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Intrahepatic growth and maturation of *Gnathostoma turgidum* in the natural definitive opossum host, *Didelphis virginiana*

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ABSTRACT

Gnathostoma turgidum is a gastric nematode parasite of opossums found in the Americas. We recently found that *G. turgidum* juveniles appear in the liver of the opossums where they become mature adults and almost synchronously move to the stomach during certain months of the year, suggesting the importance of the liver for the growth and maturation of this species in the final hosts. In this study we attempted to detect *G. turgidum* larvae in the liver of opossums, *Didelphis virginiana* that are the natural final hosts. The results show that tiny (<3 mm in length) third stage larvae (L3) appeared in the liver of opossums around November and December. Also in the liver, we found large L3 of up to about 10 mm in length together with juveniles and mature adults from February to March. In spite of their length, large L3 have 4 rows of hooklets, and their gonads remained undeveloped. Morphological features of the small and large L3 of *G. turgidum* are described including scanning electron microscope images. The seasonal switching of the several growth stages of *G. turgidum* from small L3 to adult worms in the liver and eventual migration to the stomach in opossums suggests the unique feature of *G. turgidum* utilizing the liver as the maturation site.

1. Introduction

Gnathostomosis is a food-borne zoonosis caused by vigorous migration of the advanced 3rd stage larvae (AL3) of the genus *Gnathostoma* in the human body. Infection occurs by ingesting raw or insufficiently cooked fresh fish meat contaminated with *Gnathostoma* AL3. The disease is endemic where people have the custom of consuming raw or under-cooked fish dishes. Thailand and Japan have been known as the most famous endemic areas of this disease, although patients have been found in many other Asian countries [1–4]. In Mexico, the first human gnathostomosis case was found in 1970 [5] and the country has been recently found to be heavily endemic for this disease [6–10]. Three *Gnathostoma* species, *Gnathostoma binucleatum*, *Gnathostoma turgidum*, and the newly identified *Gnathostoma lamothei* Bertoni-Ruiz et al. 2005, are recognized as native species in Mexico [11]. Among them, only *G. binucleatum* has been proven to be a pathogen for humans [12,13]. Recently we found a highly endemic area of *G. turgidum* in common opossums, *Didelphis virginiana* [14,15] in the southern part of Sinaloa State, Mexico, where *G. binucleatum* is also highly endemic and an acute outbreak of human gnathostomiasis due to *G. binucleatum* infection was recorded [9]. In spite of the co-existence of two *Gnathostoma* species in this small area, we could find only *G. binucleatum* AL3 in an array of intermediate/paratenic hosts such as estuarine fish, ichthyophagous birds, amphibians and reptiles [16,17]. In our 10 year survey in this area since 2001, we have not yet found *G. turgidum* larvae in any of those intermediate/paratenic hosts, suggesting that *G. binucleatum* and *G. turgidum* have separate natural lifecycles in the same geographic area. Moreover, we found that *G. turgidum* larvae develop to juveniles and fully mature adults in the liver of the natural final host opossum before they appear in the final tissue, the gastric wall [14,15]. This intrahepatic maturation of *G. turgidum* appears to be unique from the maturation process of other *Gnathostoma* species. Moreover, recently extremely small AL3 of *G. turgidum* were found in natural and experimental intermediate/paratenic hosts [18], and two large *G. turgidum* larvae were found accidentally in the liver of a four-eyed opossum, which were proposed as the 4th stage larvae of *G. turgidum* [19]. In the present study, we describe morphological features of small and large size *G. turgidum* larvae obtained from the liver of common opossums, *D. virginiana*. The significance of the seasonal appearance of those

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Table 1
Seasonal changes of the tissue distribution and developmental stages of *Gnathostoma turgidum* in opossums.

Month	Opossums examined (+ve/total)	Liver						Stomach		
		S–L3	L–L3	JUV	Adults			Adults		
					M	F	T*	M	F	T
January 2009	9/10	21	0	9	0	0	2	0	0	0
February 2008	37/47	0[b]	9	3	81	49	152	0	0	0
February 2009	2/2	0	0	8	4	6	10	0	0	0
March 2008	3/3	0	0	2	8	5	17	0	0	0
March 2009	7/8	3	0	2	19	7	31	0	0	0
April 2008	9/9[a]	0	1	1	20	37	57	7	5	12
April 2009	13/15	0	1	1	9	27	36	73	46	119
May 2008	6/8	0	0	1	4	9	17	17	13	30
May 2009	12/13	0	0	1	4	7	12	38	30	68
June 2008/2009	0	–	–	–	–	–	–	–	–	–
July 2008	4/6	0	0	0	0	0	1	15	13	28
August 2008	0/1	0	0	0	0	0	0	0	0	0
September 2008	0/1	0	0	0	0	0	0	0	0	0
October 2009	2/9	0	0	0	3	0	3	0	0	0
November 2008	8/12	18	0	0	0	0	0	0	1	1
November 2009	7/12	17	0	0	0	0	0	1	0	1
December 2008	0/9	0	0	0	0	0	0	0	0	0
December 2009	6/13	9	0	0	0	0	0	0	1	1
TOTAL	125/178	68	11	28	152	147	338	151	109	260

S–L3 and L–L3 stand for small- and large–L3 *G. turgidum* larvae, respectively.
JUV: juvenile form of *G. turgidum*.
M: male, F: female, and T = total.
[a] 3 adult worms in the peritoneum and 1 in the intestinal subserosa (Diaz-Camacho et al. [17]) were not included in this table.
[b] A total number of worms occasionally higher than the sum of males and females, because those of unidentified sexes were also included.

larvae is discussed in relation to the maturation/development of *G. turgidum* in the liver of natural final host opossums.

2. Materials and methods

Common opossums, *D. virginiana*, were captured and killed by local hunters at *Ojo de Agua* (22°45′28″N, 105°40′25″W), Tecualilla, Sinaloa State, Mexico, from February 2008 to December 2009. The bodies of opossums were packed in ice for transportation to our laboratory, and examined within 48 h after being killed. The thoracic and abdominal viscera were removed *en masse* and then the stomach and liver tissues were separately dissected out and examined first visually for the presence of worms. After removal of worms, the liver was cut into small pieces, compressed between two glass plates, and observed under a dissection microscope to check for very small worms. The residues were mixed with 10-fold volumes of artificial gastric juice (0.1% pepsin/0.7%HCl) for digestion at 37 °C overnight. The undigested materials were collected after extensive washing by sedimentation/decantation and examined under a dissection microscope to find larvae.

Fig. 2. Light microscopic images of one small and two large L3 of *Gnathostoma turgidum* obtained from the liver of opossums. Scale bar = 1 mm.

After observation in a light microscope, some live larvae were fixed in 90% ethanol for DNA extraction, and some specimens were fixed in Karnovsky's solution for electron microscopy and were processed as described previously [16]. In semi-thin sections of the middle portion of the larvae, 200 intestinal epithelial cells were examined to determine the number of nuclei in each cell.

Molecular genetic identification procedures for *Gnathostoma* species were as described previously [12,13]. The larvae fixed in absolute ethanol were deposited in an Eppendorf tube and genomic DNA extracted using a phenol–chloroform–isopropanol method. ITS2 region of ribosomal DNA was amplified by PCR using the primer set, LC1 (forward) 5′-CGA GTA TCG ATG AAG AAC GCA GC-3′ and 28SW (reverse) 5′-GCA ACC CGA CTC CAA GGA AC [20]. The PCR products were sequenced and aligned with the known ITS2 sequences of *G. turgidum*, *G. binucleatum* and *G. lamothei* using Clustal X v1.83 [21].

3. Results

The numbers of each developmental stages of *G. turgidum* found in the liver and stomach of opossums in the longitudinal study throughout the year are summarized in Table 1. Small size 3rd stage larvae (S–L3) up to 3 mm in length were found in the liver of opossums from November to January. From a total of 68 S–L3, only two were found encapsulated (Fig. 1). Large size 3rd stage larvae (L–L3) were found mainly in February, and juvenile worms were found from

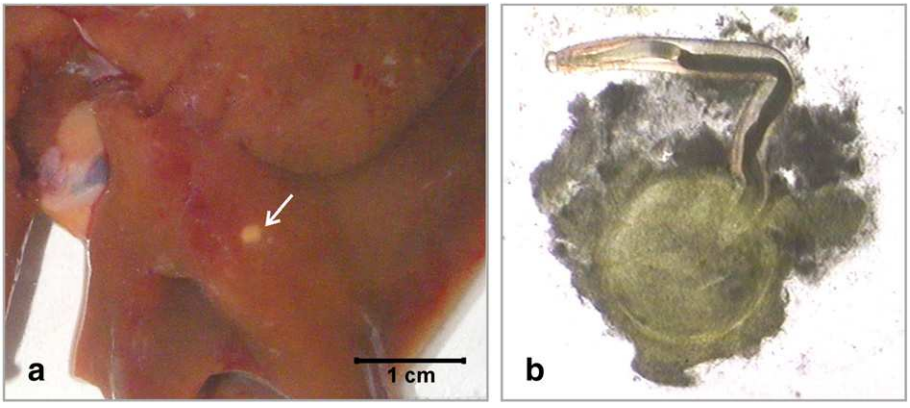

Fig. 1. Encapsulated *Gnathostoma* larva found in the liver of an opossum. a: cyst in the liver. b: a larva emerging from the cyst.

Table 2
Body length of various developmental stages of *Gnathostoma turgidum* in opossums.

Time of the year	Liver					Stomach	
	Larvae (mm)		Juveniles (cm)	Adults (cm)		Adults (cm)	
	S–L3	L–L3		M	F	M	F
Nov–Dec	2.75 ± 0.74 (28)						
Feb–Mar		9.19 ± 1.30 (9)	3.28 ± 0.61 (6)	3.48 ± 0.57 (10)	3.47 1.01 (11)		
Apr				3.55 ± 0.84 (25)	3.92 ± 0.79 (58)	4.74 ± 0.74 (77)	5.40 ± 0.89 (54)
July						5.51 ± 0.82 (11)	6.78 ± 1.00 (12)

S–L3 and L–L3 stand for small- and large-L3 *G. turgidum* larvae, respectively.
JUV: juvenile form of *G. turgidum.*
M: male and F: female.
The figures in bracket are the total number of worms examined.

February to May. Young adult worms began to appear in the liver of opossums as early as January and were continuously found in the liver until April, when the transition of worms from the liver to the stomach began. Fully mature adult worms, of which females can lay eggs, were seen in the liver around April but the majority of them moved to the stomach during May and July, and had almost completely disappeared by August. From August to October (a rainy season in the study area) only few residual adult worms were seen either in the liver or stomach.

As shown in Fig. 2, S–L3 were morphologically similar to the advanced 3rd stage larvae (AL3) of other *Gnathostoma* species, with the body length of 1.5–4 mm. In contrast, *G. turgidum* L–L3 were large, measuring about 7–10 mm in length and 0.3–0.4 mm in width. Chronological changes of the body length along with the developmental stages of *G. turgidum* are summarized in Table 2.

By scanning electron microscopic (SEM) observations (Fig. 3), the head bulb of both S–L3 and L–L3 was clearly distinguished from the rest of the body by the bulb shape and was covered with four rows of small single-pointed hooklets with an irregular polygonal or oblong base (Fig. 3a, b). While body and head bulb sizes increased from S–L3 to L–L3, their hooklet size did not change much, so that the hooklets on the head bulb of L–L3 were scanty with wide spaces between them (Fig. 3b). The headbulb of the juvenile worm has 9 rows of well-developed hooklets (Fig. 3c). On the anterior 1/5 to 1/3 of the body of S–L3 and L–L3, single-pointed tiny spines were arranged along the transverse striations (Fig. 3d, e), which were difficult to see by low power light microscopy. In contrast, the anterior half of the juvenile worms were, like the mature adults [14], densely covered with irregularly arranged multi-dentated cuticular spines (Fig. 3f). Throughout their development, the caudal end of the body of *G. turgidum* had no cuticular spines, though they had well-developed circular rings (Fig. 4a, b). The posterior 2/3 to 1/2 of the body of *G. turgidum* L–L3 had an apparently smooth surface with extremely fine striations (Fig. 4a). The caudal end of juvenile worms showed remarkable wrinkles, but no spines (Fig. 4b). A wide terminal opening was observed on the ventral surface (Fig. 4a, b). Semi-thin sections of the mid-body (Fig. 5) revealed that the intestinal epithelial cells are

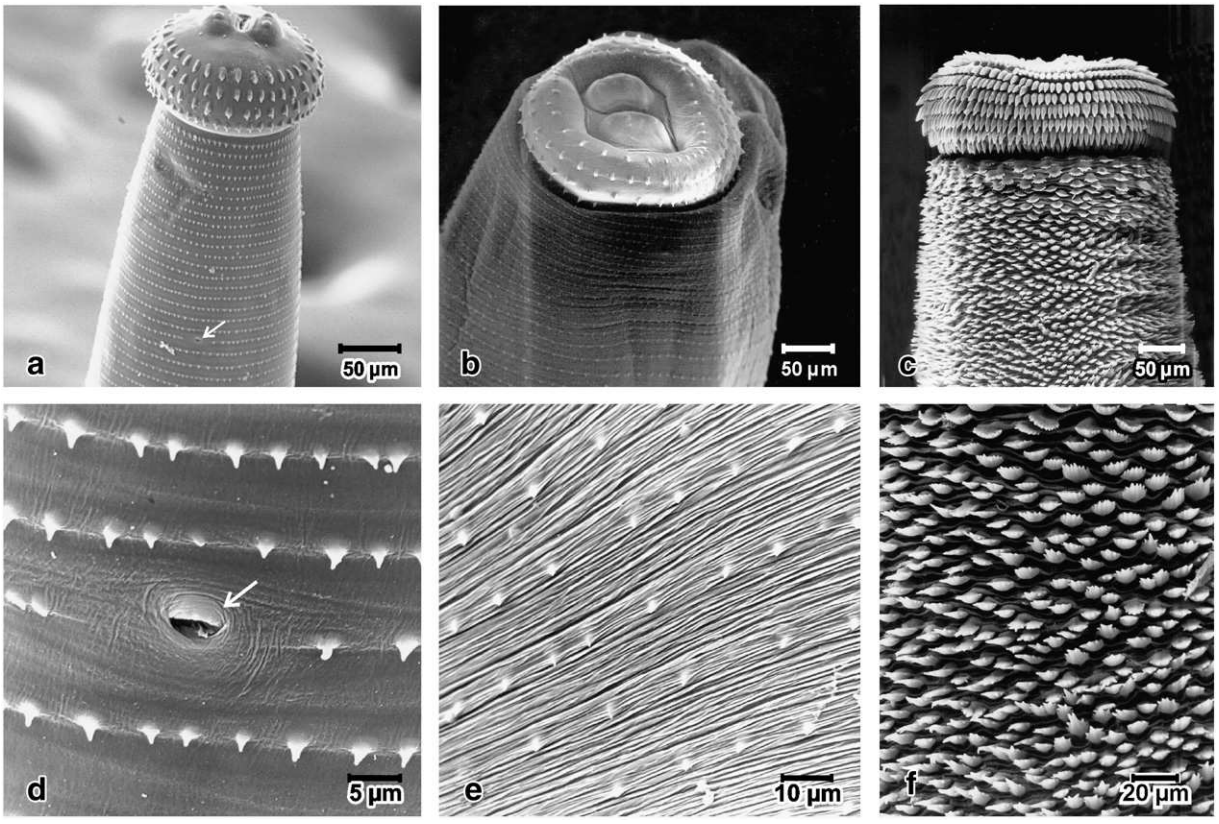

Fig. 3. SEM images of the anterior half of *Gnathostoma turgidum* L3 and a juvenile worm. The cephalic bulb and the anterior portion of (a) small-L3, (b) large-L3, and (c) juvenile worm. Panels (d), (e) and (f) illustrate the cuticular spines of the corresponding stages. White arrows in (a) and (d) indicate the excretory pore.

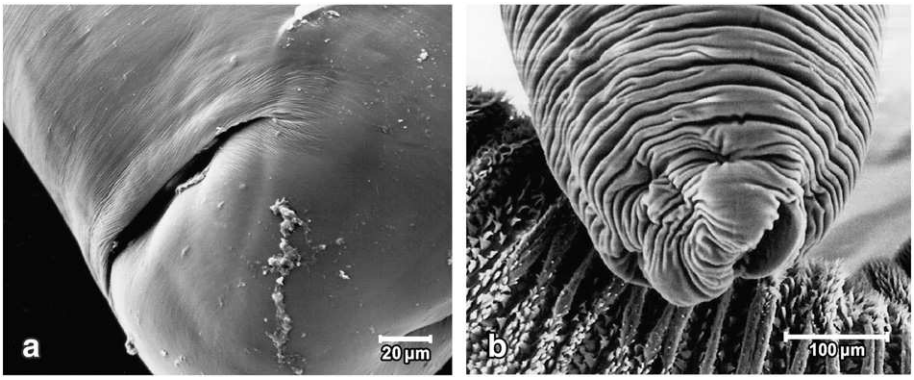

Fig. 4. SEM images of the caudal end of *Gnathostoma turgidum*. (a) Large-L3, and (b) juvenile worm.

columnar in shape and have 2–12 nuclei (mean of 4.8 nuclei/cell with the median of 4.0). Some morphological features of *G. turgidum* S–L3 and L–L3 were compared with those of other *Gnathostoma* spp. (Table 3).

By chance we found that one specimen of L–L3 was moulting (Fig. 6), in that the adult type head bulb having 9 rows of hooklets was covered with the transparent shedding cuticle of the head bulb of the larva with 4 rows of hooklets.

In addition to the morphological characteristics, the DNA sequence of ITS2 of the L–L3 in this study (the GenBank accession no. FJ524380) was identical with those of *G. turgidum* adult worms obtained from the stomach of opossums caught in the same endemic area (accession no. EU930817–EU930822) [14].

4. Discussion

G. turgidum was first described by Stossich [22] more than 100 years ago as the nematode parasite found in the stomach of an opossum, *Didelphis azarae* (=*Dracaena paraguayensis*) in Argentina. Subsequently, Travassos [23] described the morphological details of adult worms obtained from the stomach of *Didelphis aurita* in Brazil. Sporadic discovery of this species was reported from Louisiana [24] and Texas [25], USA, Panama [26], Peru [27] and Mexico [12,28–30]. In spite of the diverse distribution of this species in the Americas, almost all previous descriptions of mature or immature adult worms were accidental, so that the precise life-cycle with the sequential morphological changes for each developmental stage of *G. turgidum* remains unclear.

Recently Mosqueda-Cabrera et al. [18] described a rather small AL3 and, this year, Almeyda-Artigas et al. [19] described the morphological features of the large larvae of *G. turgidum* obtained from the liver of the four-eyed opossum, and proposed those large larvae as L4. In the present study, we found both types of *G. turgidum* L3 in the liver of opossums and large larvae were identified not only by morphology but also by the sequencing of the partial ITS2 of ribosomal DNA. Together with our previous discovery of immature and mature adult *G. turgidum* in the liver of the opossums [14,15], and as shown in Tables 1 and 2 in this study, various developmental stages of *G. turgidum* appears in the liver of opossums sequentially from S–L3 to mature adults with the close relationship to the seasons of the year and the adult worms transposed finally to the stomach. These results clearly support our previous hypothesis that *G. turgidum* is the annual parasite of opossums [15].

Speciation of the genus *Gnathostoma* is primarily based on the morphological features of mature adult worms. In the case of *Gnathostoma*, morphological features of larvae have also been

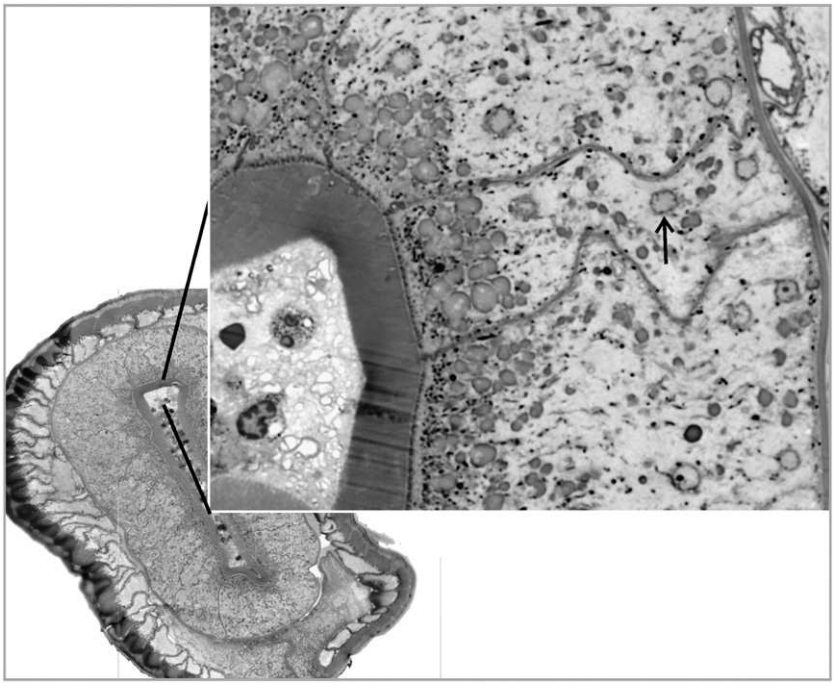

Fig. 5. Light micrograph of a semi-thin section showing intestinal epithelial cells. An arrow indicates the nucleus in a columnar cell. Note multiple nuclei in each cell.

Table 3
Morphological comparison of *Gnathostoma* AL3.

Species	Length (mm)	Hooklets					Intestine[a]		Ref.
	Mean range	I	II	III	IV	Base shape	Cells	Nuclei	
Gnathostoma spinigerum	5 4.6–5.5	43	45	47	52	Oblong	Columnar	3–7	[31]
Gnathostoma hispidum	2.9 2.5–3.3	40	41	47	48	Oblong	Cuboidal	1	[32]
Gnathostoma doloresi	3.2 2.4–3.5	37	37	34	34	Irregular	Cuboidal	1–2	[33]
Gnathostoma nipponicum	0.9 0.6–1.6	37	37	41	(−)	Oblong	Columnar	1–2	[34]
Gnathostoma procyonis	5.2 Range ND	33	37	41	45	ND	ND	ND	[40]
Gnathostoma binucleatum	4.3 2.6–5.9	38	41	44	47	Oblong	Columnar	2–3	[12]
G. binucleatum	ND 2–3.3	39	42	45	47	Oblong	Columnar	1.5–1.7	[16]
Gnathostoma turgidum S–L3[b]	2.7 1.3–4.0	34	37	36	42	Irregular	Columnar	ND	
G. turgidum L–L3[b]	9.1 7.0–11.0	31	34	36	37	Irregular	Columnar	2–12	

ND: not determined.
[a] From the reference of Akahane et al. [37] and Nawa [4].
[b] This study.

extensively studied because identification of the larvae obtained by biopsy from human gnathostomosis cases is critically important for the definitive diagnosis. Since Miyazaki [31] described in his earlier work the morphological characteristics of adults and larvae of three *Gnathostoma* species, *Gnathostoma spinigerum*, *Gnathostoma doloresi* and *Gnathostoma nipponicum*, a large amount of data on the morphology of adult and larval stages of *Gnathostoma* spp. has been accumulated by Miyazaki et al. [1–3]. In particular, scanning electron microscopic (SEM) observations facilitated the detailed observations of the fine surface structure of headbulb hooklets, cuticular spines and caudal papillae, etc. [32–36]. Moreover, Akahane et al. [37] added that the shape of the intestinal epithelial cells and the number of nuclei in cells are good morphological markers for the speciation of *Gnathostoma* spp. AL3 in tissue sections. The LM and SEM morphological features of *G. turgidum* larvae described here were markedly different from those of any other known *Gnathostoma* spp. In our study area, the southern part of Sinaloa State, at least two species, *G. binucleatum* [16] and *G. turgidum* [14], were found in wild animals. Present results clearly show that the AL3 of those two species can be easily distinguished from each other by morphology.

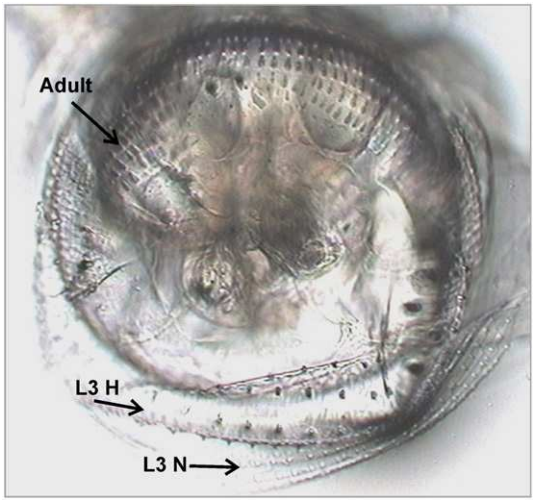

Fig. 6. Moulting of large L3 of *Gnathostoma turgidum* to adult worm. The remnant of the exsheathed cuticular surface of the head bulb of L3 (L3-H) with 4 rows of hooklets and the neck portion (L3-N) is attached to the head bulb of an adult worm (Adult) with 9 rows of hooklets.

Together with our recent discovery of mature and/or immature adult worms in the liver of opossums [14,15], the present findings of S–L3 and L–L3 of *G. turgidum* in the liver of opossums clearly indicate the requirement of a hepatic stage for the maturation of *G. turgidum* AL3 to the adult stage. Hepatic passage of *Gnathostoma* AL3 in mammalian hosts has been reported for several *Gnathostoma* species; *G. doloresi* adult worms were found in the liver of a pig in Singapore [38]; immature *G. spinigerum* worms were found in the liver of 2 out of 7 dogs in the Philippines [39]; immature *G. spinigerum* worms were also found in the livers of dogs and cats in Kyushu, Japan [31]; hepatic passage of *G. procyonis* was demonstrated by experimental infection in racoons [40]. Hepatotropic accumulation of *G. spinigerum* larvae was reported in swamp eels, the second intermediate host [41]. Although *Gnathostoma* larvae in general might have chemotactic activity towards the liver, intrahepatic growth and maturation to fully mature adults appears to be a unique feature of *G. turgidum*.

In the present study, we found a worm moulting from L–L3 to the adult stage, suggesting that L–L3 is a process of normal maturation/development. Although we examined a total of 60 S–L3 in the liver of opossums, we could not find any evidence of moulting from S–L3 to L–L3. Relatively small hooklets on the headbulb and small cuticular spines of L–L3 shown in this study also suggest the growth of the body without moulting. These findings are compatible with the previous findings of pre-moult, moulting and post-moult stages of *G. doloresi* larvae in the gastric wall of wild boars in Japan [42] and strongly support their proposed concept that *Gnathostoma*, *Spiroxys*, and presumably, all other gnathostomatoids have only four stages, i.e., three larval stages and one adult stage, in their life cycles. Intrahepatic growth without moulting of *G. spinigerum* L3 was clearly demonstrated by experimental infection in mice [43,44]. Although Almeyda-Artigas et al. [19] speculated that two large larvae of *G. turgidum* found in four-eyed opossums were the 4th stage larvae, the materials were obtained from a paratenic host, whereas our materials were all from the natural definitive hosts. We are now conducting a more extensive survey to clarify whether L4 exists in the developmental stages of *G. turgidum*.

In conclusion, our results describe the unique growth/maturation process of *G. turgidum* from L3 larvae to the mature adult stage in the liver of the definitive opossum host.

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