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ORIGINAL PAPER

Description of advanced third-stage larvae of *Gnathostoma lamothei* Bertoni-Ruiz et al. 2005 (Nematoda: Gnathostomatidae) from experimental hosts and contributions to its life cycle

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Abstract The advanced third-stage larvae ($AdvL_3$) of *Gnathostoma lamothei* was obtained from experimental hosts. Frogs *Lithobates heckscheri* and snakes *Nerodia fasciata pictiventris* were compatible hosts allowing optimal larval development. $AdvL_3$ are 4,487.94 μm long, have two lateral cervical papillae between rows 10 and 16 and an excretory pore at row 23. The average counts of the cephalic bulb hooklets from the four rows are 39.3, 43.3, 44.2, and 47.3. Larvae show an esophagus that represents 40 % of the body width. These findings indicate that amphibians and reptiles could be involved as *G. lamothei* natural hosts; nevertheless, their role as etiological agents of human gnathostomiasis is uncertain. This paper reports for the first time the taxonomic description of *G. lamothei* $AdvL_3$ obtained from experimental hosts and contributes to the understanding of its life cycle.

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Introduction

Seven valid species have been recorded for the genus *Gnathostoma* Owen, 1836 in the Americas: *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 *Gnathostoma lamothei* Bertoni et al., 2005. In Mexico, adult stages have been recorded for three species: *G. binucleatum* (Almeyda-Artigas 1991; Koga et al. 1999; Almeyda-Artigas et al. 1995; Díaz Camacho et al. 2002; Álvarez-Guerrero et al. 2010), *G. turgidum* (Lamothe-Argumedo et al. 1998; Almeyda-Artigas et al. 2000a; Almeyda-Artigas et al. 2000b; Díaz-Camacho et al. 2009, Nawa et al. 2009, Mosqueda-Cabrera et al. 2009; Díaz Camacho et al. 2010b), and *G. lamothei* (Bertoni et al. 2005).

In Mexico, except for *G. lamothei*, detailed descriptions of the advanced third-stage larvae ($AdvL_3$) of two of the referred species have been recorded: *G. binucleatum* (Almeyda-Artigas 1991; Almeyda-Artigas et al. 1994; Koga et al. 1999; Díaz Camacho et al. 2002; León-Règagnon et al. 2002; Kifune et al. 2004; Martínez-Salazar and León-Règagnon 2005; Álvarez-Guerrero and Alba-Hurtado 2007;

García-Márquez et al. 2009; Álvarez-Guerrero et al. 2010; Díaz Camacho et al. 2010a) and *G. turgidum* (Mosqueda-Cabrera et al. 2009).

The recent finding of *G. lamothei* $AdvL_3$ in commercial fish in Tabasco State (*Gobiomorus dormitor*) (Hernández-Gómez et al. 2010) suggests its inclusion as a new etiological agent of human gnathostomiasis in Mexico, besides *G. binucleatum* (Almeyda-Artigas 1991). The absence of a description of the $AdvL_3$ of the former species has contributed to the fact that, in many instances, it has been impossible to clarify the specific identity of the recovered $AdvL_3$. This paper reports for the first time the taxonomic description of *G. lamothei* $AdvL_3$ obtained from experimental hosts.

Material and methods

The process for obtaining experimental $AdvL_3$ began with the infection of cyclopoid copepods [*Acanthocyclops robustus* (G.O. Sars, 1863)] with second stage larvae (L_2) obtained from the eggs of females isolated from raccoons *Procyon lotor hernandezii* (Mosqueda-Cabrera et al., submitted for publication). Subsequently, the infected copepods with EaL_3 were used to infect ad libitum fish *Poeciliopsis gracilis* and toad *Rhinella marina* tadpoles collected in Xochimilco Lake canals and maintained according to Sukontason et al. (2001). After 3–5 days, these second intermediate hosts were used to feed per os and ad libitum different potential paratenic hosts (Table 1) that were kept under appropriate conditions as follows: fish were maintained according to Sukontason et al. (2001); amphibians and reptiles were transferred to mixed vivariums (terrarium/aquarium) and fed with chicken liver (amphibians) and live fish and tadpoles (reptiles); mammals were kept in autoclaved plastic cages and sawdust floor and fed with standard rodent food pellets and water ad libitum. All mentioned animals were purchased in local markets. All potential hosts were humanely euthanized at regular intervals, and their musculature and viscera were examined under a stereomicroscope in order to determine the number of larvae, considering days postinfection (DPI). The recovered larvae were fixed in hot 70 % ethanol and cleared in Amann lactophenol. They were studied on temporary mounts on glass slides and later stored in 70 % ethanol. 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 using a Kodak Technical Pan black and white film. Specimens were deposited at the Colección Helmintológica de la Universidad Autónoma Metropolitana Unidad Xochimilco (CHUX), Mexico City, Mexico, as follows: CHUX-G015, G048, G118, and G123 (six larvae from *P. gracilis*), CHUX-G741 (two larvae from *Dormitator maculatus*), CHUX-G915 (one larvae from *Eleotris pisonis*), CHUX-G742 (one larvae from *Ambystoma tigrinum*), CHUX-G918 (five larvae from *Lithobates berlandieri*), CHUX-G743, G744 and G760 (248 larvae from *Lithobates heckscheri*), CHUX-G730 (two larvae from *Thamnophis eques*), CHUX-G745 (18 larvae from *Nerodia fasciata pictiventris*), CHUX-

Table 1 Development of infection and amount of *G. lamothei* $AdvL_3$ recovered from experimental second intermediate and paratenic hosts

DPI	Species host (number of hosts at the beginning of the experiment)												
	ON (50)	PG (100)	DM (17)	EP (1)	AT (1)	LB (2)	LH (2)	HE (2)	PD (1)	TE (1)	NFP (1)	KB (1)	MM (5)
3		3											
5		1											
15		5											16
21										2			21
24						5							
29													29
32			2										
36											18		
56				1									
75							166					2	
85							82						
97					1								

DPI days postinfection

Pisces: *ON Oreochromis niloticus*, *PG Poeciliopsis gracilis*, *DM Dormitator maculatus*, *EP Eleotris pisonis*; Amphibia: *AT Ambystoma tigrinum*, *LB Lithobates berlandieri*, *LH Lithobates heckscheri*, *HE Hyla eximia*, *PD Pachymedusa dacnicolor*; Reptilia: *TE Thamnophis eques*, *NFP Nerodia fasciata pictiventris*, *KB Kinosternon baurii*; Mammalia: *MM Mus musculus*

G746 (two larvae from *Kinosternon baurii*), and CHUX-G724, G725, G738, and G740 (66 larvae from *Mus musculus*).

All procedures for the use of animals were approved by the Care and Use of Laboratory Animals Internal Committee of the Universidad Autónoma Metropolitana-Xochimilco, according to the Mexican Official Norm 062-ZOO-1999.

Results

In order to obtain *G. lamothei* $AdvL_3$, 13 species of potential second intermediate and paratenic hosts were infected. Larvae were recovered from ten of them, on different days postinfection. No larvae were recovered from *Oreochromis niloticus* fry, while in the primary consumer fish *P. gracilis* and omnivorous anuran, larval stage (*R. marina* tadpoles) failed to infect them after 15 DPI. Three hundred forty fully developed $AdvL_3$ were obtained encysted and embedded in the musculature of two River frogs *L. heckscheri* (n=248), one Rio Grande Leopard frog *L. berlandieri* (n=5), one Tiger salamander *A. tigrinum* (n=1), one Mexican garter snake *T. eques* (n=2), one Banded water snake *N. fasciata pictiventris* (n=18), and three mice *M. musculus* (n=66). In one river frog *L. heckscheri* with 75 DPI, the 166 larvae were found in the liver (n=12), stomach (n=5), adipose tissue (n=1), mesentery (n=1), hypodermis (n=7), and musculature (n=140). No larvae were recovered from frogs *Hyla eximia* and *Pachymedusa dacnicolor*. Besides that the recovery rate of larvae was lower in *D. maculatus*, *E. pisonis*, *A. tigrinum*, *T. eques*, and *K. baurii* (eight), these worms were smaller even though the infection was maintained for a considerable period of time (21–97 DPI) (Table 1).

Gnathostoma lamothei

The following description is based on 15 $AdvL_3$ (seven males and eight females): body 3,582.24–5,095.90 (4487.94±389.84) long and 236.60–318.24 (288.74±21.52) wide; body completely covered with 137–258 (227.07±30.99) transverse rows of simple spines; pair of lateral cervical papillae present, the right between rows 10 and 14 (11.73±1.16) and left between rows 10 and 16 (11.53±1.46); excretory pore ventral between rows 20 and 29 (23.07±2.28); pair of small caudal lateral papillae situated on the second half of the body, located right 37.84–67.88 (54.70 %±8.61) and left 55.54–75.26 (67.63 %±5.18) in relation to the total length; cephalic bulb 69.36–106.08 (84.55±9.68) long and 167.28–216.24 (188.50±11.96) wide (Fig. 1a, b); cephalic hooklets counts from rows 1–4, 34–44 (39.33±3.22), 38–47 (43.27±2.46), 40–48 (44.20±5.51), and 45–58 (47.33±3.62), respectively, [IV-I, 1–12 (7.13±2.95)]; esophagus 889.44–1162.80 (970.77±62.38) long, and 97.92–134.64 (116.96±9.71) wide; esophagus width occupies 36.36–45.21 (40.5 %±2.36) in relation to body width (Fig. 1c); cervical sacs occupy 45.76–63.79 (55.99 %±5.27) of the esophagus length; tail 36.72–75.44 (59.16±11.51) long; and, in females, genital primordium located between the two caudal papillae, below the right and above the left, 51.36–66.31 % (60.64 %±5.04) in relation to the total body length.

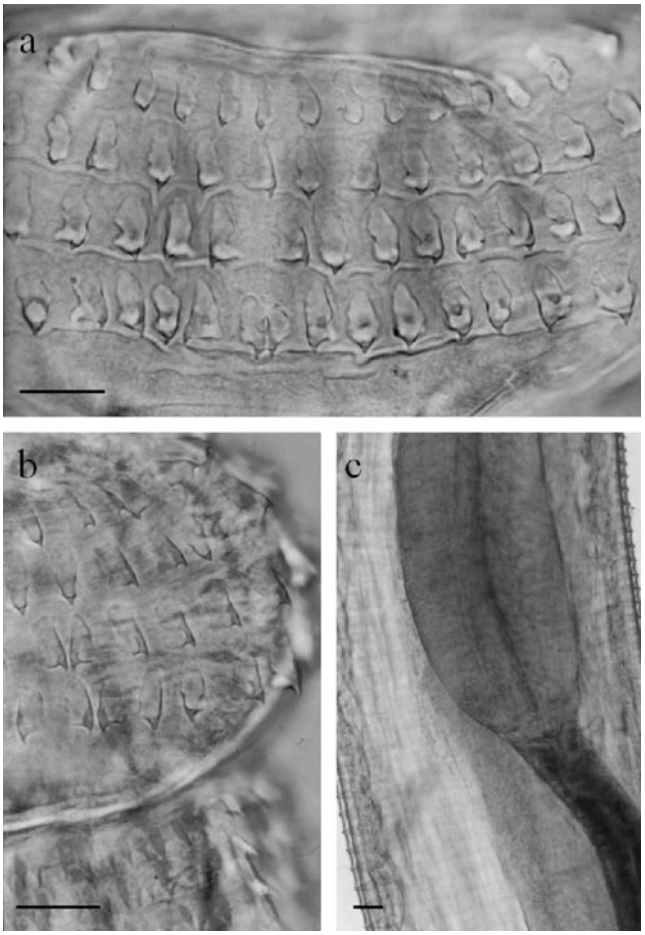

Fig. 1 Experimental *G. lamothei* $AdvL_3$ recovered from *L. heckscheri*. **a** Front view of the cephalic bulb.**b** Lateral view of the hooklets of the four rows. **c** View of the esophagus–intestine intersection area. *Scale bars*: 20 μm

Taxonomic summary

Experimental host: *Lithobates heckscheri*

Other experimental hosts: *Poeciliopsis gracilis*, *Dormitator maculatus*, *Eleotris pisonis*, *Ambystoma tigrinum*, *Lithobates berlandieri*, *Thamnophis eques*, *Nerodia fasciata pictiventris*, *Kinosternon baurii*, and *Mus musculus*

Site of infection: Musculature

Deposit of specimens: CHUX-G744

Discussion

In order to understand its potential threat to humans, it is vital to study the morphology of $AdvL_3$ of species of *Gnathostoma*, as well as its life cycle and geographical distribution. The results from the 15 DPI in fishes infected with copepods parasitized with EaL_3 were negative. Nevertheless, $AdvL_3$ were obtained in omnivorous and secondary consumer fishes *D. maculatus* and *E. pisonis* because they were infected with *P. gracilis* with 3 DPI. The infection in these fishes reveals their position as second intermediate hosts, although the amount of larvae recovered was low. Once tadpoles were infected with EaL_3, they were difficult to keep under laboratory conditions in order to extend the infection in adult frogs and obtain $AdvL_3$. The success of the $AdvL_3$ infection in the frogs supposes that they could acquire it in natural conditions by feeding on fishes and/or tadpoles, or being infected since the anuran larval stage (tadpole).

The experimental infections of the present study suggest that when the primary consumer fish *P. gracilis* and the omnivorous amphibian larval stage (tadpole) prey on the first intermediate host (copepods parasitized with EaL_3), they act as temporal second intermediate hosts in the food web between the first and the paratenic hosts (i.e., *L. berlandieri*, *L. heckscheri*, *N. fasciata pictiventris*, and *M. musculus*) where the $AdvL_3$ of *G. lamothei* develops.

Combes (2001) proposes the "compatibility filter" to eliminate the host species that does not allow the coexistence with the parasite because of morphologic or immunologic reasons. In this study, the frogs *L. berlandieri*, *L. heckscheri*, the small snake *N. fasciata pictiventris*, and the mouse *M. musculus* were the compatible species that best acted as experimental paratenic hosts because of the amount of larvae recovered and the longest DPI value. Even though the hosts *E. pisonis*, *D. maculatus*, *A. tigrinum*, *T. eques*, and *K. baurii* kept the infection longer than 21 DPI, the amount of larvae recovered was small. In contrast, in *O. niloticus* fry, the infection did not take place (Table 1).

Particularly *P. gracilis* fishes maintained the infection until 15 DPI; nevertheless, the larvae were smaller than those recovered from other hosts in similar DPI (i.e., *M. musculus*). Although the experimental hosts (*L. heckscheri* and *N. fasciata pictiventris*) used in this study are native species from the USA, they were effective for the $AdvL_3$ development. In Mexico, different species of amphibians and reptiles have been reported as natural hosts of $AdvL_3$: for *Gnathostoma* sp., the frog *Rana cf. forreri* (Martínez-Salazar and León-Règagnon 2005; Cabrera-Guzmán et al. 2007); for *G. turgidum*, the frog *Rana zweifeli* and the turtle *Kinosternon integrum* (Mosqueda-Cabrera et al. 2009); and for *G. binucleatum*, the turtles *K. integrum* and *Trachemys*

Table 2 Morphometric comparison of $AdvL_3$ of *Gnathostoma* spp

Species (Reference)	Body		Esophagus		CP[a]	EP[a]	Hooklets per row on the cephalic bulb				
	Length	Width	Length	Width			I	II	III	IV	IV-I
G. spinigerum (Miyazaki 1954)							39–49 (44.3)	42–54 (47.3)	45–56 (49.6)	45–58 (52.0)	7.7
G. procyonis (Ash 1960)	4,600–5,900 (5,200)	283–382 (342)	1,000–1,400 (1,200)	109–142 (124)	11–16 (13.5)	19–27 (24.3)	29–36 (32.70)	32–40 (36.60)	37–45 (41.0)	42–47 (45.0)	12.3
G. binucleatum (Almeyda-Artigas 1991)					10–17 (13.50)	27–37 (30.00)	35–44 (38.7)	38–47 (42.4)	40–49 (44.7)	43–52 (48.2)	9.5
G. binucleatum (García-Márquez et al. 2009)	3,120–3,140 (3,130.0)	310–320 (320)	1,220–1,230 (1,220)	190–210 (200)	14	30	36–39 (38)	40–41 (40)	44–46 (45)	44–47 (46)	8
G. turgidum (Mosqueda-Cabrera et al. 2009)	1,530.0–2,007.4 (1,670.2)	134.6–160.4 (140.8)	579.4–722.2 (648.0)	69.4–85.7 (80.8)	9–14 (11.4)	19–25 (21.6)	28–35 (31.8)	31–37 (35.0)	34–39 (37.4)	40–46 (41.4)	9.6
G. lamothei (present study)	3,582.2–5,095.9 (4,487.9)	236.6–318.2 (288.7)	889.4–1,162.8 (970.8)	97.9–134.6 (117.0)	10–14 (11.73)	20–29 (23.07)	34–44 (39.33)	38–47 (43.27)	40–48 (44.20)	45–58 (47.33)	7.13

Data are not reported in cells with no values

CP cervical papillae, *EP* excretory pore

[a] Location of CP and EP in relation to transverse rows of corporal spines

scripta (Álvarez-Guerrero and Alba-Hurtado 2007; Díaz-Camacho et al. 2010a) and the crocodile *Crocodylus acutus* (García-Márquez et al. 2009). Therefore, the group of leopard frogs and small snakes (i.e., *L. berlandieri*, *L. vaillanti*, and *N. rhombifer werleri*) living where the definitive host occur, near Tlacotalpan, Veracruz, Mexico, could constitute natural hosts of *G. lamothei*.

On the other hand, morphological differences, such as the amount and shape of the cephalic bulb hooklets and the location of cervical papillae and excretory pore, are considered as specific characteristics to distinguish between species in the genus *Gnathostoma* (Miyazaki 1954; Akahane et al. 1994). The location of the cervical papillae is unable to differ between Mexican species, since values overlap between rows 9 and 17 (Table 2).

G. lamothei $AdvL_3$ are similar to those of *G. binucleatum* in the amount and shape of the cephalic bulb hooklets. In both species, the hooklet bases have a rectangular form (Fig. 2a, c); nevertheless, the excretory pore location allows their differentiation (23 vs. 30, respectively) (Table 2). Moreover, *G. lamothei* esophagus is thinner and with the same diameter until the insertion with the intestine, meanwhile in *G. binucleatum* adopts a globular form (Fig. 2b, d).

G. lamothei larvae are different from *G. turgidum* in the amount and shape of the cephalic bulb hooklet rows, with more hooklets in rows of *G. lamothei*; besides, *G. turgidum* $AdvL_3$ are very small, 4,487 vs. 1,670, respectively (Table 2).

On the other hand, *G. lamothei* larvae have smaller corporal dimensions, being in average 15 % less long and wide than *G. procyonis* (Ash 1960) and have an esophagus 19 % smaller than those of the other species. This species differs from *G. procyonis* in the amount of cephalic bulb hooklets in the different rows: in average, *G. lamothei* has more hooklets (seven more in the first and second rows, three on the third row, and two on fourth row).

The taxonomic characters that allow the differentiation between *G. lamothei* and *G. binucleatum* are (a) the cephalic bulb average dimensions (84×188 vs. 119×246, from 12 $AdvL_3$ of *P. gracilis* with 46 DPI, data unpublished), respectively (Fig. 2a, c); (b) in *G. binucleatum*, cephalic bulb hooklets are bigger; (c) the average location of the excretory pore (Table 2); (d) on average, two hooklets less in *G. lamothei*, the difference being between the fourth and first rows of the cephalic bulb (Table 2); and (e) the proportion of space occupied by the esophagus against the body width, 40 vs. 70 %, respectively (Fig. 2b, d).

Human gnathostomiasis is caused by $AdvL_3$ of different species of the genus *Gnathostoma* and is mostly acquired by eating raw or poorly cooked fish meat (Almeyda-Artigas 1991; León-Règagnon et al. 2000; Álvarez-Guerrero et al. 2010). The etiologic agent proved in Mexico is the $AdvL_3$ of *G. binucleatum* (Almeyda-Artigas et al. 2000b; León-Règagnon et al. 2002); recently, it has been put aside the idea of the participation of *G. turgidum* as an etiologic agent (Mosqueda-Cabrera et al. 2009). Also, Hernández-Gómez et al. (2010) identified *G. lamothei* $AdvL_3$, from the fish

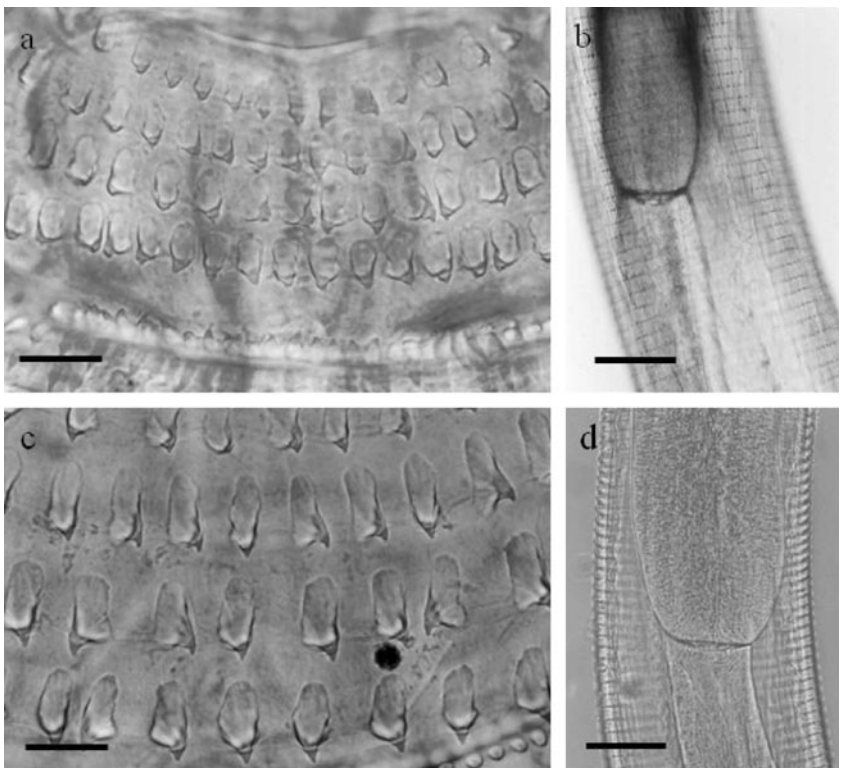

Fig. 2 Comparison between the cephalic bulb and esophagus of $AdvL_3$ of *Gnathostoma*. **a** and **b** *G. lamothei* larva from *L. heckscheri*, **c** and **d** *G. binucleatum* larva experimentally obtained from *P. gracilis* (unpublished data). *Scale bars*: **a** and **c** 20 μm; **b** and **d** 60 μm

G. dormitor, by molecular techniques. These authors presume that this species can also be responsible of this disease. Nevertheless, until now there is no record of any larvae recovered from human patients identified as different from *G. binucleatum*.

Based on the above observations and information concerning the trophic web, a postulated life cycle of *G. lamothei* is suggested as follows: adult forms are lodged in multiple nodules in *P. lotor hernandezii* stomachs. Fertilized eggs laid by gravid females are shed from definitive hosts within their feces. Once in touch with water bodies, the zygote develops into a first-stage larva (L_1) which in turn molts to a L_2; after hatching, it is swallowed by predaceous cyclopoid copepods, the first intermediate host, where the L_2 molts to EaL_3 (Mosqueda-Cabrera et al., submitted for publication). When primary consumer fishes and omnivorous tadpoles prey on copepods, they act as temporal second intermediate hosts between the first and the paratenic hosts (fish and mainly frogs and small snakes), where the EaL_3 develops into $AdvL_3$. When the latter hosts are eaten by definitive hosts, the infection is transmitted and the $AdvL_3$ molts to the adult form, thus completing the life cycle.

The finding of *G. lamothei* $AdvL_3$ in *G. dormitor* (Hernández-Gómez et al. 2010) and the results of the experimental infections reported herein support the belief that this eleotrid fish acquired the infection by the consumption of primary consumer, omnivorous, and/or secondary consumer fish species (i.e., *P. gracilis*, *D. maculatus*, and *E. pisonis*), and not by preying upon cyclopoid copepods.

Due to the high amount of *G. lamothei* $AdvL_3$ recovered from experimental frogs and small snakes, it would be expected that anuran species with similar ecological roles (i.e., *L. berlandieri*, *L. vaillanti*, and *N. rhombifer werleri*) could be the main natural hosts. However, raw meat human consumption of the latter anuran species is unusual in Mexican populations; thus, *G. lamothei* does not seem to represent a health risk for carnivore humans.

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