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ORIGINAL PAPER

Finding advanced third-stage larvae of *Gnathostoma turgidum* Stossich, 1902 in Mexico from natural and experimental host and contributions to the life cycle description

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Abstract In order to clarify the role of *Gnathostoma turgidum* as an etiological agent involved in human gnathostomiasis in Mexico, establish the taxonomic identity of the advanced third-stage larvae ($AdvL_3$), and contribute to the knowledge of its life cycle, experimental host infections, examination of potential natural hosts, and morphological comparisons were carried out. Examination of ten species of potential hosts at San Pedro las Playas and Tres Palos Lagoon in Guerrero state, Mexico revealed that two (*Kinosternon integrum* and *Rana zweifeli*) were infected by 15 $AdvL_3$ of *G. turgidum*. A specific identity was obtained comparing these larvae with those recovered from hosts experimentally infected. The $AdvL_3$ measured 1.6 mm in length, with two cervical papillae (both in 12th row) and an excretory pore on the 19th row. The average of cephalic hooklets, from first to fourth row, was 30.8, 34.0, 36.7, and 39.6, respectively. This is the first record of $AdvL_3$ of *G. turgidum* in America, and it represents a significant contribution for the understanding of the life cycle of this species.

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Introduction

Thirteen species of the genus *Gnathostoma* Owen 1836 are considered as valid records. Six were recorded in Europe (from species coming from Asia): *Gnathostoma spinigerum* Owen 1836, *Gnathostoma hispidum* Fedchenko 1872, *Gnathostoma doloresi* Tubangui 1925, *Gnathostoma nipponicum* Yamaguti 1941, *Gnathostoma vietnamicum* Le-Van-Hoa 1965, and *Gnathostoma malaysiae* Miyazaki and Dunn 1965; the other seven were identified in America: *Gnathostoma sociale* Leidy 1858, *Gnathostoma turgidum* Stossich 1902, *Gnathostoma procyonis* Chandler 1942, *Gnathostoma miyazakii* Anderson 1964, *Gnathostoma americanum* Travassos 1925, *Gnathostoma binucleatum* Almeyda-Artigas 1991, and recently *Gnathostoma lamothei* Bertoni et al. 2005.

Except for *G. malaysiae*, there are detailed descriptions of advanced third-stage larvae ($AdvL_3$) for all the species mainly distributed in Asia. However, for the species registered in America, descriptions have been documented only for *G. procyonis* (Ash 1962) and *G. binucleatum* (Almeyda-Artigas 1991; León-Règagnon et al. 2002). For *G. turgidum*, descriptions are based only on the adult stage (Stossich 1902; Travassos 1925) and occasional reports in Mexico (Lamothe et al. 1998; Almeyda-Artigas et al.

2000). Due to lack of information on $AdvL_3$, its life cycle is poorly known (León-Règagnon et al. 2002; Kifune et al. 2004).

Gnathostomes follow a three-host life cycle with a copepod as the first intermediate host harboring the development of the second larval stage and a carnivorous mammal as the definitive host (Almeyda-Artigas et al. 2000). Since there is no record in America on the infection of fish with *G. turgidum* $AdvL_3$, it may be possible for the definitive host to acquire the infection by drinking water containing copepods infected with early third stage (EaL_3). Although this is not a common means of infection, it has been observed (Golovin 1956) that the presence of a second intermediate host is not required in *G. hispidum*. The aim of this paper was to clarify the life cycle of *G. turgidum*, describing the $AdvL_3$ for the first time, and to provide key information about its life cycle.

Materials and methods

Specimens were collected during February and June 2000 and September 2001 at San Pedro las Playas and Tres Palos Lagoon in the state of Guerrero, Mexico (16°41′ to 16°50′ N and 99°37′ to 99°47′ W). The search for *G. turgidum* $AdvL_3$ was carried out by examining muscle and liver of different organisms (Pisces, Amphibia, Reptilia, Aves, and Mammalia). The muscle layer was separated and sliced and every slice was pressed between two glass plates (8.5× 11.0×0.5 cm) and examined under a stereo-microscope. The livers were digested with artificial gastric juice (7 g pepsin in 10 ml HCl/1,000 distilled water) during 4 h at 40°C to harvest any larvae overlooked. After digestion, the resulting liquid was examined using a stereo-microscope.

The process for obtaining the experimental $AdvL_3$ began with cyclopoid copepods experimentally infected with second stage larvae (L_2) obtained from the eggs of a female extracted from an opossum (*Didelphis virginiana*), following the procedure established by Almeyda-Artigas et al. (1995). Subsequently, the copepods infected with EaL_3 were utilized for infecting *per os* different second intermediate hosts: Pisces—guppies *Poeciliopsis gracilis* (30), nile tilapia *Oreochromis niloticus* (30); Amphibia—northern leopard frogs *Rana pipiens* (5) and rana montezume *Rana montezumae* (1), toads *Bufo* sp. (2); and Mammalia—white mice *Mus musculus* (3). These organisms were purchased in a local market and kept under appropriate conditions. The hosts were euthanized at regular intervals and their musculature and viscera were examined by stereo-microscopy in order to determine the number of larvae considering days post-infection (DPI). Five young opossums were born and maintained in the laboratory for infection purposes; they showed no evidence of parasite eggs in their feces. One opossum was fed 30 guppy fish *P. gracilis*, which were infected with copepods with EaL_3, on the assumption that they were infected with $AdvL_3$ over 30 DPI. The other four opossums received the copepods infected with EaL_3 orally using a catheter. They were relaxed using Rompum® and killed with a lethal dose of Anestesal® injected in their hearts.

The recovered larvae were fixed in hot 70% ethanol and cleared in Amann lactophenol. They were studied on temporary wet mounts on glass slides and later stored in 70% ethanol. All measurements are given in micrometers, unless otherwise stated, and they are presented as a range, followed by the mean±SD in parentheses. Photomicrographs were obtained using a Kodak Technical Pan black and white film. Specimens were deposited at the "Colección Helmintológica de la Universidad Autónoma Metropolitana Unidad Xochimilco (CHUAMX)", Mexico City, Mexico as follows: CHUAMX-G255 (four larvae from *K. integrum*), CHUAMX-G475 and 476 (11 larvae from *R. zweifeli*), CHUAMX-G342, 357, 387–389 (532 larvae from *R. pipiens*), CHUAMX-G416 (three larvae from *R. montezumae*) CHUAMX-G384-386, 305, 308, 315, 321, 334–335 (15 larvae from *Poecilopsis gracilis*), CHUAMX-G310 (11 larvae from *Bufo* sp.), and CHUAMX-G340, 358–359 (seven larvae from *M. musculus*). Pisces were identified according to Espinosa-Pérez et al. (1993); Amphibia and Reptilia following Flores-Villela and Canseco-Marquez (2004), and Hillis and Willcox (2005), Hillis (2007), Aves as specified by AOU (1998), and Mammalia following Emmons and Feer (1997). The infection parameters utilized are those proposed by Bush et al. (1997).

Results

Natural host

A total of 396 organisms, from ten vertebrate species, were examined. Only two species were found naturally infected with $AdvL_3$ of *G. turgidum*: one turtle *K. integrum*, with four larvae located in the musculature, and three of the five frogs, *R. zweifeli*, infected showing eight and two parasites and one parasite, respectively. All larvae were found encysted in the musculature. The prevalence was about 60%, the intensity 1–8, the mean intensity 3.6, and the abundance 2.2. Moreover, two frogs were infected with 55 and 26 $AdvL_3$ of *G. binucleatum*, respectively. Each of the eight remaining species tested positive for $AdvL_3$ of *G. binucleatum* (data not shown); the 390 individuals consist of 322 "popoyote" *Dormitator latifrons* (Pisces: Eleotridae), 17 "charra" *Cichlasoma trimaculatum* (Pisces: Cichlidae), 37 *Poecilia butleri* (Pisces: Poeciliidae), three toads *Bufo marinus* (Amphibia: Anura), one *Thamnophis valida*

(Reptilia: Colubridae), four brown pelican *Pelecanus occidentalis* (Aves: Pelecanidae), five great egret *Ardea alba* (Aves: Ardeidae), and one striated heron *Butorides striata* (Aves: Ardeidae).

Experimental host

The $AdvL_3$ were extracted from every experimentally infected organism; their number varied according to the species of the hosts. The highest infection parameters were registered in amphibians (Table 1). Only those collected from frogs were found to be surrounded by a fibrous cyst. In general terms, when fish were infected, the infection "disappeared" quickly, in contrast to the frog, *R. pipiens*, where it remained up to 279 DPI showing intensities from 23 to 419.

All the opossums experimentally infected via copepods were negative to $AdvL_3$ or immature forms after 90–180 DPI. In contrast, two worms were recovered and isolated 135 DPI from the liver of the opossum infected through fish (one of the worms was an $AdvL_3$ and the other was molting to the juvenile stage). The cuticle shed from the molting worm is of the $AdvL_3$, whereas the new cuticle completed beneath is identical to that of the adult (unpublished data), showing also the same arrangement of the cephalic bulb hooklets and body spines. Moreover, the worm possesses genital papillae and spicules. Hence, they were adults (Imai and Hasegawa 2001).

Descriptions

There were no significant differences between the $AdvL_3$ of *G. turgidum* obtained from natural and experimental hosts. However, a slight increase in the size was observed in those specimens obtained naturally. This may be attributed to the density of the parasite population on the host.

Advanced third-stage larvae Based on 11 $AdvL_3$ extracted from *R. zweifeli*. Body is 1,530–2,007.4 (1,670.2±127.8) long and 134.6–160.4 (140.8±7.5) wide. Cuticle is covered with small spines arranged in 182–202 (188.6±7.1) transverse rows over the entire body. Esophagus is 579.4–722.2 (648.0±50.3) long and 69.4–85.7 (80.8±4.8) wide, occupying 35.7–42.7% (38.9±2.4) of body length. The four cervical sacs extend back 302.9–435.0 (350.0±43.7) long in average, occupying 45.2–63.5% (54.0±5.4) of the esophagus length. Two cervical papillae, the left located at 10–14 (11.7±1.4) and the right at 11–14 (12.0±0.9) rows of the body, excretory pore is at 15–22 (19.7±2.0) rows behind head bulb. One caudal papilla located at 408.0–742.6 (563.5±129.3) to posterior extremity. Genital primordium at 979.2–1,154.6 (1,083.3±65.2, $n=7$) occupying 60.4–71.6% (66.7±3.6) of body length. Tail is 24.4–40.8 (29.2±5.5) long. Cephalic bulb is 36.7–65.0 (46.6±8.0) long and 97.9–118.3 (110.3±5.8) wide (Fig. 1a). The number of cephalic hooklets from rows 1 to 4 were 26–34 (30.8±2.8), 29–38 (34.0±2.7), 29–43 (36.7±3.6), and 33–42 (39.6±2.7), respectively. The hooklets in the fourth row are smaller than those located in the three previous rows, which show the same size (Fig. 1b). They all show a sharp point like the claw of a cat. Completely developed larvae were found inside a fibrous cyst, 0.6 mm in diameter (Fig. 1c). The encysted larva is small and shows a stumpy appearance, except the intestine, which is light brown; the body is colorless. It has no colored liquid in the body cavity.

Molting worm The worm is covered by a double cuticle; on the outer cuticle, four transverse rows of hooklets were observed on the cephalic bulb, similar to those shown at $AdvL_3$ stage (Fig. 2a). The inner cuticle is covered by nine complete and one incomplete transverse rows of hooklets on the cephalic bulb and multidentates body spines densely distributed which are overlapped in the anterior body and single in the posterior body (Figs. 2b, c), as it occurs in adult stage (unpublished data). Body is 8,249.72 long by 499.72 wide. Cephalic bulb is 179.52 long by 367.2 wide. Esophagus is 1,765.14 long by 290.16 wide, occupying 40% of the body length. One cervical papilla was observed in the 16th row of the cuticle present in the $AdvL_3$, and the other one in the 11th row of the juvenile cuticle. The four cervical sacs are 701.22, 693.16, 733.46, and 701.22 long;

Table 1 Data of infections with *G. turgidum* EaL_3 obtained from different experimental second hosts

Species	*N*	*I*(Pr)	DPI	P%	IP	*A*	Site of infection
Poecilipsis gracilis	30	6(15)	17–59	20.0	2.5	0.5	mus/li
O. niloticus	30	2(2)	12–13	6.6	1.0	6.6	mus
R. pipiens	5	5(532)	74–279	100.0	106.4	106.4	mus/li
R. montezumae	1	1(3)	292	100.0	3.0	3.0	mus
Bufo sp.	2	2(15)	30	100.0	7.5	7.5	mus
M. musculus	3	3(7)	15–51	100.0	2.3	2.3	mus

N number of host examined, *I* number of host infected, *Pr* number of parasites, *DPI* days post-infection, *P%* prevalence, *IP* mean intensity, *A* abundance, *mus* muscles, *li* liver

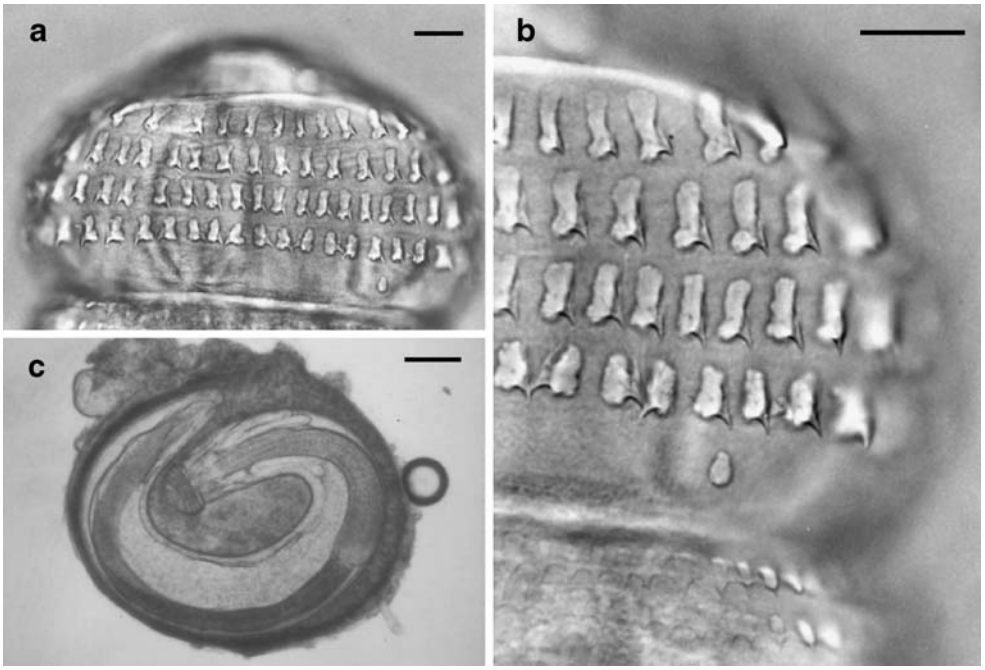

Fig. 1 Advanced third-stage larvae from *G. turgidum* collected from *R. zweifeli*. **a** Lateral view of cephalic hooklets from rows (scale, 10 μm). **b** Front view of the cephalic bulb and hooklets rows (scale, 10 μm). **c** Encyst in the host musculature (scale, 100 μm)

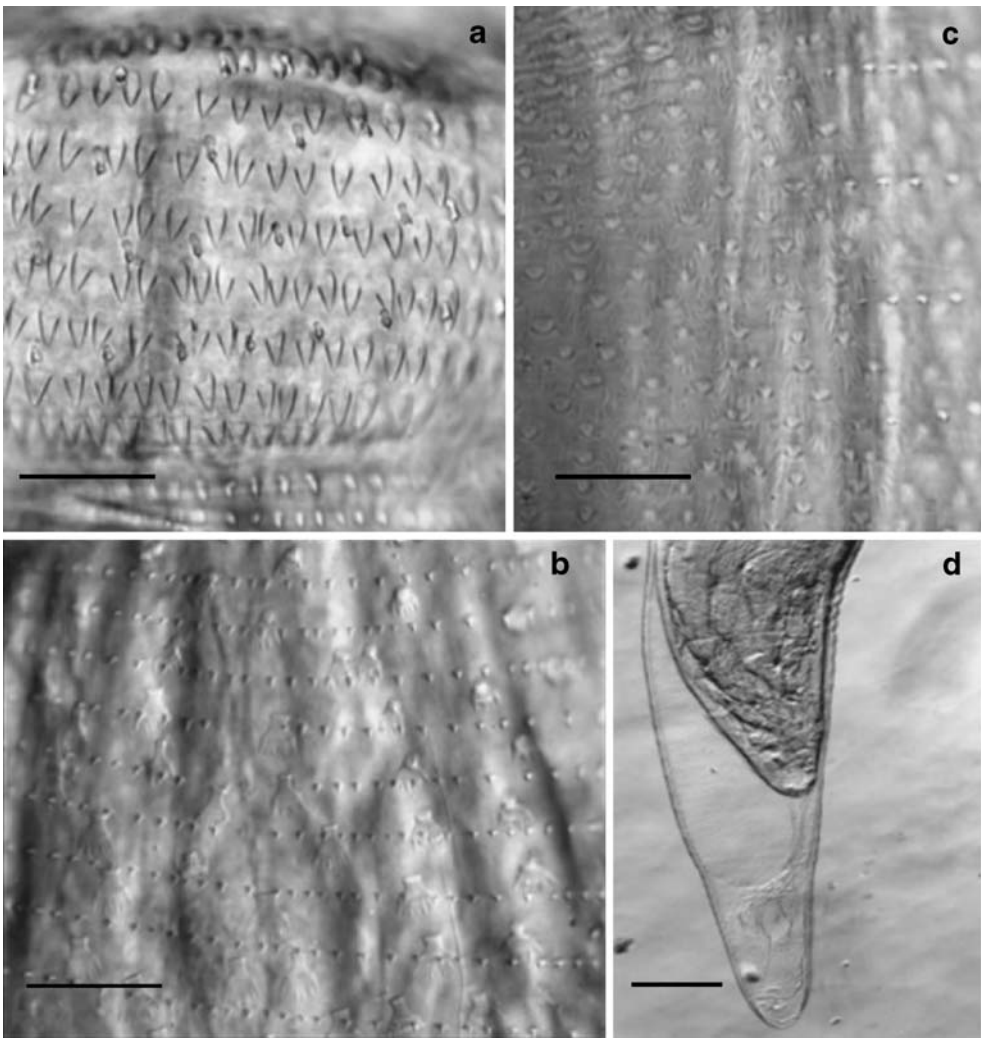

Fig. 2 Molting of *G. turgidum* $AdvL_3$ to adult stage in experimental host opossum *D. virginiana*. **a** Arrangement of hooklets on the cephalic bulb, on the outer and inner cuticle (scale, 50 μm). **b** Body spines at cervical papillae level (scale, 50 μm). **c** Body spines at esophagus–intestine junction (scale, 50 μm). **d** Detached outer cuticle at the posterior end (scale, 100 μm)

the distal extreme is noticeably wider. On the caudal extreme, the loose molt is 692.58 long, with single-tipped hooklets. Tail 163.2 long (Fig. 2d).

Discussion

On the basis of general morphology, the position of the cervical papilla and the excretory pore, the shape and number of hooklets in the rows of the cephalic bulb, and the amount of transverse striations are specific characteristics on the identification of the species of *Gnathostoma* at the larvae level (Miyazaki 1954; Koga et al. 1994; Akahane et al. 1994). The $AdvL_3$ of *Gnathostoma* have two cervical papillae (but most published records of $AdvL_3$ report only one). In *G. binucleatum*, the papilla is located between rows 12 and 13, while *G. turgidum* shows both papillae between rows 9 and 14 (12.0). Considering these intervals, there is an evident overlapping between these species, thus making it difficult to differentiate between them using this criterion. However, the position of the excretory pore presents a key character for distinguishing *G. turgidum* from *G. binucleatum*. On average, the excretory pore in *G. binucleatum* is located in row 28, while in *G. turgidum*, it appears on rows 19–25 (22.0). Moreover, the average number of transverse rows of cuticular spines is less than 200. This is another good criterion for distinguishing this species from *G. binucleatum*. An evident difference between *G. binucleatum* and *G. turgidum* is the lower quantity of hooklets in the latter, which present an average of seven hooklets on the four rows of the cephalic bulb; meanwhile, there are no convincing differences between *G. turgidum* and *G. procyonis* (Table 2). However, the main difference between the American species of *Gnathostoma* is the smaller size of *G. turgidum*, which is about two or three times smaller than *G. binucleatum* and *G. procyonis*, and the hooklets in the fourth row of the cephalic bulb are smaller than those present in the three previous rows, which, in the case of *G. turgidum*, have the same size (Fig. 1b). In contrast, as it can be observed in Fig. 3 of Almeyda-Artigas et al. (1994), the hooklets from *G. binucleatum* show the same size in all the rows of the head bulb. Furthermore, the colorless characteristic of the body of living $AdvL_3$ in *G. turgidum* and the lack of colored liquid, may be considered as another relevant characteristic for separating this species from *G. binucleatum* whose larvae have a conspicuously dark brownish alimentary tract and a celomic fluid deep bloodred throughout its length (Almeyda-Artigas et al. 1994). Because the $AdvL_3$ of *G. lamothei* has not been obtained in experimental or natural conditions yet, it is not possible to make a comparison.

Among the species of *Gnathostoma*, *G. hispidum* is the only one capable of producing an $AdvL_3$ when the

Table 2 Morphometric comparison of advanced third stage larvae of *Gnathostoma* spp.

Species (Reference)	Cervical papillae in rows[a]		Excretory pore in rows[a]	Length	Rows in body	Number of cephalic hooklets from rows				
	A	B				I	II	III	IV	IV-I
G. binucleatum (Almeyda-Artigas 1991)	12–13[b]	ND	28[b]	2,600–5,900 (4,300.0)	240[b]	35–44 (38.7)	38–47 (42.4)	40–49 (44.7)	43–52 (48.2)	9.5
G. procyonis (Ash 1962)	ND	ND	ND	(5,200.0)	ND	29–36 (32.7)	32–40 (36.6)	37–45 (41.0)	42–47 (45.0)	12.3
G. turgidum Present study[c]	10–14 (11.4)	10–20 (13.4)	19–24 (21.4)	1,374.6–1,640.2 (1,496.3)	169–194 (181.2)	29–33 (31.6)	31–37 (34.6)	35–41 (38.0)	41–46 (43.4)	11.8
G. turgidum Present study[d]	9–14 (11.8)	9–13 (11.6)	19–25 (21.6)	1,350.5–1,689.1 (1,493.8)	172–210 (195.0)	28–34 (31.3)	29–39 (34.0)	33–40 (37.0)	38–45 (41.8)	10.6
G. turgidum Present study[e]	10–14 (11.7)	11–14 (12.0)	15–22 (19.7)	1,530.0–2,007.4 (1,670.2)	182–202 (188.6)	26–34 (30.8)	29–38 (34.0)	29–43 (36.7)	33–42 (39.6)	8.80
G. turgidum Present study[f]	10–13 (11.3)	12–12 (12.0)	19–20 (19.3)	1,787.0–2,021.9 (1,876.3)	184–210 (199.0)	33–36 (33.5)	34–36 (35.0)	38–41 (38.8)	39–47 (43.0)	9.5

ND no data
[a] Ordinal numbers show position of transverse striations
[b] Almeyda-Artigas et al. 1994
[c] From five $AdvL_3$ with 18 days post-infection in *P. gracilis*
[d] From 30 $AdvL_3$ with 92 days post-infection in *R. pipiens*
[e] From 11 $AdvL_3$ collected in *R. zweifeli*
[f] From four $AdvL_3$ collected in *K. integrum*

definitive host drinks water containing infected copepods (Golovin 1956). The results obtained from this research support the existence of a second intermediary host in the life cycle of *G. turgidum*, mainly based on: (1) $AdvL_3$ recovered from naturally infected frogs of *R. zweifeli* which may act as second intermediary host that acquired the infection by consuming infected copepods during its tadpole phase; (2) the $AdvL_3$ recovered from naturally infected turtle *K. integrum* which may act as a paratenic host that acquired the infection when it consumed tadpoles, frogs, or small fish; (3) the development of $AdvL_3$ to juvenile stage in the liver of their definitive host; and (4) the negative results to the experimental infection of the opossums using copepods infected with EaL_3.

In this sense, the role of the amphibians as intermediary hosts on the life cycle takes the place of the most important intermediary host which, in other species, is performed by fish. This assessment relies on the fact that there are no records of fish in the published findings of *G. turgidum* $AdvL_3$ from Tres Palos Lagoon (León-Règagnon et al. 2000; Martínez-Salazar and León-Règagnon 2005), low Papaloapan River basin (Lamothe-Argumedo et al. 1989; Almeyda-Artigas 1991; Almeyda-Artigas et al. 1994), Pantanos de Centla, Tabasco (Kifune et al. 2004), lagoons and lake in southern Sinaloa (Díaz Camacho et al. 2002, 2003), and Laguna de Agua Brava in Nayarit, Mexico (León-Règagnon et al. 2002; Álvarez-Guerrero and Alba-Hurtado 2007). Moreover, the larvae reported in these publications do not fit, in taxonomical terms, with those described in the current paper.

Lamothe-Argumedo and Osorio-Sarabia (1998) report fishes, amphibians, reptilians, and birds from Mexico as intermediary and paratenic hosts of *Gnathostoma* spp. $AdvL_3$. These authors state that the $AdvL_3$ present differences in the number of cephalic hooklets by row, on the larval intestinal nuclear pattern, and on the extension of the armed cuticular surface. Consequently, they conclude that there is a great difference between these larvae and those of *G. binucleatum* (Almeyda-Artigas 1991); however, based on the existence of a plurispecific complex responsible for human gnathostomiasis, they decided to report these organisms as *Gnathostoma* sp. In light of the current research, we consider that the differences observed by Lamothe-Argumedo and Osorio-Sarabia (1998) on the $AdvL_3$ are due to intraspecific differences of *G. binucleatum* and not because of interspecific differences. Furthermore, thus far, there is no evidence of the participation of birds in the life cycle of *G. turgidum*. On the other hand, recovering an adult from an opossum in the experimental infection using infected fish cannot be conclusive regarding this method of infection; therefore, this finding does not confirm that it occurs in natural conditions. The high seasonality of the adult stage in stomach and in the liver found in this research in the definitive host is associated with the synchronized recruitment of $AdvL_3$ during the mating season of frogs (unpublished data). It supports the assertion that the frogs and not the fish are the second intermediate host.

In Mexico, the human gnathostomiasis has been attributed to $AdvL_3$ of *G. binucleatum* (Almeyda-Artigas 1991; Almeyda-Artigas et al. 2000; León-Règagnon et al. 2002). Although Vidal-Martínez et al. (2001) have suggested that *G. turgidum* is also involved in Mexican human cases, these authors do not provide evidence that support this statement. Because most of the human reports in Mexico are linked to the ingestion of raw fish, we consider that *G. turgidum* is not responsible for the human gnathostomiasis in this country. Thus, the possibility of *G. turgidum* $AdvL_3$ producing a larva migrans-type syndrome must be related to the ingestion of raw or undercooked frog meat.

Our evidence shows that the life cycle of *G. turgidum* begins when eggs are passed with feces of the definitive host into water. Once the eggs are in contact with the water, the development of the first-stage larva (L_1) occurs. Upon hatching, a rapidly moving L_2 develops while retaining the sheath. The cuticle smoothens and forms a spine-like structure (larval tooth) on its anterior end which is useful both for exsheathment and for penetrating the wall of the digestive tract of the copepod. Once L_2 is ingested by a copepod intermediate host, the larva loses its sheath in the intestine and migrates into the hemocoel of the copepod. After that, the larva grows and molts to EaL_3. When the infected copepod harboring the EaL_3 is eaten by a second intermediate host, such as a tadpole, the parasite develops into the $AdvL_3$ encysting in the musculature and remaining in this host even when it becomes an adult frog. In the water, before tadpoles metamorphose, the $AdvL_3$ can be transferred to a paratenic host such as turtles. Once out of the water, the offspring frogs pass the infection to the definitive host, the opossum. In the definitive host, the larva necessarily migrates to the liver to achieve its juvenile phase where it remains for a long time; later on, it passes to the stomach where it reaches the adult stage in order to begin a new cycle.

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