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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

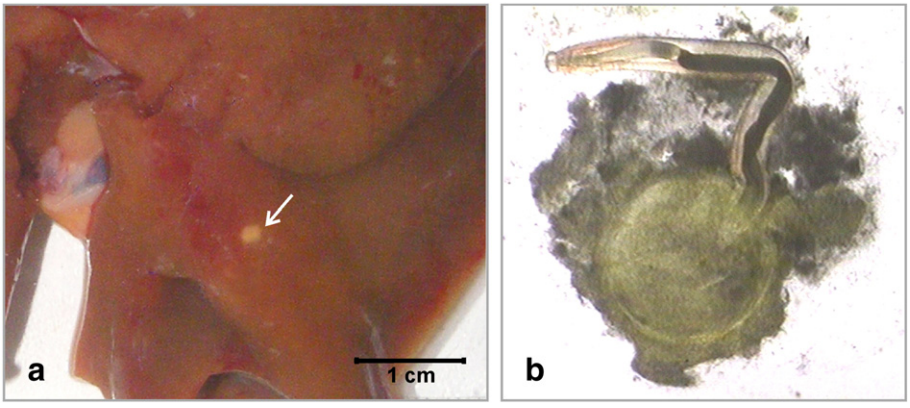

Fig. 1. Encapsulated *Gnathostoma* larva found in the liver of an opossum. a: cyst in the liver. b: a larva emerging from the cyst.