worms. In *table* 4, the measurements were made on live material for parasites n° 1 to 8 and on fixed and cleared material for parasites n° 9 to 12; it should be noted that these fixatives may cause length changes which must be taken into account (*see* Fagerholm, 1979 and Fagerholm and Lövdahl, 1984). SEM measurements were not related.

RESULTS

The description of the early larval stages includes data from Petter *et al.* (1989) with additional information.

The egg (*fig.* 1, *A* and 10, *B*)

The mature egg (*fig.* 1, *A*) has a thin shell and under the SEM its surface appears to be covered with small nodules (*fig.* 10, *B*). Their dimensions are 90-100 by 75-90 and contain second-stage larvae surrounded by the first stage cuticle.

Second-stage larvae

Free larvae (fig. 1, B)

Free second-stage larvae (*fig.* 1, *B*) retain the first-stage cuticle which forms a loosely fitted sheath around the body except in the posterior part where it forms about fifteen folds. The conical anterior end bears a larval tooth and the inside wall of the oesophagus anterior end is sclerotized for about 5. In the same region small refringent granulations are present. The oesophagus is bulbously inflated at its posterior end and the outlines of the digestive tract can be discerned with difficulty. An area of abundant refringent granulation can be seen at bout 100 from the anterior end. The tail is long and slender.

Major dimensions (10 specimens studied; mean ± standard deviation; range in parentheses): length (µm) 272.2 ± 16.27 (243-290); width (µm) 17.6 ± 0.97 (16.4-19.4).

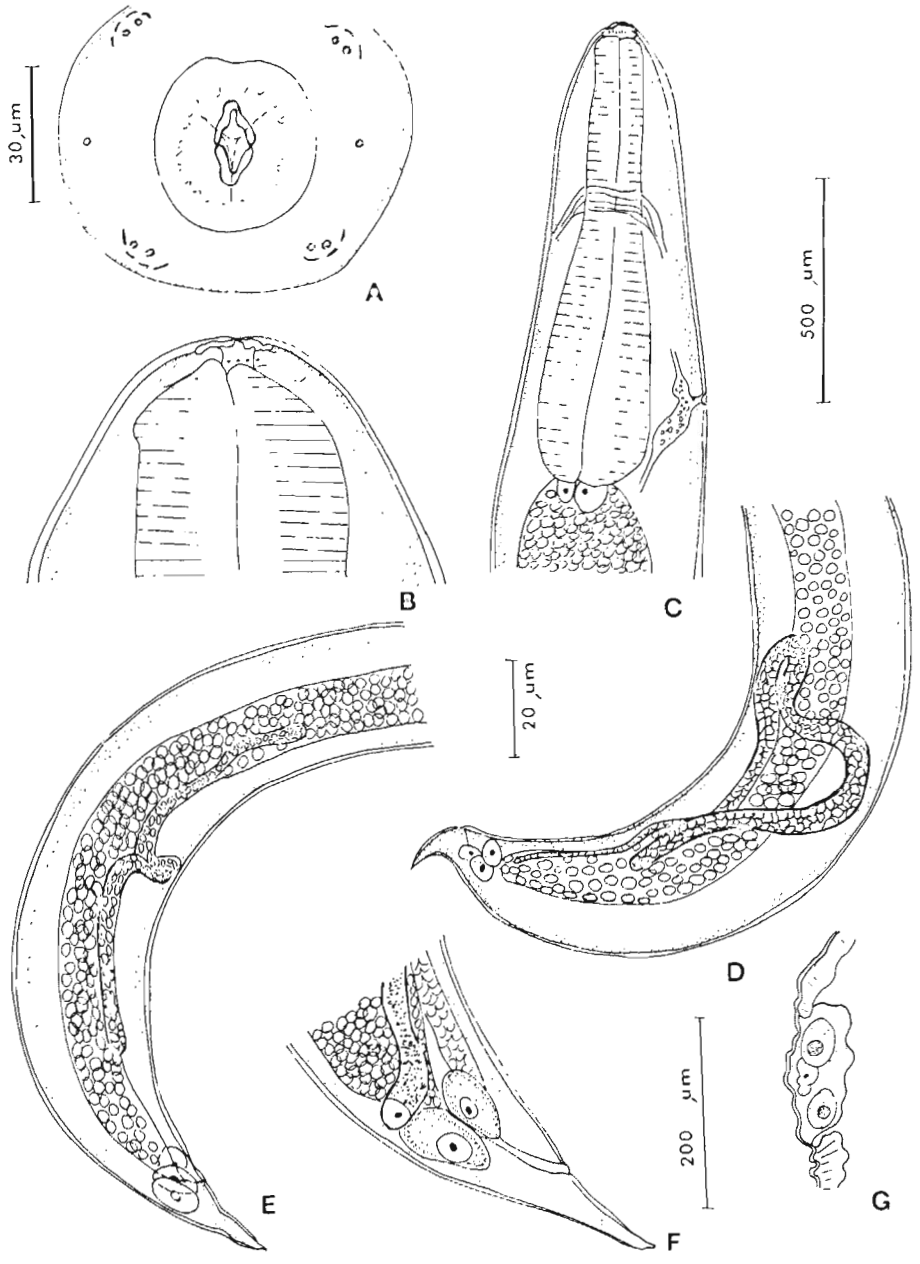

Figure 7. – *Anguillicola crassus*. Old fourth-stage larvae. *A*: *en face* view. *B*: anterior end, lateral view. *C*: anterior part, lateral view. *D*: male, posterior part. *E*: female, posterior part. *F*: male, posterior end, lateral view. *G*: transverse section of the lateral chord at the level of the oesophagus. Scale-bar: *A*, *G*, 30 µm; *B*, 20 µm; *C*, *F*, 200 µm; *D*, *E*, 500 µm.

Development of the second-stage larvae in the intermediate host (fig. 1, C-H; table 1)

In the haemocoel of the intermediate host, the second-stage larvae lose the first-stage cuticle and increase in size (*fig.* 1, *C*). The anterior end becomes globular and becomes distinct from the body by a small constriction (*fig.* 1, *D*). The larval tooth appears in the dorsal side at the base of the globular cephalic part (*fig.* 1, *E*, *F*). The mouth opens into a short sclerotized tube which is surrounded by the oesophagus in its posterior third (*fig.* 1, *E*). The oesophagus is clearly rhabditoid and the intestine is heavily lined with granulations. When the larvae are 650 long, three crescent-shaped refringent small pieces appear in the anterior third of the oesophagus and correspond to the oesophageal glands openings (*fig.* 1, *G*). Once larvae reach 690 long, the second moult begins firstly at the posterior end and then later at the apical end (*fig.* 1, *H*).

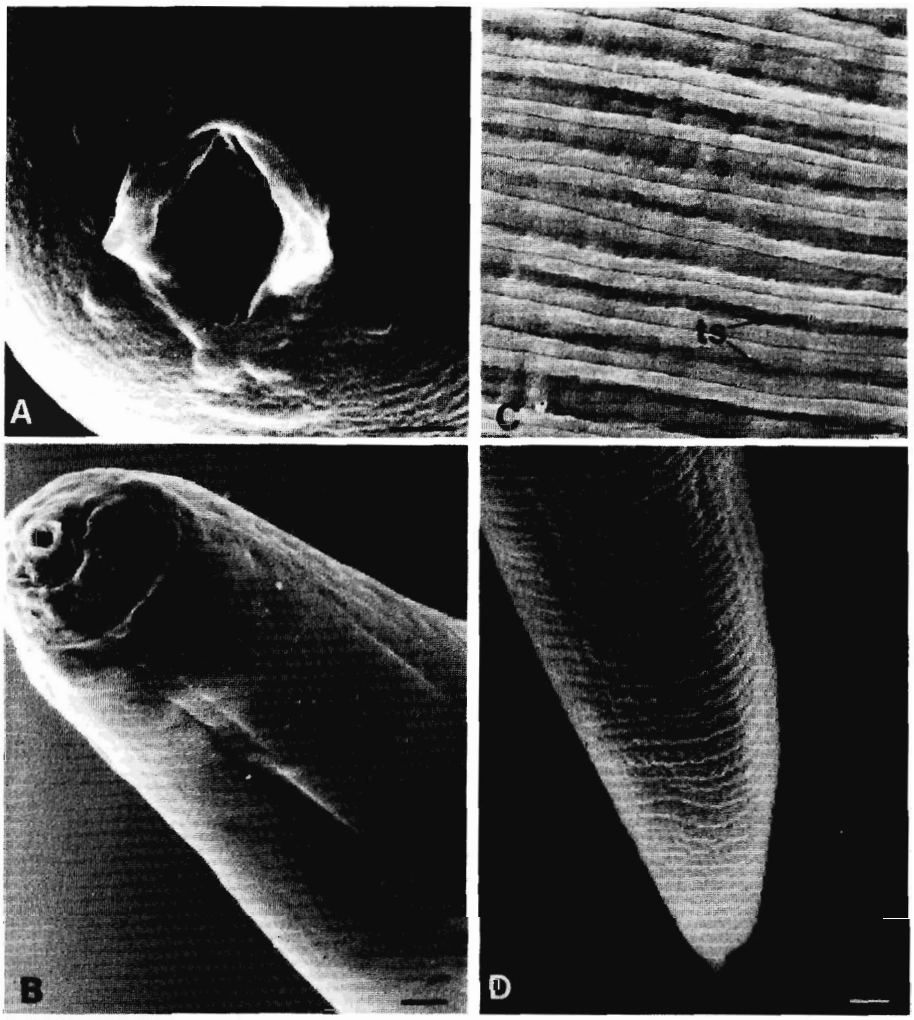

Figure 8. – *Anguillicola crassus*. Fourth-stage larvae; scanning electron micrographs. *A*: apical view: (see *fig.* 3). *B*: anterior extremity. *C*: cuticle (slightly behind the posterior end of the oesophagus): *ts*, transverse striae. *D*: posterior end. Scale-bar: *A*, *C*, *D*, 1 µm; *B*, 10 µm.

Third-stage larvae

Third-stage larvae from the intermediate host (fig. 2 and 3; table 2)

The infective third stage larvae, collected in the intermediate and paratenic hosts, have narrow lateral alae which begin at 10 from the anterior end and stop at 20 from the posterior end (*figs.* 2, *C* and 3, *B*, *C*, *D*). Tiny deirids which can hardly be seen with light microscopy are present at the level of the posterior third of the oesophagus (*fig.* 3, *C*). Examined with SEM, the cuticle appears transversely striated with longitudinal ridges present between transverse striations (*fig.* 3, *C*). The conical anterior end has a sclerotized ornamentation made up of two median U-shaped pieces joined laterally (*figs.* 2, *A*, *B*, *C* and 3, *A*, *B*). Four submedian papillae and two amphids can be observed in *en face* view (*figs.* 2, *A* and 3, *A*). The oesophagus has three anterior lobes and is composed of a short hyalin anterior part and a longer granular posterior part. Numerous granulations are present in the wall of the intestine, except in a small region near the rectum (*fig.* 2, *D*).

A small oval genital primordium is located in the posterior part of the body (*fig.* 2, *E*). The conical tail has a small terminal spike and small nodules at its end (*fig.* 3, *D*).

Third-stage larvae from the definitive host (fig. 4 and 5 A; table 3)

In the eels, it is difficult to distinguish between third- and fourth-stages because the third moult has never been observed and there are no obvious characters differentiating the two stages; the shape of the body and proportions change allometrically in conjunction with larval growth. However, observations

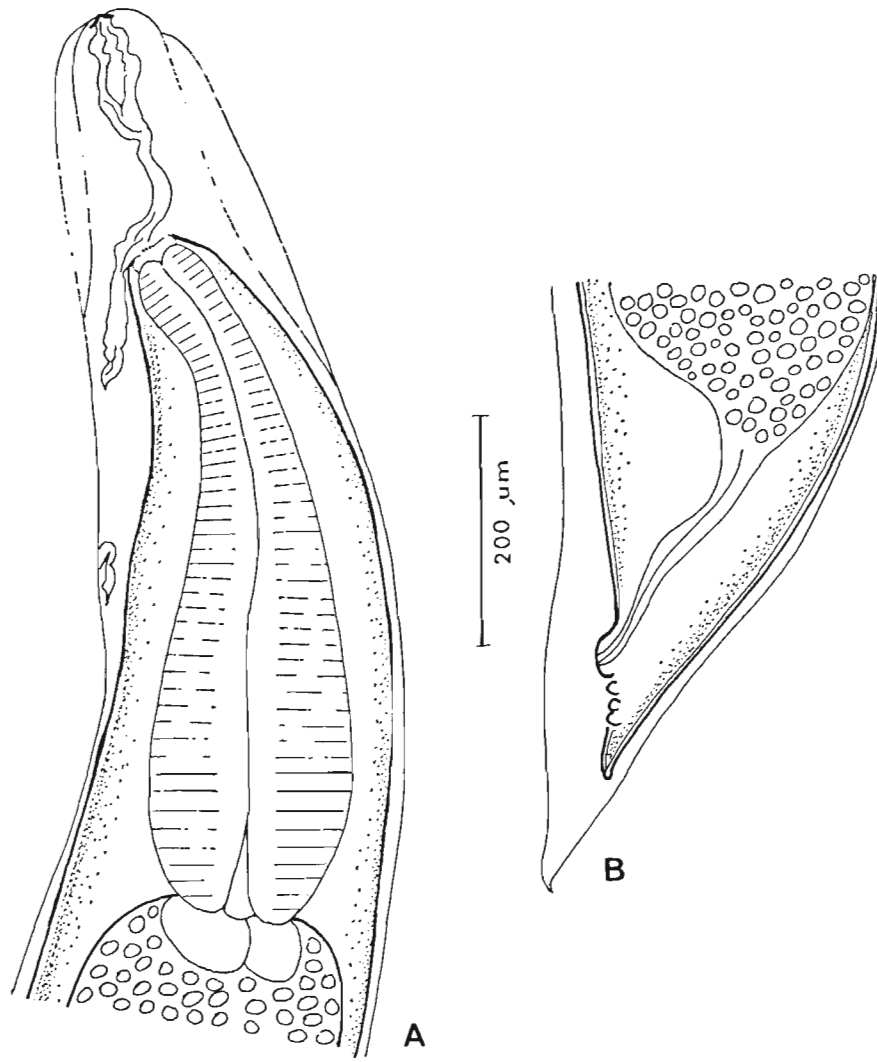

Figure 9. – *Anguillicola crassus*. Adults in the fourth-stage cuticle. *A*: anterior part. *B*: posterior part.

using the SEM indicate that neither deirids nor lateral alae can be seen beyond a certain size. In addition, the longitudinal crests of the cuticle disappear. These differences are therefore considered here as stage-specific. The observations of deirids in 1 600 but not in 1 200 long larvae lead us to believe that the third moult takes place when the size is between these two lengths.

As the third-stage larva grows, its body thickens and its apical end becomes rounded; it exhibits the same sclerotized ornamentation and papillae arrangement as the infective third-stage larva (*fig.* 4, *A*, *B*, *C*). The lateral alae becomes narrower, being hardly visible in cross-section by LM, but can be seen by their V-shaped sclerotized support (*figs.* 4, *G* and 5, *A*). The deirids have the same appearance as the infecting third-stage larvae. Lastly the genital primordium lengthens (*fig.* 4, *F*). Histological examinations were conducted on 11 third-stage larvae, none of which had erythrocytes in their digestive tract.

Fourth-stage larvae (*fig.* 5, *B*;, 6, 7, 8, 9; *table* 4)

The fourth-stage larvae appear thicker than the third-stage larvae. The anterior end flattens out and the posterior end curves ventrally. The same third-stage sclerotized ornamentation is present at the apical end. Given the flatness of the anterior end, the branches of the two pieces look aligned in lateral view (*fig.* 6, *B*). The cephalic papillae have the same pattern as the third-stage larvae (*figs.* 6, *A* and 8, *A*). In the largest larvae, the adult circumoral teeth appear under the larval buccal opening (*fig.* 7, *A*). Deirids and lateral alae are not present and there is no trace left of the V-shaped sclerotized support (*figs.* 5, *B;* 7, *G* and 8, *B*). The buccal cavity is devoid of a slerotized wall (*fig.* 6, *B*, *C*). The cuticle is transversely striated but longitudinal crests are lacking, except on the tail of young larvae (*fig.* 8, *C*). The oesophagus is shorter in relation to the body length. Lastly, as the larvae grow, the excretory pore more

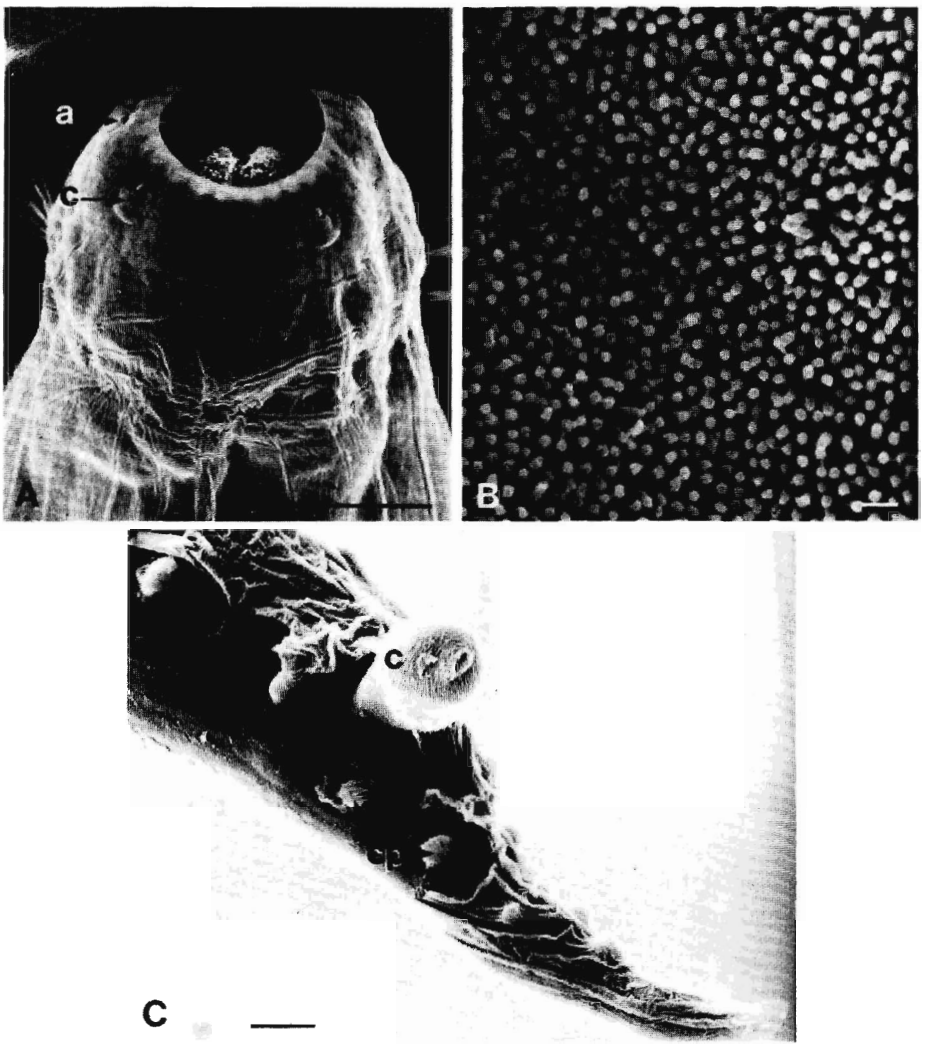

Figure 10. – *Anguillicola crassus*. Adults and egg; scanning electron micrographs. *A*: anterior extremity: *a*, amphid; *c*, cephalic papilla. *B*: egg shell. *C*: male tail: *c*, cloaca; *cp*, caudal papillae. Scale-bar: *A*, *C*, 10 µm; *B*, 0.1 µm.

closely approaches the posterior end of the oesophagus (*figs.* 6, *D* and 7, *C*). The tail, as in the third-stage larvae, has a terminal spike but the nodules covering the posterior end tend to disappear (*fig.* 8, *D*). The tail length is clearly shorter in relation to body length as the larval size increases.

In the male larvae, the genital primordium is elongate and recurved at its anterior end (*fig.* 6, *F*). In the largest larvae, the genital tract reaches as far as the rectum (*fig.* 7, *D* and *F*).

In the female larvae, the anlage of the vulva can be seen under the cuticle and opens out into a short tube leading to the middle of the genital primordium (*figs.* 6, *G* and 7, *E*). The last moult was observed in 1.9 to 3.9 mm length larvae (*fig* 9, *A* and *B*). Lastly, the absence of erythrocytes in the intestine of five specimens was established by histological examinations.

Adults (*fig.* 10)

Immature and mature adults have been described by several authors (Kuwahara *et al.*, 1974; Moravec and Taraschewski, 1988). They differ from the fourth-stage larvae by their well-sclerotized buccal capsule provided anteriorly with one row of 17 to 22 relatively large circumoral teeth and by the absence of the U-shaped sclerotized ornamentation (*fig.* 10, *A*). In the female the vulva is open, and in the male 5 to 6 pairs of caudal papillae are present and the cloaca is prominent (*fig.* 10, *C*).

DISCUSSION-CONCLUSION

As far as we are aware, no detailed description of *Anguillicola crassus* larvae in the eel has already been

published. Boon *et al.* (1990) have defined the different larval stages and have distinguished the third-stage from the fourth-stage larvae by the presence of erythrocytes in the intestine of the fourth-stage larvae. These observations has also been made by Haenen *et al.* (1989) and De Charleroy *et al.* (1990). However, in this study, erythrocytes in the fourth-stage larvae were not observed and the dark coloration of their intestinal tract was due to the presence of brown granulations in the enterocytes.

As found in the present study, the morphological characters that can be used to distinguish the third and fourth stages are the presence or absence of deirids, the presence or absence of lateral alae and the structure of the cuticle. However, these characteristics can only be observed clearly by using SEM. Furthermore, deirids and lateral alae may gradually be reduced in size as a result of larval growth without the occurrence of moulting. In order to be certain, the observation of the third moulting is required.

The second, third and fourth larval stages of *Anguillicola crassus* are distinguishable by morphological features from both one another and the larvae of other nematodes parasitizing copepods or eels and other fishes acting as paratenic hosts, thus enabling epidemiological studies to be carried out at these stages.

Acknowledgements

The authors are grateful to Professor M. Marjolet and Mrs P. Avranche for providing scanning electron microscopy facilities and their skillfull assistance in this field. This work was supported by an operating grant from the FDAAPP.

REFERENCES

Anderson R. C., 1958. Méthode pour l'examen des Nématodes en vue apicale. *Ann. Parasitol. Hum. Comp.*, Notes et Informations, **33**, 171-172.

Bonneau S., G. Blanc, A. J. Petter, 1991. Étude sur la biologie des premiers stades larvaires d'*Anguillicola crassus* (Nematoda, Dracunculoidea): spécificité de l'hôte intermédiaire et influence de la température sur la durée du développement. *Bull. Fr. Pêche Piscic.*, **320**, 1-6.

Boon J. H., H. Augustin, V. M. H. Cannaerts, C. J. A. Lokin, M. A. M. Machiels, F. Ollevier, 1990. The suitability of experimental inoculations with infective larvae of *Anguillicola crassus* and their effects on the growth and mortality of the European Eel (*Anguilla anguilla*). *Aquaculture*, **87**, 111-120.

Decharleroy D., L. Grisez, K. Thomas, C. Belpaire, F. Ollevier, 1990. The life cycle of *Anguillicola crassus*. *Dis. Aquat. Org.*, **8**, 77-84.

Fagerholm H.-P., 1979. Nematode Length and Preservatives, with a Method for determining the length of live specimens. *J. Parasitol.*, **65**, 334-335.

Fagerholm H.-P., M. Lovdahl, 1984. Induced morphometric variation in the preparation of nematode parasites for the LM and SEM. *Syst. Parasitol.*, **6**, 245-247.

Haenen O. L. M., L. Grisez, D. Decharleroy, C. Belpaire, F. Ollevier, 1989. Experimentally induced infections of European eel *Anguilla anguilla* with *Anguillicola crassus* (Nematoda, Dracunculoida) and subsequent migration of larvae. *Dis. Aquat. Org.*, **7**, 97-101.

Kennedy C. R., D. J. Fitch, 1990. Colonization, larval survival and epidemiology of the nematode *Anguillicola crassus*, parasitic in the eel, *Anguilla anguilla*, in Britain. *J. Fish Biol.*, **36**, 117-131.

Kuwahara A., A. Niimi, H. Itagaki, 1974. Studies on a Nematode Parasitic in the Air Bladder of the Eel I. Descriptions of *Anguillicola crassa* n. sp. (Philometridea, Anguillicolidae). *Jpn. J. Parasitol.*, **23**, 275-279.

Moravec F., H. Taraschewski, 1988. Revision of the genus *Anguillicola* Yamaguti, 1935 (Nematoda: Anguillicolidae) of the swimbladder of eels, including descriptions of two new species, *A. novaezelandiae* sp. n. and *A. papernai* s. n. *Folia Parasitol.*, **35**, 125-146.

Petter A. J., J. Casone, N. Le Belle, 1990. Observations sur la biologie des premiers stades larvaires d'*Anguillicola crassus*, nématode parasite d'Anguille. *Ann. Parasitol. Hum. Comp.*, **65**, 28-31.

Petter A. J., Y. A. Fontaine, N. Le Belle, 1989. Étude du développement larvaire de *Anguillicola crassus* (Dracunculoidea, Nematoda) chez un Cyclopidae de la région parisienne. *Ann. Parasitol. Hum. Comp.*, **64**, 347-355.

Richardson K. C., L. Jarret, E. M. Finke, 1960. Embedding in epoxy resin of ultrathin selectioning in electron microscopy. *Stain Technol.*, **35**, 313-323.