Proc. Helminthol. Soc. Wash.
52(1), 1985, pp. 98–113

Morphogenesis of Developmental Stages of *Dirofilaria immitis* (Nematoda) in the Dog

J. R. LICHTENFELS,[1] P. A. PILITT,[1] T. KOTANI,[2,3] AND K. G. POWERS[2,4]

[1] Biosystematic Parasitology Laboratory, Animal Parasitology Institute, Agricultural Research Service, USDA, Beltsville, Maryland 20705 and

[2] Bureau of Veterinary Medicine, Food and Drug Administration, Beltsville Agricultural Research Center, Beltsville, Maryland 20705

ABSTRACT: Morphogenesis of the dog heartworm, *Dirofilaria immitis,* through the third and fourth molts to the fifth stage is described. Specimens were collected from mosquitoes 14 or 15 days after infection (DAI) or from dogs 3–79 DAI. Infective larvae from mosquitoes were 0.7–1.0 mm long with a tapered blunt anterior end, bilayered cuticle, refractile granules in the anterior ⅔ of the glandular esophagus, and a pair of small, broadly based, subventral bumps near the larger conical tail tip. Sexes were distinguished by the position of the oval-shaped genital primordium (GP) (near the anterior part of the glandular esophagus in females and just anterior to midbody in males), and by the presence of spicular primordia in males. When larvae were first collected from dogs 3 DAI most had completed the third molt. Fourth-stage larvae (L-4) at 3–6 DAI were 1.0–1.5 mm long, had untapered anterior ends, and had sharply defined, angular submedian papillae near a button-like tail tip. Posterior growth of the male GP was seen first in L-4's 9 DAI and it reached the rectum 30 DAI. Genital papillae and developing spicules of the fifth stage were evident 41 DAI. Formation of the vagina was initiated 9 DAI by hypodermal invasion of the GP at its attachment point. By 12 DAI the female GP was enlarged and swollen posteriorly with two large nuclei near the posterior tip. By 21 DAI two branches of the GP developed, each with a large nucleus at its posterior tip; by 30 DAI each branch extended beyond the junction of the esophagus and intestine (E–I). The vulva was located near but anterior to the E–I 41–58 DAI. The fourth molt occurred 50–58 DAI. Some specimens of both sexes completed ecdysis by 58 DAI. Early fifth-stage specimens at 58 DAI were 12–14.8 mm long with a thick, finely striated cuticle. Spicules were almost completely sclerotized and as large as in mature males by 79 DAI. Spermatozoa were present in the testis but not in the vas deferens 79 DAI.

The development of the dog heartworm, *Dirofilaria immitis,* in the dog has been described by Kume and Itagaki (1955), Orihel (1961), and Kotani and Powers (1982). The only description of the morphogenesis of *D. immitis* in the dog was by Orihel (1961). Because of the high cost of dogs for research and humane considerations much current research on *D. immitis* is carried out in model animal systems or in vitro rather than in dogs. Studies by Taylor (1960), Yoeli et al. (1964), Sawyer (1965), Sawyer and Weinstein (1965), Wong et al. (1982), and Lok et al. (1984) in vitro; and by P. Supakorndej, J. J. Jun, and J. W. McCall (pers. comm.) in a model animal system all have reported developmental differences from that described by Orihel (1961) in the dog, especially in the time of the third molt. The objective of this study was to redescribe the morphogenesis of *D. immitis* in its normal definitive host, the dog. The results should be useful for evaluating the development of the nematode in vitro and in model animal systems.

Materials and Methods

Specimens

Specimens of *Dirofilaria immitis* for study were collected in a previous project on development in dogs reported by Kotani and Powers (1982). Nematodes were dissected from two beagle dogs 3, 6, 9, 12, 15, 21, 30, 41, 50, 70, and 79 days after infection (DAI) as described previously (Kotani and Powers, 1982). Some specimens collected by Kotani and Powers (1982) were used in other studies and were unavailable for this study. All available specimens 3–15 DAI were studied. Fewer than the total number available from 21 to 79 DAI were studied. The numbers of specimens studied (*N*) are given with the Results. Kotani and Powers (1982) also collected specimens from 84–196 DAI, but those specimens were not part of this study.

Infective larvae (third stage) were allowed to emerge from the mouthparts of cold-inactivated mosquitoes, *Aedes aegypti* (Liverpool strain), into Hanks' balanced salt solution 14 or 15 DAI (Kotani and Powers, 1982). Additional specimens were collected for study from *A. aegypti* (Liverpool selected strain) at 12 DAI (Lok et al., 1984) and from in vitro cultures (Wong et al., 1982;

[3] Present address: Department of Veterinary Pathology, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 591, Japan.

[4] Present address: National Cancer Institute, National Institutes of Health, Westwood Building, Bethesda, Maryland 20205.

Table 1. Measurements* of *Dirofilaria immitis*: infective larvae from mosquitoes; third molt from in vitro cultures; and third, fourth, and early fifth stages from dogs by stage of development and days after infection.

Stage of development (age of infection)	Total length (mm)	Diameter at level of			Distance from anterior extremity to			Length of			
		Anterior extremity	Base of esophagus	Anus	Nerve ring	Genital primordium			Reproductive system		
						Male	Female	Esophagus	Male	Female	Tail
Infective third (14 or 15 DAI)	0.74–1.04	8–13	22–49	17–22	74–100	336–528	112–216	256–400	14–22	11–23	29–42
Late third (6 DAI)†	0.83	9	–	15	69	–	–	–	–	–	28
Third molt (2 or 3 days)	0.72–1.17	10–20	19–38	14–28	71–95	365–496	144–200	256–400	16–20	15–20	32–46
Early fourth (3 DAI)	0.98–1.30	14–20	21–30	18–23	79–100	352–592	144–288	272–448	20–28	15–42	34–46
(6 DAI)	1.14–1.56	15–22	15–36	16–28	82–121	464–640	200–224	256–560	19–23	20–38	29–52
Middle fourth (9 DAI)	1.33–1.66	17–27	25–35	20–29	72–110	448–688	200–240	256–432	22–105	27–43	35–47
(12 DAI)	1.33–1.84	19–25	24–38	19–28	83–101	416–784	192–236	288–448	36–275	25–52	35–52
(15 DAI)	1.69–2.21	19–31	29–38	21–31	89–113	672–864	196–256	304–448	273–496	41–55	37–54
(21 DAI)	1.32–3.24	28–40	37–50	24–37	88–120	704–1,490	224–288	272–495	1.09–1.64 mm	74–129	37–57
(30 DAI)	3.12–5.63	34–58	41–74	29–52	99–131	1.37–2.69 mm	265–368	320–496	1.38–2.30 mm	239–425	41–68
Late fourth (41 DAI)	5.63–9.20	39–84	60–98	36–79	110–137	2.24–3.27 mm	300–432	368–560	2.90–6.96 mm	1.01–2.46 mm	41–74
(50 DAI)	8.65–12.8	50–84	90–138	48–92	125–147	1.75–2.24 mm	424–520	448–624	7.20–9.20 mm	4.08–7.19 mm	60–82
(58 DAI)	8.71–12.8	60–68	103–126	42–84	128–152	0.73–1.04 mm	453–528	435–608	8.48–8.77 mm§	6.24–7.36 mm	58–92
Fourth molt (50 DAI)‡	8.29–10.4	58–74	85–119	63–84	126–157	1.27–2.35 mm	–	480–560	6.77–8.35 mm	–	63–79
(58 DAI)	8.89–13.2	46–89	90–145	52–87	120–140	0.85–1.94 mm	454–665	540–665	7.93–9.26 mm	8.13–11.1 mm	63–84
Early fifth (58 DAI)	11.4–14.8	59–89	115–144	58–84	126–152	2.84–4.42 mm	448–588	540–797	8.47–10.8 mm	8.93–10.2 mm‖	71–89
(70 DAI)	19.2–29.4	59–96	147–229	76–102	128–168	7.92–11.8 mm	496–693	666–896	12.0–14.6 mm	12.7–20.6 mm	66–92
(79 DAI)	34.5–47.1	82–114	178–256	85–108	166–220	18.6–22.5 mm	726–1,400	787–975	20.8–24.6 mm	20.3–28.8 mm	80–145

* Measurements in micrometers unless noted otherwise.
† One specimen.
‡ Males only.
§ Two males measured.
‖ Four females measured.

Lok et al., 1984). Terminology for the phases of development follows that used by Douvres et al. (1969) for *Ascaris suum*. At least 10 specimens of each sex were measured for larvae collected 0–41 DAI. Five specimens of each sex were measured for specimens collected 50–79 DAI. Additional morphometrics of specimens from this study were reported by Kotani and Powers (1982). Voucher specimens have been deposited in the USNM Helminthological Collection, USDA, Beltsville, Maryland 20705 (Nos. 78343–78365).

Fixing and clearing

Infective larvae were studied alive after heat inactivation at 37°C for 1 hr on a slidewarming table; the remaining specimens were fixed in hot (60°C) 5% neutral buffered formalin; hot 70% ethanol-glycerine (20:1), or cold (4°C) 3% glutaraldehyde buffered with 0.2 M potassium phosphate at pH 6.8. Specimens for light microscopy were cleared for study in tinted glycerine (<0.01% cottonblue). Specimens studied by electron microscopy were postfixed 2 hr in 2% buffered osmium tetroxide, dehydrated in ethanol, and critical-point-dried in liquid CO_2 (Humphreys, 1975).

Microscopy

Most specimens were studied and photographed as whole mounts in glycerine with an oil immersion lens and interference-contrast microscopy. SEM specimens were attached with adhesive to a stub, coated with gold/palladium, and viewed at 5–20 kV (Madden and Tromba, 1976). TEM specimens were stained with uranyl acetate and lead citrate and viewed at 60 kV with a 20-μm aperture (Endo and Wergen, 1973; Wergin and Endo, 1976).

Results

All available *Dirofilaria immitis* specimens (80) recovered from the two dogs necropsied 3 DAI had completed the third molt. Among the 84 available specimens collected at 6 DAI was a single specimen in the third stage; all others were in the fourth stage through 41 DAI. Some specimens collected at 50 and most at 58 DAI were in the fourth molt; and some specimens of both sexes ecdysed to fifth stage by 58 DAI. By 70 DAI all specimens were fifth stage. Sexes could be distinguished in infective larvae by the position of the genital primordia (GP) and by the presence of spicular primordia in males. The morphogenesis of *D. immitis* will be described in seven phases: infective third stage, third molt, early fourth stage, mid-fourth stage, late fourth stage, fourth molt, and early fifth stage.

Infective third stage (Figs. 1-9) ($N = 73$)

Anterior fifth of infective *D. immitis* larvae (emerged from mouthparts of mosquitoes) tapers gradually (Fig. 1) so anterior extremity 50–70% of width at level of nerve ring. Fixed infective larvae 0.74–1.04 mm long. (Additional morphometrics listed in Table 1.) Tail tapers slightly to bluntly rounded posterior end that bears three prominences: conical tail tip and two smaller subventral bumps (Fig. 9). Buccal capsule consists of thin-walled narrow tube. Glandular esophagus filled with fine refractile granules except for posterior fifth; esophageal–intestinal (E–I) valve absent (Figs. 2, 3). Intestine narrower than glandular esophagus (Fig. 3), sometimes with prominent lumen and thin walls. Male GP oval-shaped, located ventrally just anterior to mid-body (Fig. 4); female GP attached ventrally by anterior end just posterior to junction of muscular and glandular esophagus (Fig. 2). In males, spicular primordia dorsolateral to rectum (Figs. 6, 7), absent in females (Fig. 8). Cuticle with transverse striations, thick and bilayered in some specimens (Fig. 3). Lateral chord nuclei large, oval-shaped, in two rows (one row in each of two hypodermal columns of each chord) (Fig. 5).

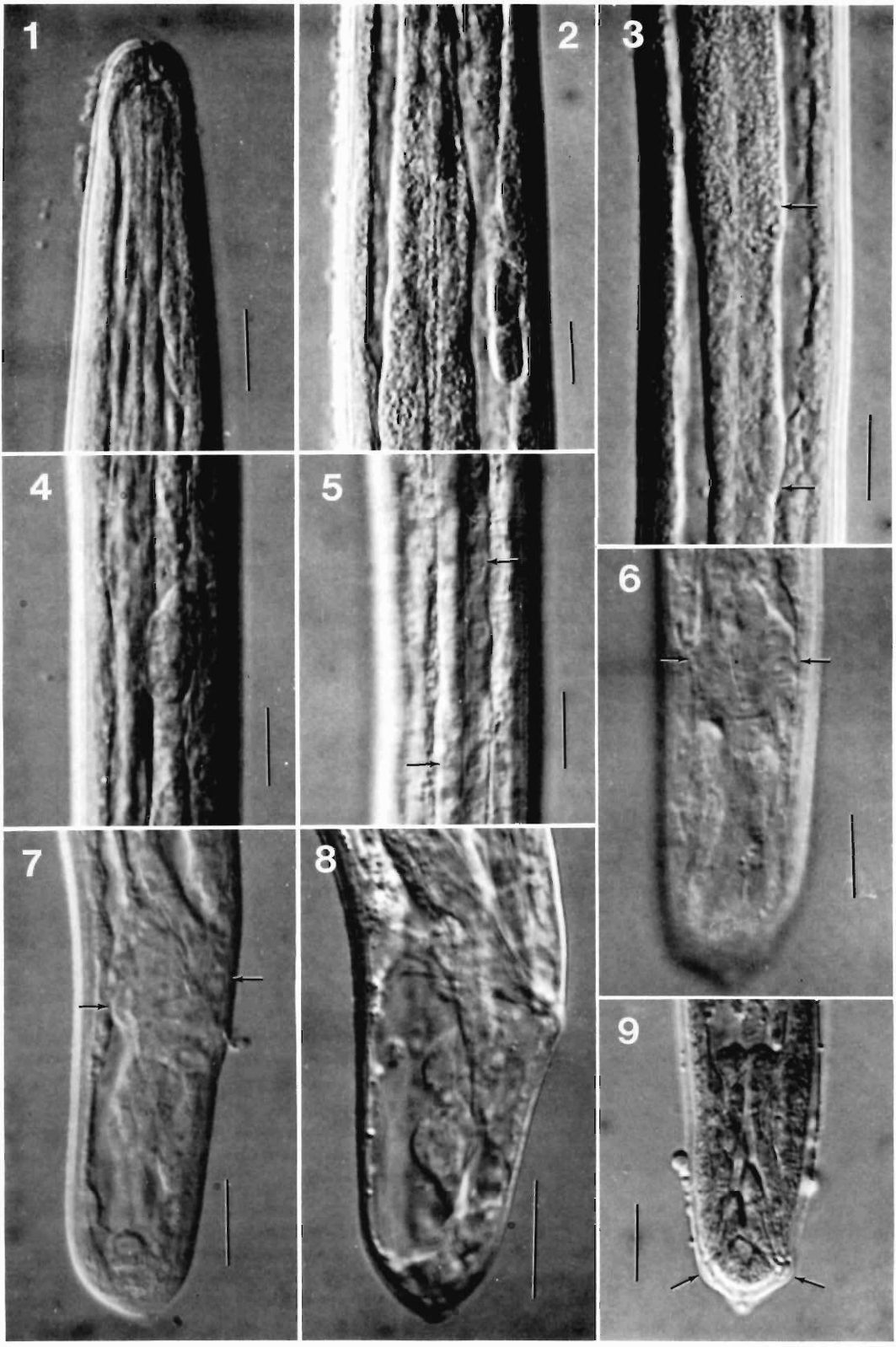
Third molt (Figs. 10-15) ($N = 34$)

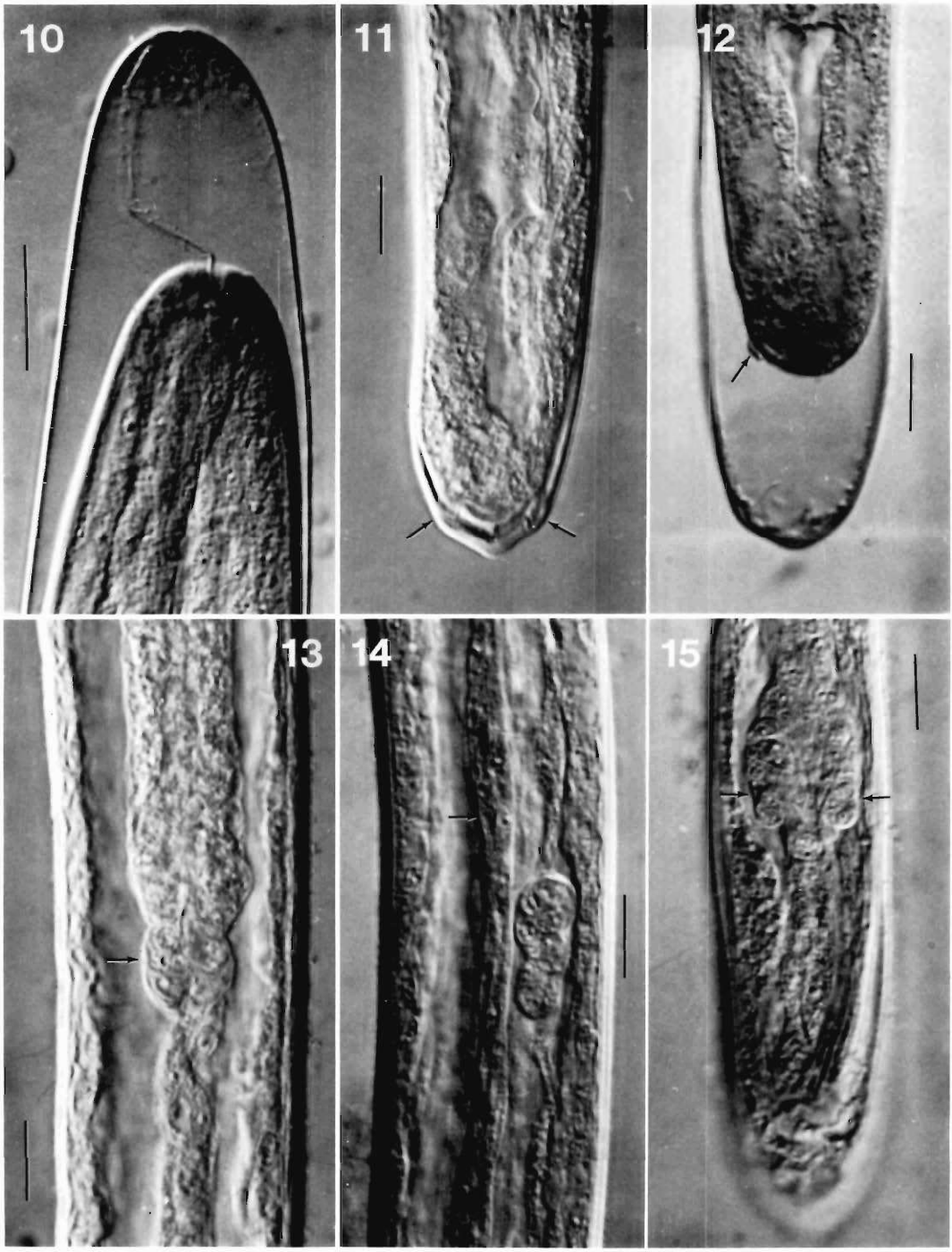
Only a single third-stage larva (collected 6 DAI) was among the *D. immitis* specimens collected from dogs; all others had completed the third molt to fourth stage by 3 DAI when the earliest collections were made. The following description is based on in vitro grown specimens (Wong et al., 1982) from cultures that were 2 and 3 days old. After 2 days in culture the cuticle of molting specimens was separated from the underlying cuticle on one or both ends (Figs. 10–12). After 3 days in culture some specimens had completed ecdysis.

Anterior end tapers only slightly (Fig. 10). Larvae (not including sheaths) 0.73–1.17 mm long. Tail of fourth stage beneath cuticle of third stage

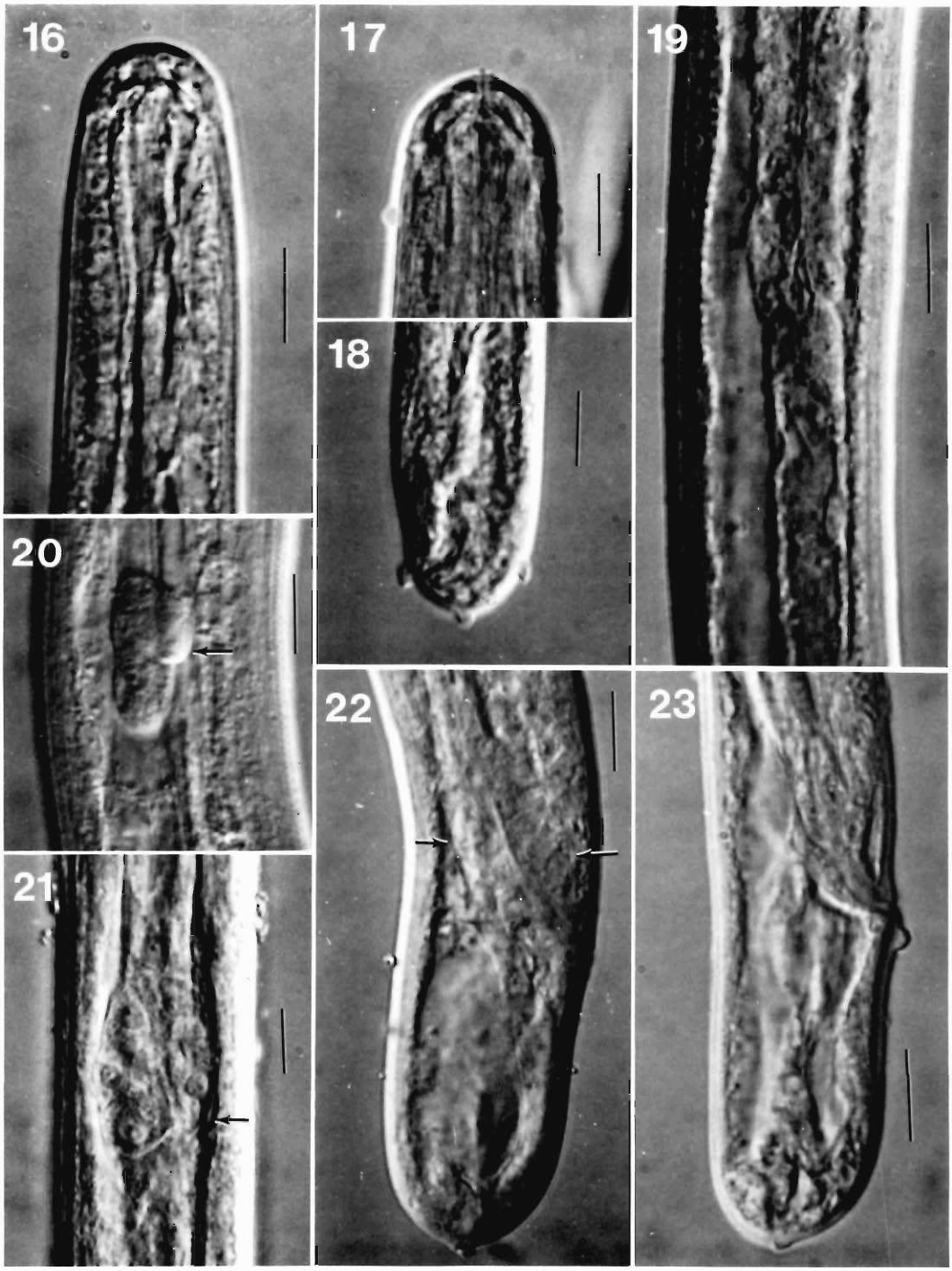
→

Figures 1-9. ***Dirofilaria immitis,*** **infective, third-stage larvae, from** ***Aedes aegypti*** **14 or 15 DAI. Scale bars 10 μm. 1. Anterior fifth of female. 2. Genital primordium of female. 3. Glandular esophagus with granules (arrow) and E-I junction (arrow). 4. Genital primordium of male. 5. Lateral chord showing oval-shaped nuclei (arrows). 6. Spicular primordia, dorsal view (arrows). 7. Spicular primordia, lateral view (arrows). 8. Female tail, lateral view. 9. Tail of male, ventral view (arrows at subventral bumps).**





Figures 10–15. ***Dirofilaria immitis,*** **third molt from in vitro cultures 2 or 3 days after inoculation. Scale bars 10 μm. 10. Anterior end with separated third-stage cuticle. 11, 12. Posterior ends showing submedian papillae (arrows) of fourth-stage larvae within cuticle of third stage. 13. Esophageal–intestinal junction showing valve (arrow). 14. Genital primordium of male with anterior end reflexed slightly, and intestine (arrow) without a lumen. 15. Spicular primordia (arrows), dorsal view.**



Figures 16–23. ***Dirofilaria immitis,*** **early fourth stage, from dogs. Scale bars 10 μm. 16. Anterior ⅙ of female, 3 DAI. 17. Anterior extremity of male, 6 DAI, showing buccal capsule. 18. Tail of female, ventral view, 3 DAI. 19. Esophageal-intestinal junction of female, 6 DAI. 20, 21. Male genital primordia, anterior end partially reflexed (arrow), and with anterior end completely reflexed (arrow), 6 DAI. 22. Tail of male, showing spicular primordia (arrows), 6 DAI. 23. Tail of female, 6 DAI.**

more bluntly rounded than in infective stage, with smaller tail button and larger well-defined submedian papillae (Figs. 11, 12). Buccal capsule still thin but wider posteriorly than anteriorly. Glandular esophagus still contains refractile granules; E–I valve present (Fig. 13). Intestine narrower than glandular esophagus, thick walled, lumen closed (Fig. 13). Male GP with anterior end reflexed (Fig. 14). Each spicular primordium consists of about eight cells (Fig. 15). Key morphological features for separating third- and fourth-stage larvae of *D. immitis* are given in Table 3.

Early fourth stage (Figs. 16–23) (*N* = 164: 80, 3 DAI; 84, 6 DAI)

At 3 and 6 DAI all (except one third-stage) specimens were in an early phase of fourth-stage development identical to *D. immitis* specimens having ecdysed a third-stage cuticle at 2 or 3 days in vitro. The following description is based on specimens collected from dogs 3 and 6 DAI.

Anterior body with almost parallel sides, untapered, with bluntly rounded anterior end (Fig. 16). Fixed larvae 0.98–1.30 mm long 3 DAI and 1.14–1.56 mm long 6 DAI. Tail bluntly rounded with three prominent projections—the button-like tail tip and the slightly larger angular submedian papillae (Fig. 18). Buccal capsule narrow, tubular, wider posteriorly; walls thicker (Fig. 17) than in third stage. Glandular esophagus without refractile granules; E–I valve present (Fig. 19). Intestine almost as wide as glandular esophagus, thin-walled with lumen open (Fig. 19). Male GP almost round (Figs. 20, 21) from anterior end reflexing to level of its posterior end. Spicular primordia evident as mass of cells around rectum of male (Fig. 22), absent in female (Fig. 23). Nuclei of lateral chords still oval-shaped with single row in each hypodermal column.

Mid-fourth stage (Figs. 24–35) (*N* = 815: 115, 9 DAI; 358, 12 DAI; 235, 15 DAI; 59, 21DAI; 48, 30 DAI)

Development from 9 through 30 DAI in *D. immitis* specimens collected from dogs is described as mid-fourth stage.

Shape of body 9 DAI uniformly cylindrical with bluntly rounded ends, but in some specimens anterior and posterior extremities slightly swollen or broader than rest of body. Swollen ends more common 12, 15, and 21 DAI and most prominent 30 DAI (Figs. 24–29). Fixed larvae 1.3–1.7 mm long 9 DAI to 3.1–5.6 mm long 30 DAI (Table 1). Tail papillae unchanged from early fourth stage but appear smaller in relation to larger body. Buccal capsule unchanged from earlier phase (Fig. 25). Glandular esophagus without refractile granules, about as wide as intestine. Male GP in shape of shepherd's crook 9 DAI due to posterior growth of its anterior end (Fig. 30). Original anterior end (now posterior end) reaches rectum 30 DAI, not joined to rectum. Vagina formation by hypodermal invagination began 9 DAI at attachment point of GP (Fig. 31). Female GP enlarged and swollen posteriorly with two large nuclei near posterior tip that each extend posteriorly in branch at 21 DAI (Fig. 32) and extend beyond the E–I junction 30 DAI (Fig. 35). Lateral chord nuclei large, oval-shaped, and in a single row through 21 DAI (Fig. 33); smaller, round, and numerous 30 DAI (Fig. 34).

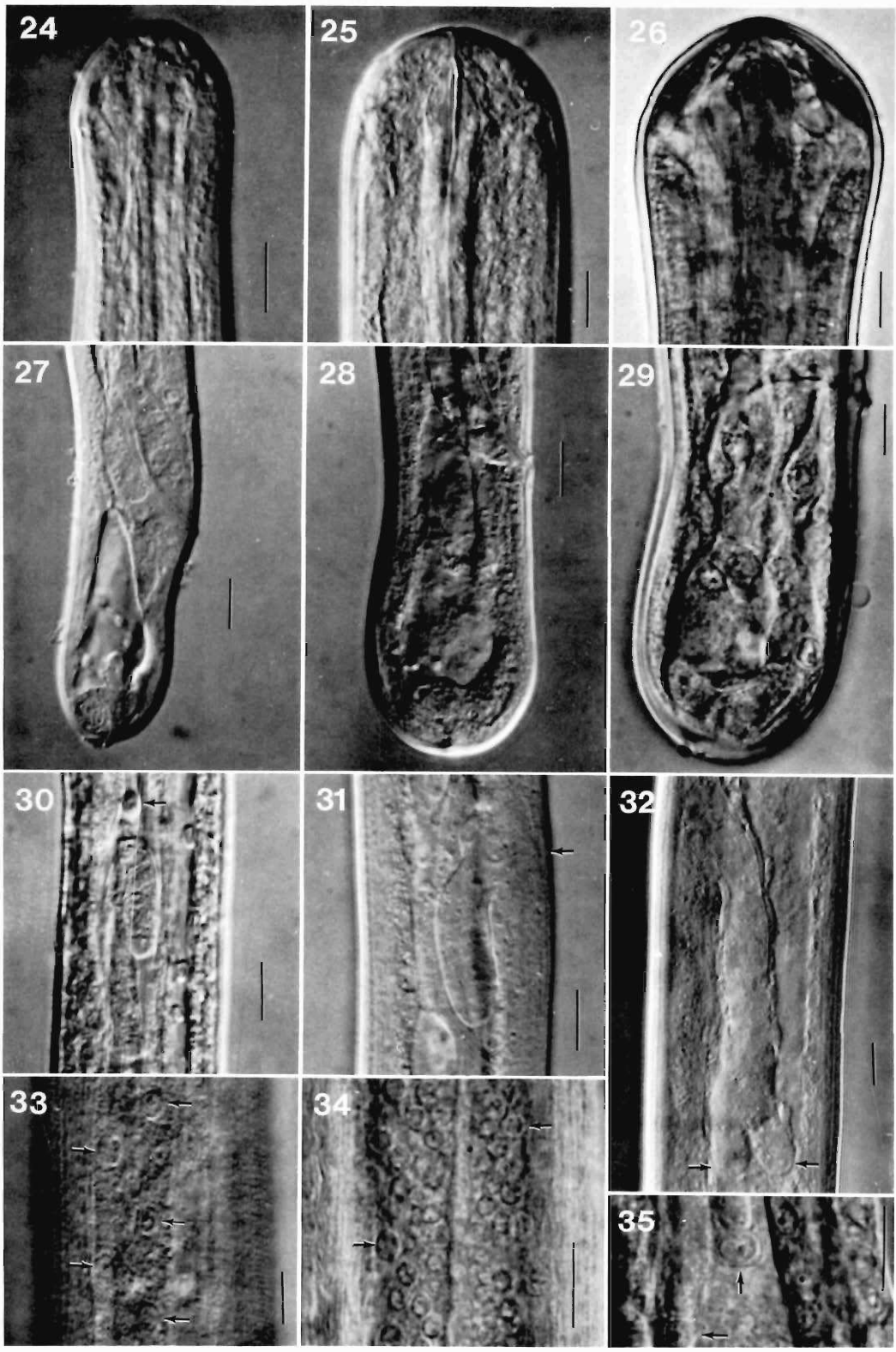
Late fourth stage (Figs. 36–42) (*N* = 129: 54, 41 DAI; 68, 50 DAI; 7, 58 DAI)

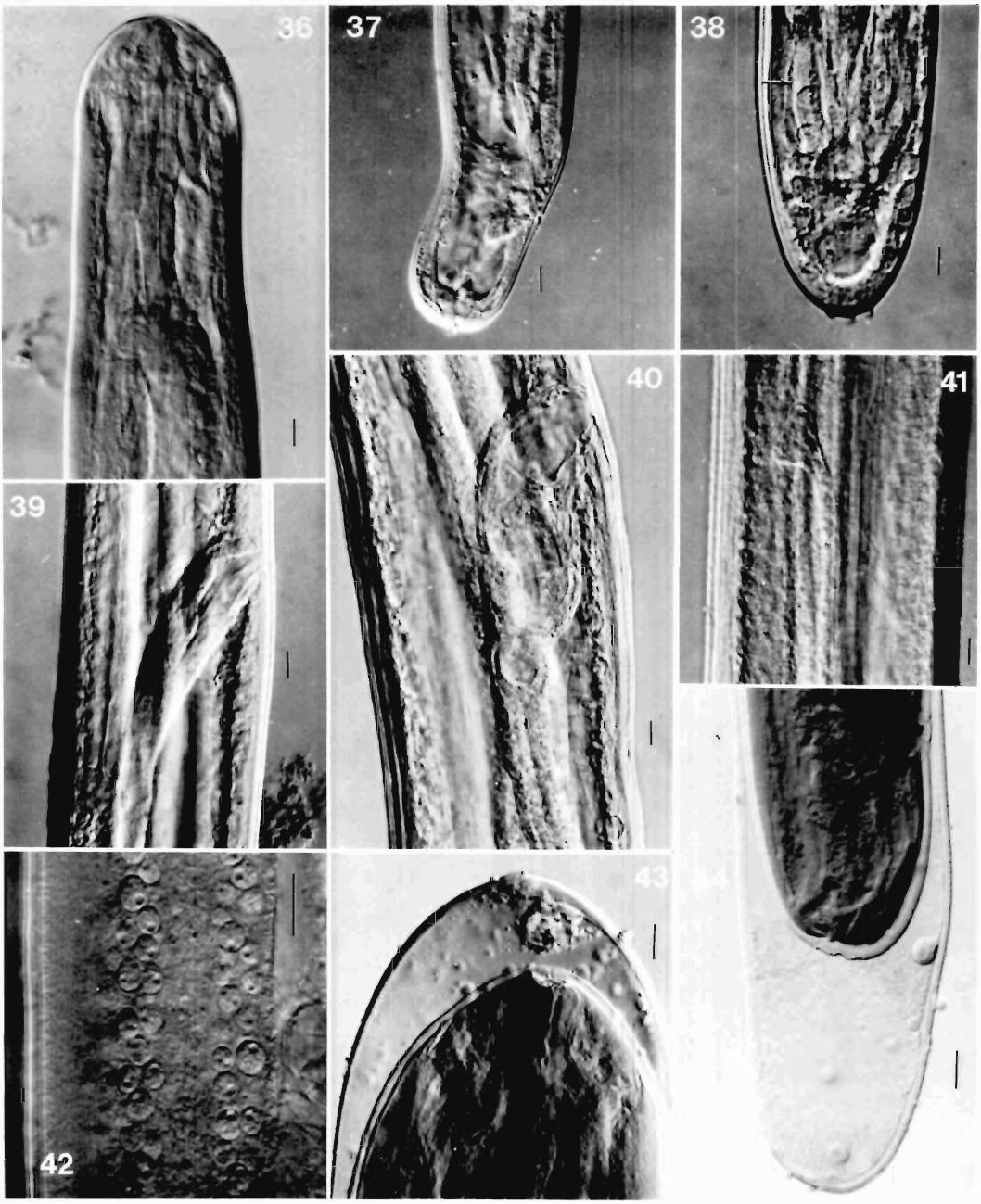
According to Kotani and Powers (1982) all *D. immitis* specimens collected from dogs 41 DAI, 98% of 317 collected 50 DAI, and 35% of 152 collected at 58 DAI were in the late phase of fourth stage.

Head and tail slightly narrower than rest of body (Figs. 36, 37). Tail tip and subventral papillae clustered at posterior extremity, proportionally smaller because of increase in size of nematode (Fig. 38). Fixed larvae 41 DAI 5.6–9.2 mm long and 50 DAI 8.6–12.8 mm long. Posterior extension of male GP joined ventrally to rectum, anterior end of GP (original posterior end) triangular- or knob-shaped, full of round cells (Fig. 41). Spicules and genital papillae partially formed and visible in cleared specimens (Fig. 38). Vagina lumen lined with cuticle 41 DAI (Fig. 39); large cells line posterior ⅔ expanded

→

Figures 24–35. ***Dirofilaria immitis*, mid-fourth stage, from dogs. Scale bars 10 μm. 24–26. Anterior extremities of male, female, and female, 9, 21, and 30 DAI, showing extremity broader than adjacent body. 27–29. Posterior extremities of male, female, and female 9, 21, and 30 DAI showing extremity broader than adjacent body. 30. Male genital primordium and pseudocoelomocyte (arrow), 9 DAI. 31. Female genital primordium (arrow at attachment), 9 DAI. 32. Female genital primordium (arrows at branches), 21 DAI. 33, 34. Nuclei (arrows) in lateral chord, 21 DAI and 30 DAI. 35. Posterior extremities (arrows) of branches of female genital primordium, 30 DAI.**





Figures 36–42. ***Dirofilaria immitis,*** **late fourth stage, from dogs. Scale bars 10 μm. 36, 37. Anterior extremity of male, and posterior extremity of female, 41 DAI. 38. Male tail, ventral view showing the genital papillae and spicule (arrow) of fifth stage beneath the cuticle of the fourth stage, 41 DAI. 39. Vulva and vagina vera, 41 DAI. 40. Vulva, expanded vagina vera, and tubular vagina uterina, 50 DAI. 41. Proximal end of male reproductive system, 50 DAI. 42. Nuclei in lateral chord of male, 50 DAI.**

Figures 43, 44. ***Dirofilaria immitis,*** **fourth molt, 58 DAI. Scale bars 25 μm. 43. Anterior extremity of female. 44. Posterior extremity of female.**

Table 2. Measurements* of spicules and the female reproductive system of *Dirofilaria immitis* from dogs in the late fourth, fourth molt, and early fifth stages.

Characteristic lengths of	Late fourth		Fourth molt	Early fifth stage		
	50 DAI	58 DAI	58 DAI	58 DAI	70 DAI	79 DAI
Left spicule (μm)	nd†	nd	nd	209–399	304–332	340–388
Right spicule (μm)	nd	nd	nd	164–220	188–192	200–222
Vagina vera (μm)	105–147	128–144	115–144	126–132‡	100–120	111–170
Vagina uterina (μm)	176–640	400–688	384–688	560–624	704–1,090	940–1,110
Uterus (mm)	2.14–3.82	4.36–5.44	4.36–5.44	4.59–4.86	5.84–6.84	4.64–5.84
Oviduct and ovary (mm)	1.23–2.96	3.21–4.36	3.21–4.36	3.60–4.84	7.30–8.21	14.4–16.6

* Other measurements of these stages in Table 1.
† nd = Not done; spicules not formed sufficiently.
‡ Four females measured.

muscular vagina vera 50 DAI (Fig. 40). Branches of female GP ½–¾ body length. Additional morphometrics are given in Table 2. Lateral chord nuclei small and numerous (Fig. 42). Lateral excretory tubule evident between columns of lateral chords. Cuticle appears to be bilayered in some specimens (Fig. 41).

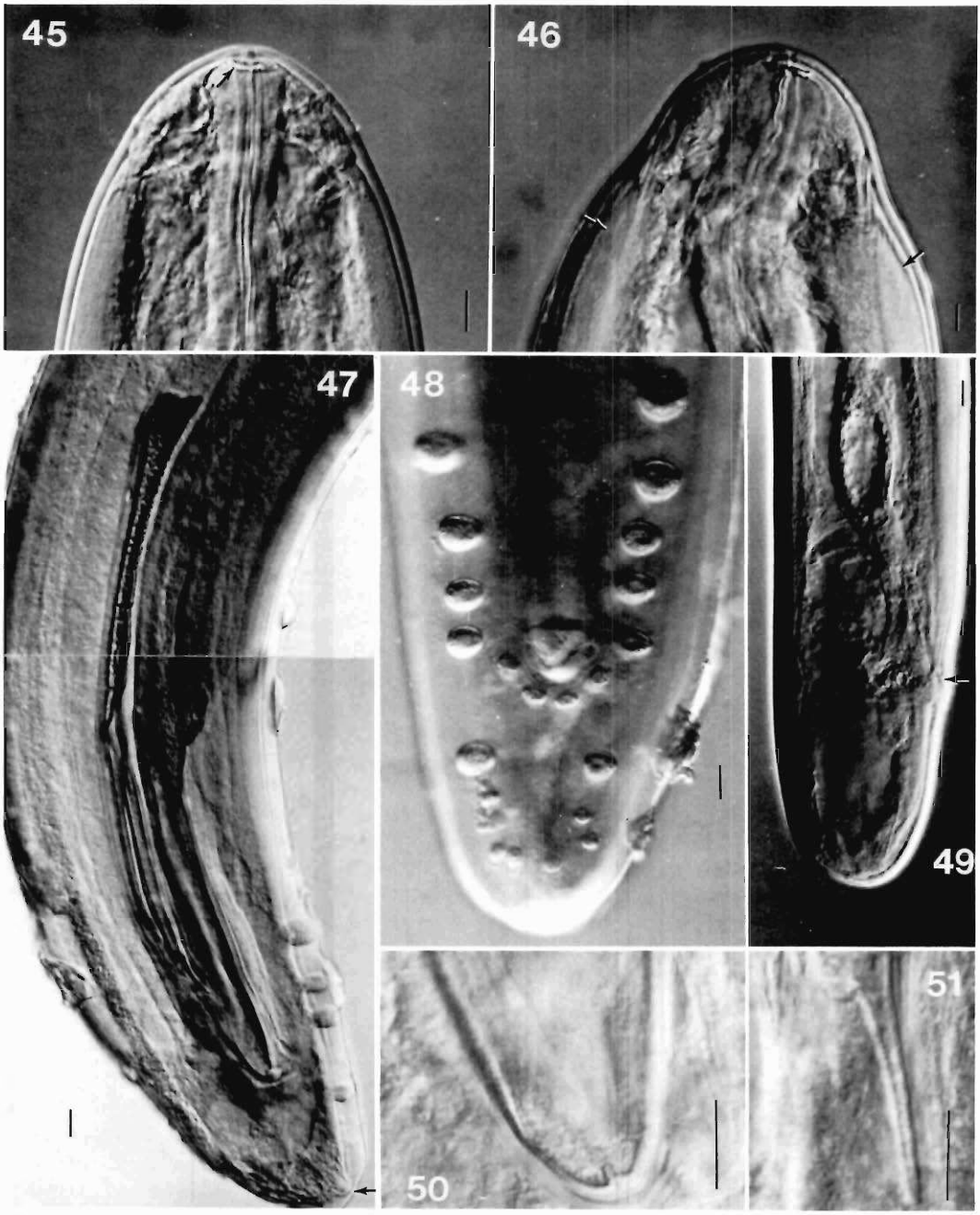
Fourth molt (Figs. 43–44) ($N = 17$: 6, 50 DAI; 11, 58 DAI)

Specimens of *D. immitis* with the fourth-stage cuticle separated from the underlying fifth-stage cuticle at either or both extremities were designated as fourth molt (Figs. 43, 44). This phase of development was in 2% of 317 specimens at 50 DAI and in 20% of 152 at 58 DAI (Kotani and Powers, 1982). Molting specimens were 8.3–12.8 mm long exclusive of fourth-stage cuticle. Female reproductive tract extended to within 1 mm of posterior extremity. Spermatocytes present in anterior portion of testis.

Early fifth stage (Figs. 45–59) ($N = 58$: 23, 58 DAI; 17, 70 DAI; 18, 79 DAI)

This phase extended from 58 DAI through 79 DAI. At 58 days 45% of 152 *D. immitis* specimens were in fifth stage; at 70 and 79 DAI 100% were fifth stage (Kotani and Powers, 1982).

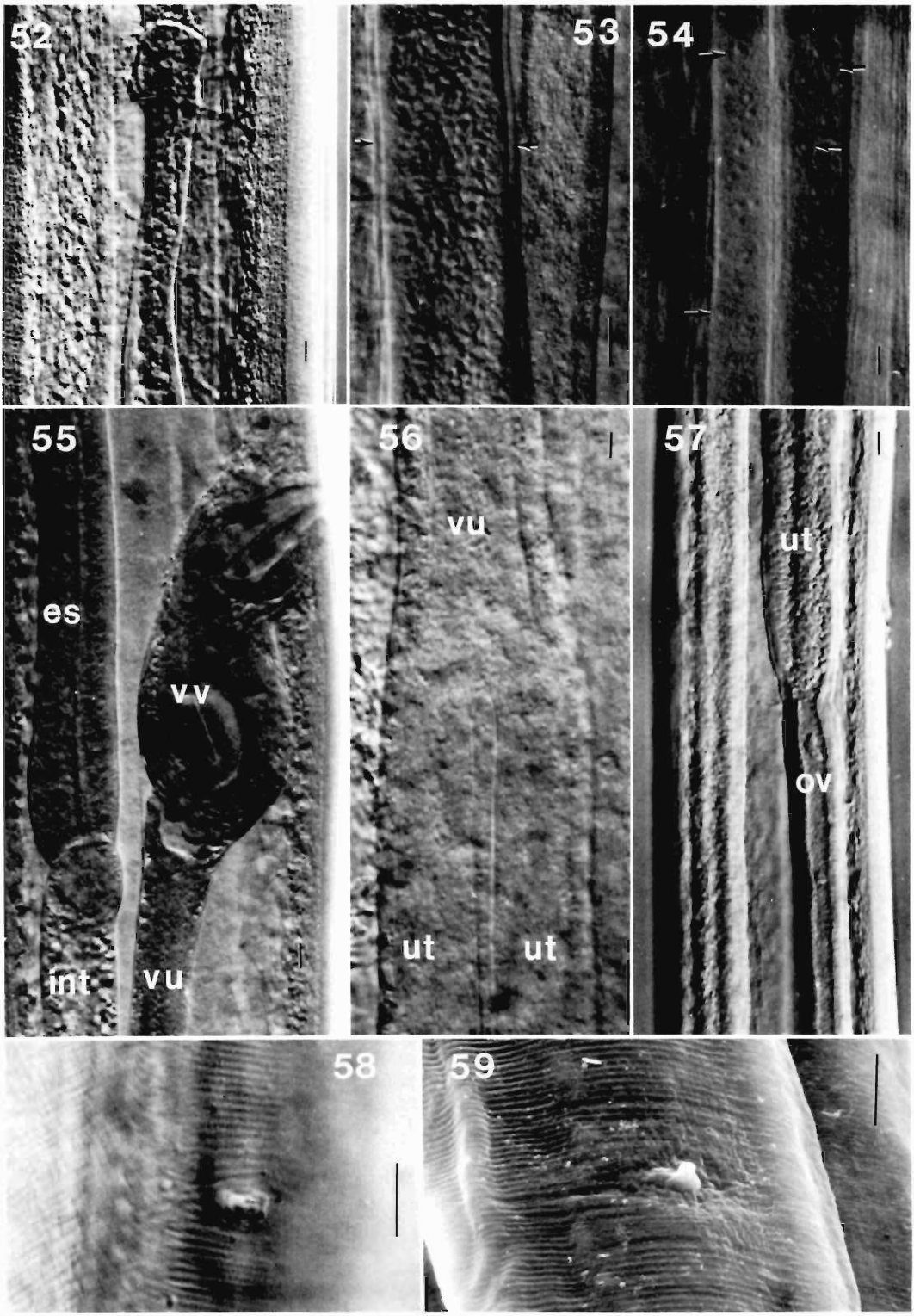
Anterior extremity bluntly rounded at ecdysis (Fig. 45), but narrower than adjacent body by 70 DAI (Fig. 46) because of thick dorsal and ventral somatic musculature; head shape persists in mature specimens. Buccal capsule absent, but peribuccal ring present at anterior end of esophagus (Figs. 45, 46). Female tail short, tapers from anus, bluntly rounded, phasmids near tip (Fig. 49). Male tail spirally coiled 79 DAI with diagonal cuticular ridges on ventral surface of posterior ⅛ of body, gradually become almost transverse before ending anterior to genital papillae. Genital papillae of male usually asymmetrically arranged, left side usually anterior to right side; arranged in three to five pairs, pedunculate, beginning preanally and ending adanally; single pedunculate pair in middle of tail; single large ventral papilla on anterior edge of semilunar-shaped vent; two pairs of smaller unstalked papillae in semicircle around posterior edge of vent; three pairs of small stalked papillae in semicircle in posterior ½ of tail (Fig. 48); phasmids at tip of tail (Fig. 47). Spicules, especially proximal ½ of left spicule, not completely sclerotized 70 DAI; sclerotization nearly complete 79 DAI. Right spicule 164–222 μm long (Table 2), thick, boat- or scoop-shaped with narrow ventral groove in tapered, rounded distal tip (Figs. 47, 50). Left spicule longer (304–399 μm), thinner, divided at about 60% of length by bend in shaft, distal tip spike-shaped (Figs. 47, 51). Testis ends in knob-shape near middle of body (Fig. 52), contains round cells (spermatocytes); male reproductive tract long, usually straight tube except for some reflexing posteriorly, of uniform diameter with epithelial covering that is clear in cleared specimens (Fig. 53); contains spermatozoa in proximal portion 79 DAI. Vulva usually near, but anterior to E–I junction; vagina vera lined with cuticle, reflexed within thick muscular capsule (Fig. 55); vagina uterina elongate (Table 2), undivided with thinner muscle coat (Figs. 55, 56); uterus divided into two long posteriorly directed branches, with muscle coat (Fig. 56); oviduct and ovaries abruptly narrower than uteri, lack muscle coat (Fig. 57). Nuclei of lateral chord numerous, round with prominent nucleoli (Fig. 54). Posterior deirids laterally 0.1–3.0 mm at differing levels



Figures 45–51. ***Dirofilaria immitis,*** **early fifth stage. Scale bars 10 μm. 45. Anterior extremity of male showing peribuccal ring (arrow), 58 DAI. 46. Anterior extremity of male with narrow head anterior to somatic muscles (arrows), 70 DAI. 47. Montage showing spicules and male tail, lateroventral view, 79 DAI (arrow indicates phasmid). 48. Male tail, ventral view, 70 DAI. 49. Female tail, lateral, (arrow at anus), 79 DAI. 50. Distal tip of right spicule, ventral view, 58 DAI. 51. Distal tip of left spicule, lateral view, 58 DAI.**

→

Figures 52–59. ***Dirofilaria immitis,*** **early fifth stage. Scale bars 10 μm. 52. Proximal end of testis, 70 DAI. 53. Male reproductive tract (between arrows), in proximal half, containing spermatozoa, 79 DAI. 54. Nuclei (arrows) of lateral chord, 79 DAI. 55. Vagina vera (vv), anterior portion of vagina uterina (vu), esophagus (es) and intestine (int), lateral view, 79 DAI. 56. Junction of vagina uterina (vu) and uteri (ut), 79 DAI. 57. Junction**



of uterus (ut) and oviduct (ov), 70 DAI. 58. Right postdeirid of female, 70 DAI. 59. Scanning electron micrograph of left postdeirid of male, 70 DAI.

Table 3. Key morphological features* of early developmental stages of *Dirofilaria immitis*.

Morphological feature	Third stage	Early fourth stage
Anterior extremity	Tapered	Untapered
Esophageal–intestinal valve	Undeveloped	Developed
Submedian caudal papillae	Broadly based bumps formed by papillae beneath cuticle	Sharply defined, angular papillae project from surface of tail
Tail tip	Conical, larger than submedian papillae	Button-like, smaller than submedian papillae

* See text for additional details.

from posterior extremity on both sides of males and females (Figs. 58, 59). Usually the left postdeirid is more anterior than the right. Cuticle with fine transverse striation (Figs. 58, 59).

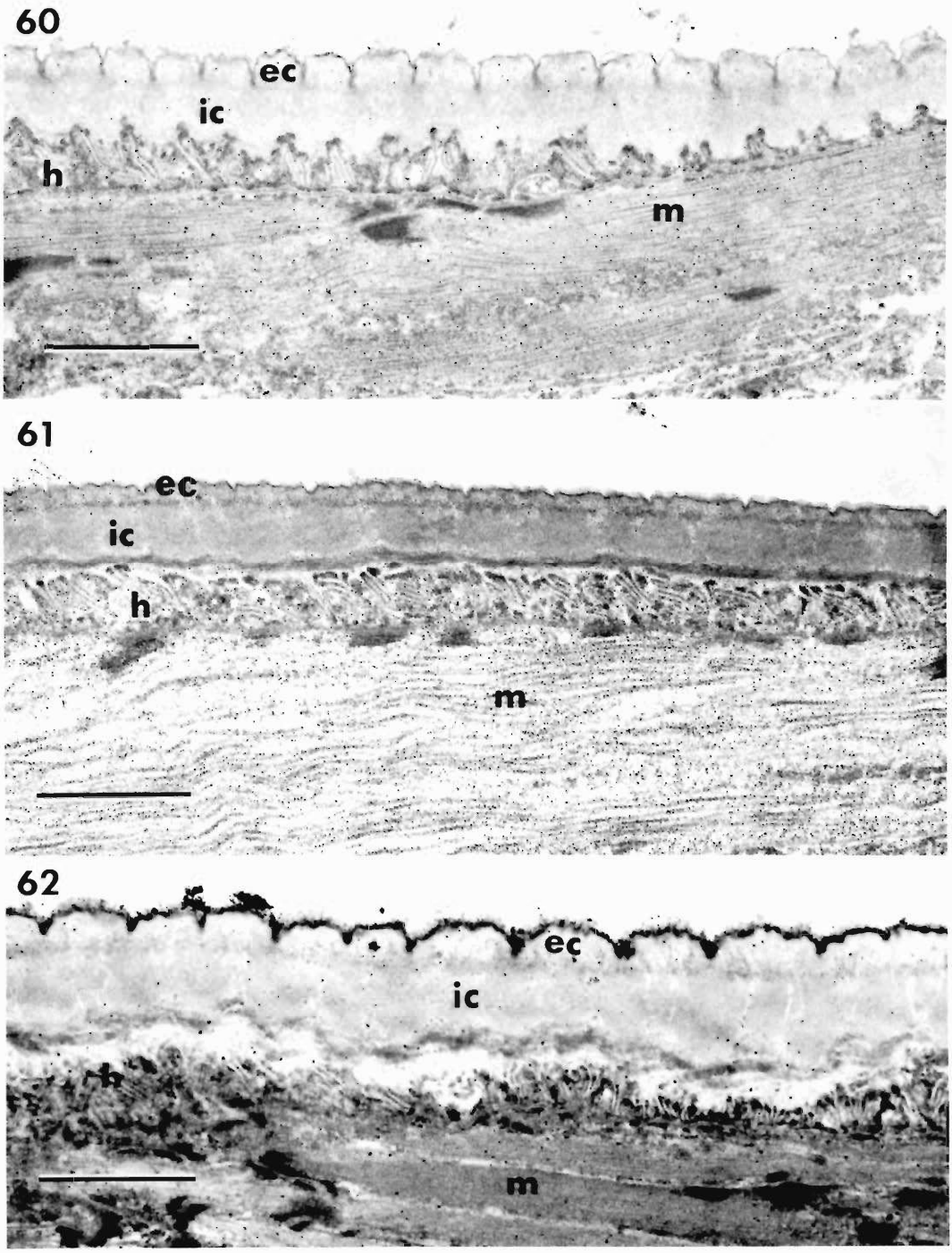
Discussion

Apparently, investigators of the development of *Dirofilaria immitis* in dogs have not observed the third molt because they have not looked early enough. Kume and Itagaki (1955) examined dogs no earlier than 23 DAI. Orihel (1961) examined dogs no earlier than 5 DAI; and the earliest examinations by Kotani and Powers (1982) were 3 DAI. In vitro studies, however, demonstrated that a molt occurs from 2 to 5 days after third-stage larvae emerge from mosquitoes (Yoeli et al., 1964; Sawyer, 1965; Sawyer and Weinstein, 1965; Wong et al., 1982; Lok et al., 1984). In addition, P. Supakorndej, J. J. Jun, and J. W. McCall (pers. comm.) reported the third molt in ferrets occurred 3 DAI. In in vitro and model animal systems all observers of the third molt reported it to occur much earlier than the 9–12 DAI third molt in the dog reported by Orihel (1961).

We conclude that the third molt was completed in dogs earlier than 3 DAI. Although the third molt was not observed in dogs in this study, all available specimens collected 3 DAI from dogs were early fourth-stage larvae identical with those that emerged from the third molt 2 and 3 days in vitro. We did not find molting larvae at 3, 6, 9, 12, 15, 21, 30, or 41 DAI. A single third-stage larva in poor condition was found 6 DAI, and we observed a few fourth-stage larvae at 6, 9, and 12 DAI that were in poor condition with cellular debris adhered to slightly swollen cuticles. We believe these were dead or moribund specimens, but such specimens resemble a premolting condition as seen in infective larvae. They were identified as early fourth-stage larvae by the presence of sharply defined submedian tail papillae and untapered anterior ends (Table 3). However because some specimens collected at 3–15 DAI were unavailable for this study, it is possible that additional specimens in third stage or third molt were present at those times and were not seen by us. In any case, all of the 872 available specimens collected from dogs at 3–15 DAI (except one third-stage larva) were in the fourth stage, indicating that those specimens had already completed the molt observed at 2 or 3 days in vitro.

Because of the differences between our observations and those of Orihel (1961) regarding the third molt of *D. immitis* in dogs, we conducted two additional studies. If the molt reported by Orihel (1961) at 9–12 DAI in dogs was a different molt from the one we observed at 2–3 days in vitro, then one or both of the following must also be true: (a) because more than four molts would have been accounted for (two in the mosquito and three in the dog), the molt observed in vitro and in ferrets would have to be an ecdysis of an earlier molt in the mosquito; and (b) molting lethargic specimens at 9–12 DAI may have been retained in the tissues of the dog and missed in the present study. Therefore, to determine whether (a) and/or (b) might be true: (1) we reexamined the development of *D. immitis* in *Aedes aegypti,* as described by Taylor (1960), by dissecting developing larvae from mosquitoes fixed in alcohol 12 DAI; and (2) we examined with transmission electron microscopy the cuticle of specimens collected at the time Orihel (1961) reported the third molt to occur to determine whether any indication of a molt, as described by Howells and Blainey (1983) for *Brugia pahangi* (Buckley and Edeson, 1956), could be seen.

The results of our reexamination of the development of *D. immitis* in *A. aegypti* found it to be as described by Taylor (1960) and by Bartlett (1984a) for *Dirofilaria scapiceps* (Leidy, 1886). We observed a first-stage larva with a pointed cap over the stoma, a first molt and ecdysis



Figures 60–62. Electron micrographs of early and middle phases of fourth-stage larval *Dirofilaria immitis* from dogs. Scale bars 5 μm. 60. Early fourth phase, 3 DAI, showing an annulated, convoluted external cuticle (ec) with deep striae separated from the thicker internal layer of cuticle (ic) by a thin electron-dense line; a thin, convoluted hypodermis (h) with electron-dense beads adjacent to the cuticle, and somatic musculature (m). 61. Mid-fourth phase, 9 DAI, showing cuticle with broader annules and more shallow striae, and a less convoluted hypodermis than in the earlier phase of development. 62. Mid-fourth phase, 12 DAI, with the cuticle somewhat thicker than earlier and with an electron-lucent layer between the cuticle and the hypodermis.

(sometimes simultaneously with the second ecdysis), a second-stage larva with elongate buccal capsule and anal plug, and clear evidence that both the buccal capsule and anal plug are shed with the ecdysis of the second molt in the mosquito as described by Taylor (1960) for *D. immitis* and by Bartlett (1984a) for *D. scapiceps*. We concluded, therefore, that the molt observed by several workers at 2–5 DAI in vitro and in ferrets 3 DAI by P. Supakorndej, J. J. Jun, and J. W. McCall (pers. comm.) is the third molt and not an ecdysis of the second molt.

The results of our transmission electron microscope study of the cuticle of developmental stages (3, 9, and 12 DAI) of *D. immitis* in the dog showed no indication of either a recent molt or an impending molt at 9–12 DAI (Figs. 60–62); rather the cuticle was typical of an intermolt period (Lee, 1970; Howells and Blainey, 1983). Furthermore, with the molt at 2 DAI, the previous two molts in the mosquito (Taylor, 1960) and the fourth molt at 50–58 DAI, all molts in the life cycle of *D. immitis* are accounted for.

The third molt in *D. scapiceps* in rabbits was recently reported by Bartlett (1984b) to occur 6 DAI. Bartlett (1984b) described a buccal capsule more strongly developed in the fourth stage than in the third stage. We also observed thicker walls in the buccal capsule of the fourth stage of *D. immitis* than in the third stage. Spicular primordia were visible in the third stage of both species. The anterior development of a narrow germinative portion of the male reproductive tract as described for *D. scapiceps* by Bartlett (1984b) was not seen in *D. immitis* which retained the knob-shaped anterior end of the original genital primordium as described by Orihel (1961).

From a survey of the literature it appears that there are considerable differences among the larvae of the Onchocercidae in the presence of submedian papillae and tail tip. Unlike *D. immitis,* in which the submedian caudal papillae of the fourth stage are more prominent than in the third stage, the submedian caudal papillae of *D. scapiceps* are more prominent in the third stage (Bartlett, 1984a, b, and a study of specimens by us). However, the submedian caudal papillae of third-stage *D. scapiceps* are smaller than the tail tip (Bartlett, 1984a) as in *D. immitis.* The cuticles of both third- and fourth-stage *D. scapiceps* are much thicker than in *D. immitis,* which may account for the difference in prominence of the papillae. In *Brugia pahangi* and *Wuchereria bancrofti* (Cobbold, 1877) the caudal papillae of the third stage are three in number, but in the fourth stage the terminal papilla or tail tip is absent or greatly reduced in size leaving only the two submedian papillae (Aoki et al., 1980; Franz and Zielke, 1980). This difference was used by Aoki et al. (1980) to identify the larvae of *B. pahangi* to stage.

In the present study refractile granules were present in the glandular esophagus of infective larvae and larvae in the third molt, but were not seen at any other times. Refractile granules were observed in the glandular esophagus of late fourth and early fifth stages of *D. scapiceps* in rabbits by Bartlett (1984b). She suggested that the granules may be secretory and of use to the migrating early fifth stage of the nematode.

Our description of sexual dimorphism in third-stage *D. immitis* from mosquitoes agrees with the earlier description by Orihel (1961). Sexual dimorphism has been described also in third-stage *D. scapiceps* from mosquitoes by Bartlett (1984a).

A single pair of bilateral postdeirids were present in all stages of *D. immitis* in the present study; they were described in adults previously by Uni (1978). Bartlett (1984b) also reported postdeirids in *D. scapiceps.* A similar lateral papilla is present on the tail of all stages of *B. pahangi* according to Aoki et al. (1980). The function of the postdeirids is unknown.

The principal contribution of the present study is the redescription of the morphogenesis of *Dirofilaria immitis* in dogs. With this basic information on the morphogenesis in the normal definitive host, the success of in vitro and model animal systems and treatment programs can be evaluated, and a series of reference specimens is available for the identification of developmental stages of the dog heartworm.

Acknowledgments

This study was supported in part by an Interagency Agreement (Nos. 1001-0110) between the Agricultural Research Service, U.S. Department of Agriculture, and the Bureau of Veterinary Medicine, Food and Drug Administration. We thank: Drs. Robert B. Grieve and David Abraham, University of Wisconsin, Madison, for providing specimens and a prepublication copy of a manuscript; Dr. Ming Ming Wong, University of California, Davis, for providing specimens; Dr. Cheryl M. Bartlett, University of Guelph,

Ontario, Canada, for providing specimens and a prepublication copy of a manuscript; and Dr. William P. Wergin, Ms. Norita Chaney, and Ms. Gretchen Kaminski, EM Laboratory, Agricultural Research Service, USDA, Beltsville, Maryland, for the electron micrographs. The review of this paper was handled by Dr. George J. Jackson, Editor, Experimental Parasitology, as a courtesy, to protect the confidentiality of the review process and to avoid any appearance of a conflict of interest because two authors of this paper are editors of the Proceedings.

Literature Cited

Aoki, Y., A. L. Vincent, L. R. Ash, and D. Katamine. 1980. Scanning electron microscopy of third- and fourth-stage larvae and adults of *Brugia pahangi* (Nematoda: Filarioidea). J. Parasitol. 66:449–457.

Bartlett, C. M. 1984a. Development of *Dirofilaria scapiceps* (Leidy, 1886) (Nematoda: Filarioidea) in *Aedes* spp. and *Mansonia perturbans* (Walker) and responses of mosquitoes to infection. Can. J. Zool. 62:112–129.

———. 1984b. Development of *Dirofilaria scapiceps* (Leidy, 1886) (Nematoda: Filarioidea) in lagomorphs. Can. J. Zool. 62:965–979.

Douvres, F. W., F. G. Tromba, and G. M. Malakatis. 1969. Morphogenesis and migration of *Ascaris suum* larvae developing to fourth stage in swine. J. Parasitol. 55:689–712.

Endo, B. Y., and W. P. Wergin. 1973. Ultrastructural investigation of clover roots during early stages of infection by the root-knot nematode, *Meloidogyne incognita.* Protoplasma 78:365–379.

Franz, M., and E. Zielke. 1980. Scanning electron microscope study on larvae of *Wuchereria bancrofti* from the vector and from experimental rodent hosts. Tropenmed. Parasitol. 31:345–356.

Howells, R. E., and L. J. Blainey. 1983. The moulting process and the phenomenon of intermoult growth in filarial nematode *Brugia pahangi.* Parasitology 87:493–505.

Humphreys, W. J. 1975. Principles and techniques of scanning electron microscopy. Pages 707–714 *in* O. Johari and I. Corvin, eds. Scanning Electron Microscopy. IIT Research Institute, Chicago.

Kotani, T., and K. G. Powers. 1982. Developmental stages of *Dirofilaria immitis* in the dog. Am. J. Vet. Res. 43:2199–2206.

Kume, S., and S. Itagaki. 1955. On the life-cycle of *Dirofilaria immitis* in the dog as the final host. British Vet. J. 111:16–24.

Lee, D. L. 1970. Moulting in nematodes: the formation of the adult cuticle during the final moult of *Nippostrongylus brasiliensis.* Tissue & Cell 2: 139–153.

Lok, J. B., M. Mika-Grieve, R. B. Grieve, and T. K. Chin. 1984. *In vitro* development of third- and fourth-stage larvae of *Dirofilaria immitis*: comparison of basal culture media, serum levels and possible serum substitutes. Acta Tropica 41:145–154.

Madden, P. A., and F. G. Tromba. 1976. Scanning electron microscopy of the lip denticles of *Ascaris suum* adults of known ages. J. Parasitol. 62:265–271.

Orihel, T. C. 1961. Morphology of the larval stages of *Dirofilaria immitis* in the dog. J. Parasitol. 47: 251–262.

Sawyer, T. K. 1965. Molting and exsheathment in vitro of third-stage *Dirofilaria immitis.* J. Parasitol. 51:1016–1017.

———, **and P. P. Weinstein.** 1965. Third molt of *Dirofilaria immitis* in vitro and in vivo. J. Parasitol. 51(sect. 2):48.

Taylor, A. E. R. 1960. The development of *Dirofilaria immitis* in the mosquito, *Aedes aegypti.* J. Helminthol. 34:27–38.

Uni, S. 1978. Scanning electron microscopic study of *Dirofilaria* species (Filarioidea, Nematoda) of Japan and a review of the genus *Dirofilaria.* [In Japanese, with English summary.] J. Osaka City Med. Center 27:439–458 + plates 1–7.

Wergin, W. P., and B. Y. Endo. 1976. Ultrastructure of a neurosensory organ in a root-knot nematode. J. Ultrastruct. Res. 56:258–276.

Wong, M. M., R. Knighton, J. Fidel, and M. Wada. 1982. *In vitro* cultures of infective-stage larvae of *Dirofilaria immitis* and *Brugia pahangi.* Ann. Trop. Med. Parasitol. 76:239–241.

Yoeli, M., S. R. Upmanis, and H. Most. 1964. Studies on filariasis. III. Partial growth of the mammalian stages of *Dirofilaria immitis* in vitro. Exper. Parasitol. 15:325–334.