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ORIGINAL PAPER

Description and development of the early third-stage larva of *Gnathostoma turgidum* Stossich, 1902 (Nematoda: Gnathostomatidae) and contributions to its life cycle

Miguel Ángel Mosqueda-Cabrera · Roberto Javier Almeyda-Artigas · Elizabeth Sánchez-Miranda · Laura Carranza-Calderón · Edmundo Sánchez-Núñez

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Abstract The egg and larval stages of *Gnathostoma turgidum* were examined using light microscopy. Fertilized uterine eggs are 65.97 long and 32.28 wide, oval, brownish, with two cap-like thickenings. The eggshell surface is covered with numerous irregularly shaped pits of various sizes and depths. A sheathed second-stage larva emerges from the egg, measures 178×9; the sheath measures 243×21. Development to early third-stage larva in the coelomic cavity of cyclopoid copepods is similar to that described for other gnathostome species. After 10 days at 27°C, the larvae undergo a molt (the second for gnathostomes) and develop to early third stage. The body of this stage measures 412.3×40.1, with evident hemispherical cephalic bulbs. Cephalic bulbs measure 25×40, armed with four transverse rows of sharp hooklets. The average number of hooklets in each row is 31, 34, 37, and 42, respectively. The whole body is covered with 193 transverse rows of small single-pointed cuticular spines. One pair of cervical papillae and an excretory pore are present on the anterior part of the body. On the other hand, potential species-specific features regarding the latter larval stage are discussed. Finally, some *G. turgidum* life cycle considerations are portrayed.

M. Á. Mosqueda-Cabrera (✉)
Departamento El Hombre y su Ambiente,
Universidad Autónoma Metropolitana-Xochimilco,
Calzada del Hueso No. 1100, Col. Villa Quietud,
Delegación Coyoacán,
04960 México, D. F., Mexico
e-mail: zitzitl@correo.xoc.uam.mx

R. J. Almeyda-Artigas
Laboratorio de Sanidad Acuícola y Parasitología Molecular,
Departamento El Hombre y su Ambiente,
Universidad Autónoma Metropolitana-Xochimilco,
Calzada del Hueso No. 1100, Col. Villa Quietud,
Delegación Coyoacán,
04960 México, D.F., Mexico
e-mail: almeyda@correo.xoc.uam.mx

E. Sánchez-Miranda
Departamento de Farmacobiología,
Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional,
Calzada de los Tenorios No. 235, Col. Granjas Coapa,
Delegación Tlalpan,
14330 Tlalpan, Mexico
e-mail: smirandae@gmail.com

L. Carranza-Calderón · E. Sánchez-Núñez
Asesores en Medio Ambiente Educación y Tecnología,
Edificio 51A-201, Lindavista-Vallejo,
Delegación Gustavo A Madero,
07720 México, D.F., Mexico

E. Sánchez-Núñez
e-mail: edmundo@asemet.com

Introduction

Gnathostoma turgidum Stossich, 1902 is a common nematode in the Americas. Adults live within the stomach wall of opossums. Recently, natural advanced third-stage larvae ($AdvL_3$) encysted in the musculature of *Rana zweifeli* were documented in Mexico (Mosqueda-Cabrera et al. 2009).

Gnathostomes life cycles have been reproduced experimentally (Prommas and Daengsvang 1933; Miyazaki 1954, 1960; Ash 1962; Daengsvang 1972, 1973, 1980; Koga and Ishii 1981; Koga et al. 1987; Ando et al. 1989; Almeyda-Artigas et al. 1995). In summary, sheathed second-stage larvae (L_2) hatch from eggs and swim actively in freshwater. After that, they are ingested by cyclopoid copepods and rapidly move toward the coelomic cavity of

the crustaceans, where the development and molting to the next stage—early third stage (EaL_3)—takes place.

Although three species of gnathostomes have been reported in Mexico: *Gnathostoma binucleatum* Almeyda-Artigas, 1991 (Almeyda-Artigas 1991), *G. turgidum* (Lamothe-Argumedo and Osorio-Sarabia 1998; Almeyda-Artigas et al. 2000), and *Gnathostoma lamothei* Bertoni-Ruiz, García-Prieto, Osorio-Sarabia, and León-Règagnon, 2005 (Bertoni-Ruiz et al. 2005), the description of the larval stages has only been made for the former species (Almeyda-Artigas et al. 1995). Therefore, the current study includes the descriptions of the egg, L_2 and EaL_3, and the development from L_2 to EaL_3 of *G. turgidum* in the first intermediate host.

Material and methods

Eggs teased from the uteri of adult *G. turgidum* females [collected from the stomach of one opossum *Didelphis virginiana* from San Pedro las Playas town near Tres Palos Lagoon, in the state of Guerrero, Mexico (16°49′06.59″ N, 99°43′13.18″ W)], were allowed to embryonate and hatch for 2 weeks in Petri dishes containing a shallow layer of aerated dechlorinated water (culture water) with gentamicin (50 μg/ml). Water was daily changed until hatching. The sheathed larvae in the Petri dishes were fed to cyclopoid copepods, *Mesocyclops* sp. and *Thermocyclops* sp., collected from Xochimilco Lake in Mexico City (19°17′10.22″ N, 99°06′07.67″ W). The crustaceans were maintained in the laboratory for two more weeks, in groups of five, in small glass vials containing 25 ml of culture water, by providing them wild type *Paramecium* spp. collected from a small permanent pond near to the Universidad Autónoma Metropolitana-Xochimilco campus (99°17′59.81″ N, 99° 05′58.39″ W) and cultured on hay infusion following Knudsen (1972). Every day ten larvae were studied to record morphological changes. All the above experiments involving eggs, L_2, copepods, and ciliates were conducted at 27°C. In order to collect the larvae, hosts were dissected using fine needles. The larvae recovered were washed in physiological saline and fixed in hot 70% ethanol and cleared in Amann lactophenol. All measurements are given in micrometers, unless otherwise stated, and are presented as a range, followed by the mean±standard deviation in parentheses. Photomicrographs were obtained by using a Kodak® Technical Pan® black and white film. Voucher specimens were deposited at the "Colección Helmintológica de la Universidad Autónoma Metropolitana, Unidad Xochimilco (CHUAMX)," Mexico City, Mexico as follows: CHUAMX-G132 (eggs), CHUAMX-G280 (second-stage larvae), and CHUAMX-G283 (106 early third-stage larvae from *Mesocyclops* sp.).

Results

Egg Fertilized uterine eggs are oval, brownish in color and are in the one-cell stage. Measurements of ten eggs are 64.96–66.99 (65.97±2.17) μm in length and 30.45–32.48 (32.28±1.43) μm in width. The eggshell surface is covered with numerous irregularly shaped pits of various sizes and depths. There are cap-like thickenings in both ends through which the L_2 escapes at the time of hatching (Fig. 1a). Eggs hatch spontaneously between the eighth and the 11th day.

Second-stage larvae The newly hatched L_2 show very active flexible movements and are enveloped in a delicate, transparent, smooth and very voluminous sheath, much longer and wider than themselves. The anterior extremity of the larvae is round and armed with a minute solid spine-like structure, sometimes called "larval tooth." The anterior half of the body is uniform in thickness while the posterior half gradually tapers and terminates in a rounded tip. The body is (178) μm long and (9) μm wide. The sheath is (243) μm long and (21) μm wide (Fig. 1b). This partly chitinized

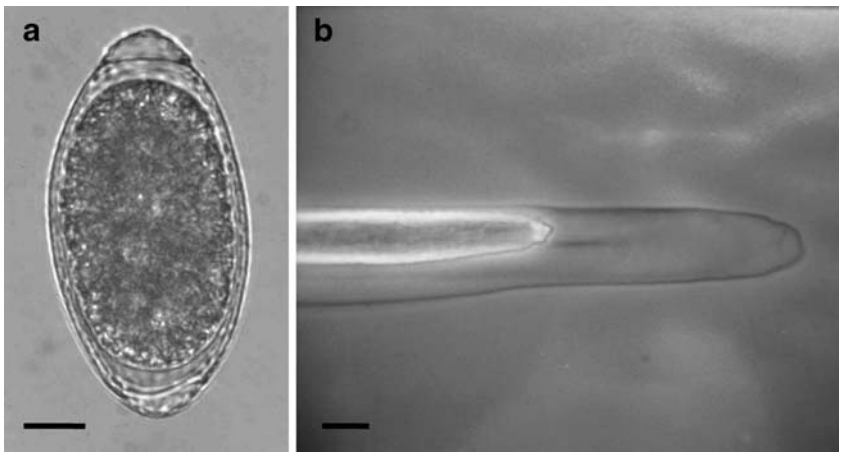

Fig. 1 *Gnathostoma turgidum*. **a** One-cell stage fertilized uterine egg, showing the two polar cap-like thickenings. **b** *Anterior end* of a second-stage larva within a very voluminous sheath, showing the partly chitinized tooth-like structure (*scale bars*—10 μm)

structure is employed, in the first place, to cast off their sheaths, and then, to pierce the gastric wall of the crustacean first intermediate host after it is ingested. We observed them in the stomach cavity immediately following ingestion by the cyclops without sheath and beginning to molt. However, we observed sheathed embryos within the intestine of copepods some moments after ingestion and within the body cavity of their hosts. We also observed copepods expelling larvae through their anal openings some minutes after they had ingested them, using intestine contraction movements. This suggests that the L_2 must pierce and leave immediately the digestive system of the host and find protection in its coelomic cavity, where they can continue their development.

Development from L_2 to EaL_3 In the body cavity of a cyclops, the L_2 undergo metamorphosis. Two hours after the copepod ingests the L_2, the latter become slightly shorter; the most evident morphological change takes place in the anterior extremity, where the larval tooth is replaced by two small unequal transparent lips. The growth of these larvae occurs very quickly and can be observed since the first day. Indeed, they reach a size of (280) μm by (24) μm at the end of the fourth day. Maximum growth occurs between the fifth and the sixth days from (316) μm to (360) μm. The lips continue to expand, and behind that area two small groups of proliferating cells, representing the primordial of the cervical sacs start to appear. Both the esophagus and the intestine begin as regions of large granules. The posterior end tapers towards the tail; the anus is subterminal. By the eighth day, the posterior extremity is wide and round, with a clear space between the body wall and the transparent and loose external cuticle (L_2 molted epicuticle). The anterior extremity is more bulb-like in appearance and the lips become proportionally smaller. The cuticle posterior to the future cephalic bulb bears minute rudimentary spines. The cervical sacs extend to the level of the nervous ring. The esophagus and intestine are less granular and more tubular; in this day, larvae are (364) μm long by (36) μm wide. Towards the end of eighth day and during the ninth day within the copepod, the larvae complete their development and molt to EaL_3. The early phase of this final phase is marked by the appearance of a cephalic bulb with hooklets in four transverse rows and a pair of prominent trilobed symmetrical lips. The larval movements inside the body cavity of the host (which is at first very active gradually diminishes as it becomes older) is almost imperceptible at this moment, except for occasional slow shifts of its anterior end. Once the larvae reach maturity, they show no further change in morphology even when kept in the crustacean for up to 22 days.

Early third-stage larvae The following data are based on 30 specimens. The body is 336.98–470.96 (412.26±33.2) μm long and 36.54–46.69 (40.86±2.9) μm wide. The cephalic bulb is 22.33–28.42 (25.0±1.3) μm long and 36.54–42.69 (40.39±2.3) μm wide. The number of cephalic hooklets per row are 27–34 (30.76±2.0), 30–39 (34.14±1.9), 30–43 (36.97±2.4), and 37–46 (41.55±2.2), respectively. The hooklets exhibit rectangular base; those of the fourth row are smaller (25×1) than those of the three previous rows (35×2; Fig. 2). The cuticle is covered with small single-pointed spines arranged in 180–204 (186.8±10.4) transverse rows over the whole body. Two cervical papillae are present, the left one located at rows 10–13 (12.0±0.9) and the right one at rows 10–13 (11.86±0.9) of the body; the excretory pore is located between rows 19–24 (21.48±1.3) behind the cephalic bulb. The esophagus is 152.25–205.03 (180.87±13.1) μm long and 18.27–32.53 (21.17±2.9) μm wide, occupying 39.10–50.30% (43.65±3.1) of the body length. Two pairs of fully developed transparent contractile cervical sacs are present, in the anterior half of the body, one on each side of the esophagus; one of them over two thirds of the esophagus total length (145.96±15.9) and (156.85±19.8) μm and the other, longer (188.54±13.3) and (203.53±17.7) μm. The tail is 10.15–24.00 (14.95±3.9) μm long. The coelomic fluid is colorless, and the intestine is yellowish in color. An undifferentiated genital primordium is localized beyond the esophagus–intestine junction.

Discussion

In broad terms, larval development of *G. turgidum* is similar to that described for other gnathostome species: *G. binucleatum* (Almeyda-Artigas et al. 1995), *Gnathostoma hispidum* (Daengsvang 1972; Daengsvang 1980; Koga et al. 1987), *Gnathostoma nipponicum* (Ando et al. 1989), *Gnathostoma procyonis* (Ash 1962), and *Gnathostoma*

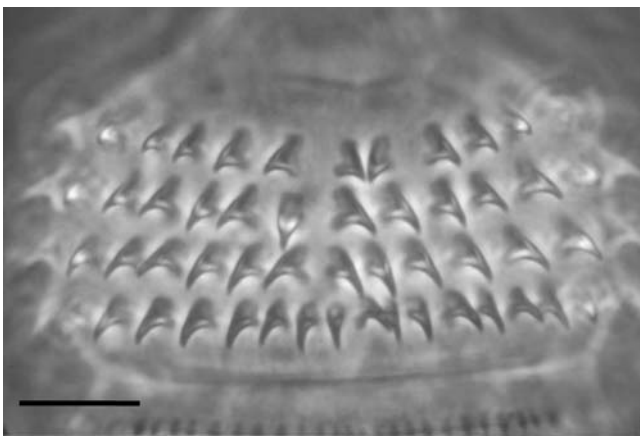

Fig. 2 *Front view* of the cephalic bulb of an experimental early third-stage larva of *Gnathostoma turgidum* recovered from *Mesocyclops* sp., showing the hooklets of the four rows in *lateral view* (*scale bar*—10 μm)

spinigerum (Miyazaki 1954; Daengsvang 1980). In our observations, the traits shown by the eggs and the larval stages made it possible to easily differentiate *G. turgidum* from other species. The size of the eggs in the case of *G. turgidum* is similar to those recorded for other species. However, the presence of two polar cap-like thickenings (similar to those of *Gnathostoma doloresi*) is considered a significant difference between species (Table 1). The shape and depth of the pits on the eggshell surface have been useful in order to differentiate them from other species. In fact, pits show irregular shapes and variable depths in the cases of *G. spinigerum* (Zaman 1987; Koga et al. 1991) and *G. nipponicum* (Koga and Ishii 1981), while in the case of *G. hispidum* pits are more rounded and of relatively equal depth (Koga et al. 1984); in the case of *G. binucleatum*, the eggshell surface is plain and without pits, and this trait is considered as a characteristic of this species and the major feature that allows its differentiation from *G. spinigerum* (Koga et al. 1999). The eggshell of *Gnathostoma vietnamicum* is regarded by Daengsvang (1973) as being superficially pitted. In our observations, the eggshell of *G. turgidum* is similar to that of *G. spinigerum*.

With regard to the L_2, there are no significant differences between species. However, *G. turgidum* corporal dimensions are the smallest of the eight species considered (Table 1). Moreover, compared to *G. binucleatum* (Almeyda-Artigas et al. 1995), the sheath of *G. turgidum* is also smaller (314×26 vs. 243×21, respectively).

Although it is difficult to separate species at level of the EaL_3 stage, Miyazaki (1954, 1960) established that the total length, the location of the cervical papillae and the excretory pore, the number of transverse rows of corporal spines, and the number of hooklets in the rows of the cephalic bulb can be considered useful species-specific features. According to Koga et al. (1988) it seems these EaL_3 traits remain unchanged after developing and growing to $AdvL_3$. As shown in Table 2, EaL_3 of *G. turgidum* are only bigger than those of *G. doloresi* and *G. procyonis*, the only species that can be discriminated from the rest by using the location of the cervical papillae is *G. doloresi*, and *G. binucleatum*. In the case of *G. doloresi* it cannot be discriminated from *G. turgidum* by using the position of the excretory pore. Finally, *G. turgidum*, together with *G. doloresi* and *G. hispidum*, bear less than 200 transverse rows of corporal single-pointed spines.

Regarding the number of cephalic hooklets per row, *G. turgidum* EaL_3 are similar to those of *G. doloresi*, *G. hispidum*, *G. nipponicum*, and *G. procyonis* because they bear, in average, less than 40 hooklets in the first three rows; although *G. nipponicum* has only three rows of hooklets, *G. doloresi* has more hooklets in the first row than in the last row, and *G. hispidum* and *G. procyonis* bear more hooklets in their fourth row (Table 2).

Furthermore, Almeyda-Artigas et al. (1995) document that *G. binucleatum* hooklets have a rectangular base, the ones from the first and fourth rows having the same size (2.4×1.6) and slightly shorter than those of the second and third rows (3.2×1.6). In *G. turgidum* the hooklets of the fourth row are smaller than those of the three previous rows, which have the same size. Interestingly, these traits remain unchanged after they continue their development and become fully grown advanced third-stage larvae in the muscles of second intermediate or paratenic hosts (Mosqueda-Cabrera et al. 2009; Table 2).

G. turgidum life cycle considerations

The life cycle of *G. turgidum* starts when the eggs are passed in the feces of the definitive host and continues when—carried by fluvial currents— reach a water body. If salinity, pH, temperature, depth, and hydrodynamics, among other conditions, are appropriate, the development is completed and hatching occurs, resulting in free L_2. The copepodid stage is more susceptible to infection, and when

Table 1 Comparisons between eggs and second-stage larvae of *Gnathostoma* spp.

Gnathostoma species	Egg size[b]	Cap-like thickenings	Larval size[b]	Reference
G. doloresi	64×32	2	234×11	Daengsvang (1980)
G. hispidum	66×38	1	265×16[a]	Daengsvang (1980)
G. nipponicum	74×42	1	280×13	Ando et al. (1989)
G. spinigerum	69×37	1	265×16	Prommas and Daengsvang (1933)
G. vietnamicum	78×43	1	262×14	Daengsvang (1973)
G. binucleatum	64×38	1	231×12	Almeyda-Artigas et al. (1995)
G. procyonis	71×39	1	276×19	Ash (1962)
G. turgidum	66×32	2	178×9	Present study

[a] According to Daengsvang (1980), the size and morphology of the second-stage larvae of this species are similar to that of *G. spinigerum* as reported by Prommas and Daengsvang (1933).

[b] Mean values

Table 2 Taxonomic comparison between early third-stage larvae of *Gnathostoma* spp. from experimentally infected cyclops

Gnathostoma species (no. of examined larvae)	Corporal dimensions µm	Cervical papilla	Excretory pore	Transverse rows of spines	Number of hooklets per row on the cephalic bulb					Reference
					I	II	III	IV	IV − I	
G. doloresi (15)	260–338×—(315×—)	15–19[a] (—)	25–28[a] (—)	176–211[a] (—)	34–42 (37.6)	35–43 (37.9)	33–39 (36.5)	33–41 (37.0)	(−0.6)	Miyazaki (1954)
G. hispidum (25)	387–720×—(530×49)	9–13 (—)	19–20 (—)	175–217 (—)	31–40 (37.0)	33–41 (36.0)	34–40 (38.0)	40–45 (43.0)	(6.0)	Koga et al. (1987)
G. nipponicum (10)	—(520×51)	8–12[b] (—)	—	213–232 (222)[b]	—(34.8)	—(36.1)	—(39.7)	[c]	([c])	Ando et al. (1989)
G. nipponicum (6)[d]	—(674×114)	—	—	215–248 (229)	32–38 (34.5)	34–39 (36.7)	38–43 (39.7)	[c]	([c])	Sohn et al. (1993)
G. spinigerum (23)	467–559×—(505×44)	11–16 (14)[e]	22–28 (25)[e]	225–256 (244)[e]	40–47 (43.2)	37–49 (44.8)	42–52 (46.7)	48–58 (52.3)	(9.1)	Miyazaki (1954)
G. vietnamicum (6)	411–777×34–57 (573×44)	—	—	—	—	—	—	—	(—)	Daengsvang (1973)
G. binucleatum (23)	518–558×44–58 (531×48)	12–16 (15)[f]	25–29 (27.4)[f]	242–285 (271)[f]	37–41 (38.3)	39–44 (41.7)	41–47 (44.1)	44–53 (47.9)	(9.6)	Almeyda-Artigas et al. (1995)
G. procyonis (5)	348–436×43–53 (401×48)[g]	—	—	—	—(31.7)	—(36.2)	—(39.5)	—(44.6)	(12.9)	Ash (1962)
G. turgidum (30)	337–471×37–47 (412×41)	10–13 (12)	19–24 (21.5)	180–204 (187)	27–34 (30.8)	30–39 (34.1)	30–43 (37.0)	37–46 (41.6)	(10.8)	Present study
G. turgidum (30)[h]	1,351–1,689×114–242 (1,494×133)	9–14 (12)	19–25 (22)	172–210 (195)	28–34 (31.3)	29–39 (34.0)	33–40 (37.0)	38–45 (41.8)	(10.6)	Mosqueda-Cabrera et al. (2009)

— not described

[a] From Koga and Ishii (1987) based on measurements of ten advanced third-stage larvae from naturally infected snakes *Trimeresurus flavoviridis* from Japan

[b] From Han et al. (2003) based on measurements of ten advanced third-stage larvae [1,262–1658 (1,460) in length] from naturally infected grass snakes *Rhabdophis tigrina* from Republic of Korea

[c] Cephalic bulb with three rows of hooklets

[d] From loaches (*Misgurnus anguillicaudatus*)

[e] From Koga et al. (1994) based on measurements of eight advanced third-stage larvae [3.0–3.6 mm in length] from naturally infected eels *Fluta alba* from Thailand

[f] From RJ Almeyda-Artigas (unpublished data) based on measurements of ten larvae

[g] Based on measurements of ten larvae

[h] Advanced third-stage larvae from one experimentally infected *Rana pipiens* from Mexico

it occurs in an older age, the latter does not guarantee the completion of the L_2 development. Our observations support the hypothesis that copepod infection occurs during the day, when they are in the bottom of the water column and the L_2 has a higher motility. Hence, in natural conditions, the infective capacity of the L_2 to the first intermediate host depends on: (a) the depth and dynamics of the water body, (b) salinity, (c) temperature, (d) pH, (e) transparency, (f) population density, feeding habits, and species of the crustacean host; and (g) the life expectancy of the L_2 in freshwater. L_2 prefer to reach its first host in shallow water bodies within the habitat of the final host; thus, lakes shores, river tributaries, and permanent and temporary small water bodies would be appropriate for such purposes. In this sense, further investigation regarding this issue is required.

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