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Polystoma knoffi n. sp. and *Polystoma travassosi* n. sp. (Monogenea: Polystomatidae): naming museum-archived specimens from Brazil

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Abstract In 1978, Kohn and co-workers deposited several polystome (Monogenea) specimens infecting several Brazilian anurans [*Trachycephalus mesophaeus* (Hensel), *T. nigromaculatus* Tschudi and *Leptodactylus pentadactylus* (Laurenti)] within the Helminthological Collection of the Instituto Oswaldo Cruz, Brazil. No specimen was formally described but we herein identified three morphotypes and formally describe two of them (*Polystoma knoffi* n. sp. and *P. tavassosi* n. sp.). These are respectively the 12th and 13th species of *Polystoma* described from South America. For both species, the intestine forms a reticulated network, a characteristic unique to most Neotropical species of *Polystoma*.

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Introduction

Brazil is by far the largest country in the Neotropics, with a surface area of 8,456,510 km^2 and occupying 42% of the surface of the Neotropical Realm (Ohler & Dubois, 2009). With 49% of the known amphibian species described from the Neotropical Realm (Stuart, 2008), 1,159 of them (14.4% of the global diversity) are known from Brazil (Frost, 2019), making Brazil the country with the richest amphibian diversity (Segalla et al., 2012). The size of Brazil and the large tropical or sub-tropical areas are contributing factors to Brazil's megadiversity.

The first studies on amphibian parasites were conducted by European naturalists in the early decades of the 19th century (Travassos et al., 1969; Vicente et al., 1991). Nearly a hundred years ago, Lauro Pereira Travassos (1890–1970), a Brazilian researcher and pioneer in the field of parasites of wildlife, entered the scene and contributed significantly to the knowledge of amphibian parasites. In the years to follow, several publications focused on amphibian parasite diversity, e.g. Travassos (1919, 1926a, b, c, d), Travassos et al. (1969) and Vicente et al. (1991). In a recent

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checklist of the helminth parasites of amphibians of South America, 289 helminth species from 186 amphibian species were reported (Campião et al., 2014). Of South American studies reporting on helminths of amphibians, 88 (55%) taxa were based on work conducted in Brazil (Campião et al., 2014). In spite of this known amphibian diversity, only a fraction of the parasitic fauna of Brazil's amphibians has been studied. One would expect that such a diversity of amphibians would cater for a great variety of undiscovered parasites.

Polystomatids (Monogenea: Polystomatidae) are globally represented by 26 genera comprising at least 180 described species. Within the Neotropical Realm, polystomes are represented by one species of each of *Mesopolystoma* Vaucher, 1981, *Parapseudopolystoma* Nasir, Fuentes-Zambrano, 1983, *Riojatrema* Lamothe, 1963 and *Wetapolystoma* Gray, 1993 plus 11 species of *Polystoma* Zeder, 1800. Except for the Australian realm, species of *Polystoma* have a widespread occurrence in all zoogeographical realms. Eleven of the 62 currently recognised species of *Polystoma* are described in South America: *Polystoma andinum* Combes & Laurent, 1978; *P. borelli* Combes & Laurent, 1974; *P. guevarai* Combes & Laurent, 1979; *P. lopezromani* Combes & Laurent, 1979 and *P. praecox* Combes & Laurent, 1978 from Argentina; *Polystoma cuvieri* Vaucher, 1990 and *P. diptychi* Vaucher, 1986 from Paraguay; *P. napoensis* Vaucher, 1987 and *P. touzeti* Vaucher, 1987 from Ecuador; and *P. naevius* Caballero & Cerecero, 1941 from Mexico and *P. stellai* Pérez-Vigueras, 1955 from Cuba.

Kohn et al. (1978) reported three species of *Polystoma* in *Trachycephalus mesophaeus* (Hensel) (syn. *Hyla mesophaea* Hensel), *Trachycephalus nigromaculatus* Tschudi (syn. *T. geographicus*) and *Leptodactylus pentadactylus* (Laurenti) from Brazil. However, these species were never formally described or assigned to a species. In the Helminthological Collection of the Instituto Oswaldo Cruz (CHIOC) we also found two specimens of *Polystoma* from *T. nigromaculatus*. We examined these specimens, compared them with information provided by Kohn et al. (1978), and describe and name the two species for which sufficient materials exist.

Materials and methods

From the Helmintological Collection of the Instituto Oswaldo Cruz (CHIOC) we obtained permanent slides and fixed specimens of the following: (i) Host 1 (*Leptodactylus pentadactylus*): a single specimen (no. 214360 collected by Dr Travassos from Para State, Brazil on 14 April 1956 and stained and mounted by Dr Anna Kohn and numbered 31423. This specimen stained dark and since only a single parasite is available it will not be formally described in the present study; (ii) Host 2 (*Trachycephalus mesophaea* (syn. *Hyla mesophaeus*)): five mounted polystome specimens (nos 31420, 31421 and 31422a-c). One formalin fixed specimen was mounted in Canada balsam (no 10151). We formally describe this polystome species herein; (iii) Host 3 (*Trachycephalus nigromaculatus*): five mounted specimens (nos 31420, 31421 and one slide with three specimens 31422a-c) and three additional formalin-fixed specimens. A single specimen (no. 21436) collected by Travassos was mounted by Anna Kohn and numbered 31423. Thee polystomes retrieved from *T. nigromaculatus* collected by an unknown person on 10 October 1983 at Nova Iguaçu, RJ (35 km north of Rio de Janeiro) were sent to Professor Claude Combes, France. These specimens were forwarded to us. We formally describe this polystome species herein.

Prior to staining, formalin-fixed parasites were rinsed in tap water for one hour, agitating the Petri dish every 10 min and replacing the water after 30 min. Parasites were stained for six hours in a weak solution of acetocarmine, dehydrated, cleared in xylene, and mounted in Dammar gum. All measurements provided are in micrometres and are given with the range followed by the mean in parentheses. The dimensions of organs and other structures represented here were measured in dorsoventral view. Illustrations were prepared with the aid of a drawing tube on a Leica DM 2500 microscope using differential interference contrast and phase contrast optics and LEICA M205A stereomicroscope. Type-specimens remain in the Helminthological Collection of the Instituto Oswaldo Cruz (CHIOC), Rio de Janeiro, RJ, Brazil. Host scientific names were validated according to the Amphibian Species of the World database (Frost, 2019).

To comply with the regulations set out in article 8.5 of the amended 2012 version of the *International Code*

of Zoological Nomenclature (ICZN, 2012), details of all new taxa have been submitted to ZooBank. For each new taxon, the Life Science Identifier (LSID) is reported in the taxonomic summary.

Class Monogenea Van Beneden, 1858
Order Polystomatidea Lebedev, 1988
Family Polystomatidae Gamble, 1896

***Polystoma knoffi* n. sp.**

Type-host: *Trachycephalus nigromaculatus* Tschudi (Anura: Hylidae). Specimens CHIOC 25701a-c were collected from *T. geographicus* but this species is regarded as a junior synonym of *T. nigromaculatus*.
Type-locality: Jacarepagua, Rio de Janeiro, Brazil.
Type-material: The holotype (CHIOC 25702a) collected on 19.v.1924 by Adolfo Lutz and 4 paratypes (CHIOC 25701a-c collected on 5.viii.1922 and CHIOC 25702b collected on 19.v.1924 by Adolfo Lutz), are housed in the Helminthological Collection, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil, and 3 paratypes (NMB P 506-508) received from Professor Claude Combes, Perpignan, France, collected by an unknown person on 10.x.1983 at Nova Iguaçu, Rio de Janeiro, Brazil, are housed in Parasitic Worm Collection, National Museum, Charles Street, Bloemfontein 2930, South Africa.
Site in host: Urinary bladder.
ZooBank registration: The Life Science Identifier (LSID) of the new name *Polystoma knoffi* Du Preez & Domingues is urn:lsid:zoobank.org:act: 5F0454AD-0BDB-4D9B-AC17-A7E6DB142C54.
Etymology: The species is named after Dr Marcelo Knoff, in recognition for his devotion to the Helmintological Collection of the Instituto Oswaldo Cruz in Rio de Janeiro, Brazil.

Description (Figs. 1, 2)

[Based on 8 sexually mature specimens; measurements of flat fixed specimens, see also Table 1.] Body elongate, with an anterior mouth and posterior haptor with 3 pairs of suckers and pair of hamuli posteriorly between posterior-most sucker pair (Fig. 1). Total body length 5,198–10,625 (7,386); greatest width 1,590–3,409 (2,494); width at vagina 852–1,818 (1,339). Haptor length 1,191–1,818 (1,473); haptor width 1,654–2,840 (2,207); haptor length to body length ratio 0.17–0.25 (0.21); haptoral suckers 6, with mean diameter 320–470 (398).

Hamulus with deep cut between handle and guard (Fig. 2A1–3) whereas hamulus of subadult parasites (Fig. 1D) lacks deep cut (Fig. 2A4–5); hamulus length to tip of handle (X) 420–571 (509); hamulus length to tip of guard (Y) 386–571 (451); handle longer than guard: X/Y ratio 1.00–1.20 (1.13); hamulus hook length 84–100 (94). Most marginal hooklets not in flat orientation; marginal hooklet pairs 1 and 2 marginal, along periphery of haptor between posterior-most pair of suckers; marginal hooklet pairs 6–8 anterior in haptor (between sucker pair 3); marginal hooklet pairs 3–5 imbedded in suckers, obscured such that they could not be reliably measured; posterior-most marginal hooklet 1 (Fig. 2B) 31–34 (31) long; marginal hooklets 2–8 (Fig. 2c) 18 long (n = 1).

Mouth subterminal, ventral. False oral sucker 207–345 (264) wide; pharynx 240–430 (318) long, 210–325 (266) wide. Intestine a dense reticulated network (Fig. 1A) extending into haptor.

Testes and vas deferens obscured by intestinal caeca; seminal vesicle a dilatation of vas deferens, sigmoid, crossing midline, dorsal to oötype and uterus. Genital pore opening mid-ventral, posterior to intestinal caeca bifurcation; genital atrium muscular; genital bulb 75–130 (107) in diametre, armed with 8 genital spines, each 29–42 (34) long (Fig. 1B). Ovary small, elongated and doubled over, dextral, submedian, anterior in body, constricted in middle portion, distal portion subovate, ascendant, proximal portion elongate, ovary length 235–1,095 (577), ovary width 132–670 (378). Vagina comprising double vaginal aperture with marginal opening; vaginal vestibule cup-shaped, with soft tissue. Vitello-vaginal canal descends from both vaginae and joins in middle of body and posterior to ovary. Mehlis' glands bilateral to oötype. Genito-intestinal canal prominent, joining intestinal caeca posterior to and on same side as ovary. Uterus tubiform, convoluted (Fig. 1A). No egg observed *in utero*. Vitellarium distributed throughout body except around mouth, ovary, uterus and haptoral suckers (Fig. 1a).

***Polystoma travassosi* n. sp.**

Type-host: Trachycephalus mesophaea (Hensel) (Anura: Hylidae).
Type-locality: Angra dos Reis, Rio de Janeiro, Brazil.

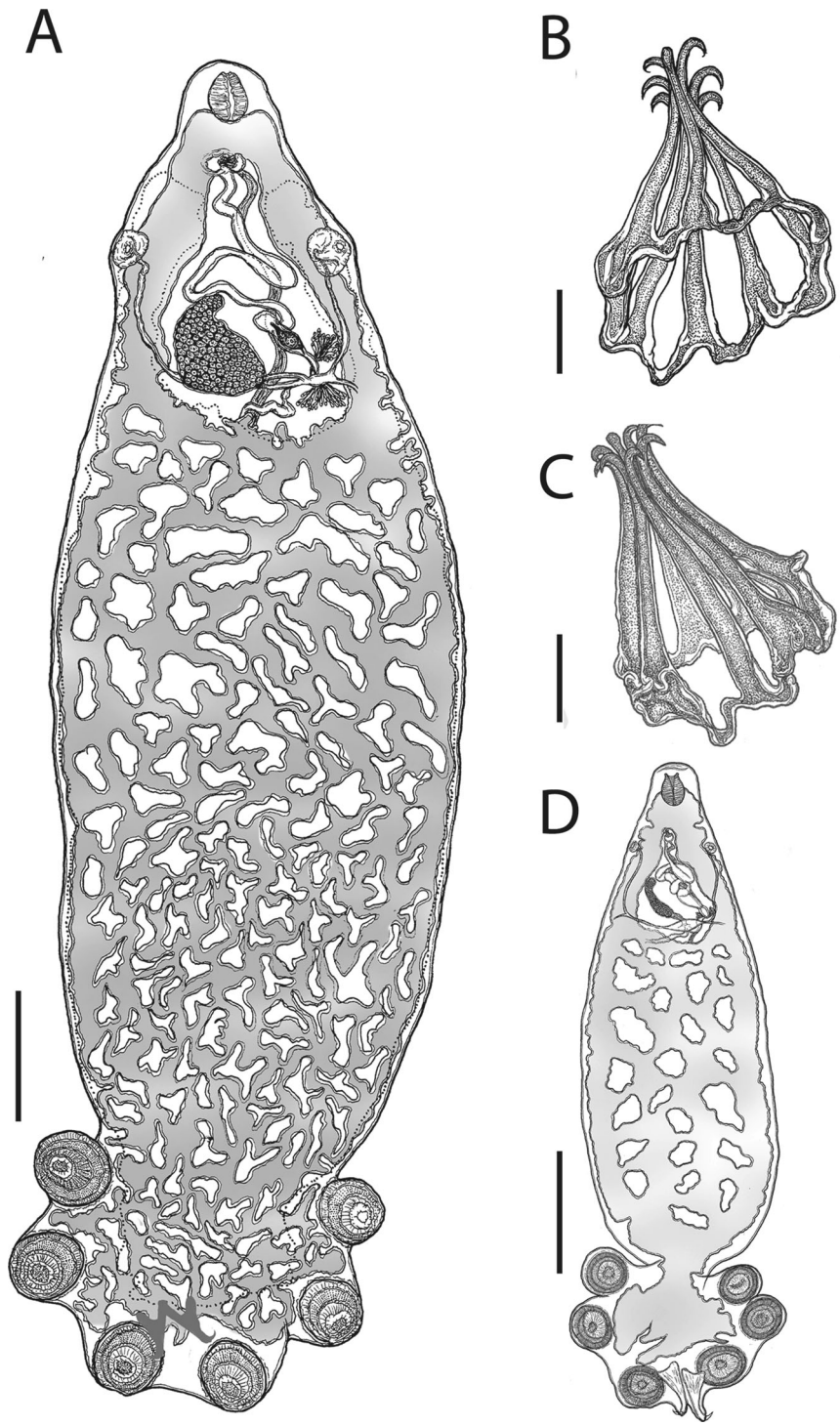

Fig. 1 *Polystoma knoffi* n. sp. A, Ventral view of the holotype; B, Genital spines of the holotype; C, Genital spines of a paratype; D, Ventral view of an immature paratype. *Scale-bars*: A, D, 1,000 μm; B, C, 10 μm

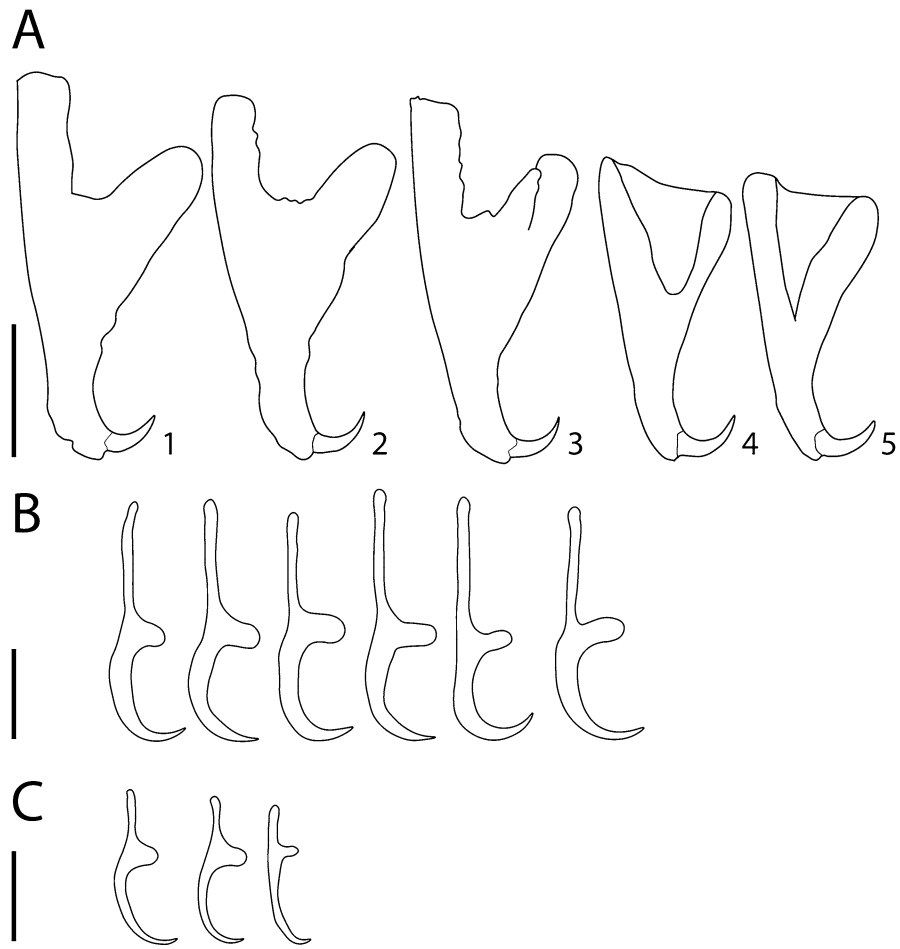

Fig. 2 *Polystoma knoffi* n. sp. A, Hamuli (1 and 2 from the holotype, 3, from a paratype and 4 and 5 from an immature paratype); B, Marginal hooklet C1; C, Marginal hooklets C2–7. *Scale-bars*: A, 200 μm; B, C, 10 μm

Type-material: Holotype (CHIOC 10151) and 5 paratypes (CHIOC 31420, 31421, 31422a, 31422b, 31422c) collected on 23 i.1925 and 24.i.1925 by Dr Lauro Travassos, all housed in the Helminthological Collection, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil.

Site in host: Urinary bladder.

ZooBank registration: The Life Science Identifier (LSID) of the new name *Polystoma travassosi* Du Preez & Domingues is urn:lsid:zoobank.org:act:D08CB059-8E32-4B44-A033-55E508A9C145.

Etymology: The species is named after Dr Lauro Travassos in recognition of his immense contribution to the knowledge of Neotropical parasites.

Description (Fig. 3)

[Based on 6 sexually mature specimens; measurements of flat fixed specimens, see also Table 1]. Body elongate, with an anterior mouth and posterior haptor with 3 pairs of suckers and a pair of hamuli posteriorly between posterior-most sucker pair (Fig. 3A). Total body length 4,980–7,820 (5,869); greatest width 1,500–2,360 (1,852); width at vagina 1,012–1,420 (1,268). Haptor length 800–1,373 (968); haptor width 1,640–2,000 (1,741); haptor length to body length ratio 0.12–0.17 (0.15); haptoral suckers 6, with mean diameter 315–360 (338).

Hamulus with a deep cut between handle and guard (Fig. 3C); hamulus length to tip of handle (X) 390–505 (436); hamulus length to tip of guard

Table 1 Metrical data for Neotropical *Polystoma* spp.

Species	*P. knoffi* n. sp.	*P. travassosi* n. sp.	*P. andinum*	*P. borelli*	*P. cuvieri*	*P. diptychi*
Source	Present study	Present study	Combes & Laurent (1978)	Combes & Laurent (1974)	Vaucher (1990)	Vaucher (1986)
Body length (BL)	5,198–10,625 (7,386)	4,980–7,820 (5,869)	4,900–8,000 (6,100)	4,200–5,600 (5,100)	4,230–3,600 (2,400)	8,300
Body maximum width	1,590–3,409 (2,494)	1,500–2,360 (1,852)	1,500–2,400 (1,900)	2,000–3,200 (2,500)	900–1,700 (1,400)	2,600
Haptor length (HL)	1,191–1,818 (1,473)	800–1,373 (969)	1,300–2,300 (1,600)	1,300–1,700 (1,500)	900–1,400 (1,300)	2,900
Haptor width	1,654–2,840 (2,207)	1,640–2,000 (1,741)	1,400–3,300 (2,500)	2,300–3,200 (2,600)	1,200–2,100 (1,800)	3,400
HL/BL ratio	0.21	0.15	0.26	0.29	0.36	0.35
Sucker diameter	320–470 (398)	315–360 (338)	380–585 (490)	510–550	295–434	755–836
Hamulus length	420–571 (509)	390–505 (436)	–	350–530 (430)	278–413	970–980
Hamulus hook length	84–100 (94)	75–98 (84)	–	–	48–68 (59)	–
Hamulus shape	Deep cut	Deep cut	Solid to shallow cut	Solid to deep cut	Solid	Shallow cut
Pharynx length	240–430 (318)	280–325 (303)	200–305 (243)	229–274 (250)	164–245 (214)	–
Pharynx width	210–325 (266)	212–270 (237)	195–270 (223)	200–285 (240)	131–205 (183)	330
Anastomoses	Network	Network	Network	Network	Network	Network
Ovary length	235–1,095 (577)	710–1,360 (915)	570–940 (725)	–	–	–
Ovary width	132–670 (378)	310–580 (396)	340–600 (430)	–	–	–
Egg length	–	–	230–283 (246)	230	165	–
Egg width	–	–	125–135 (133)	120	90–106	–
No. of genital spines	8	8	8	8	8	–
Genital spine length	31.0–42.0 (34.4)	41–45 (44)	54	–	13–28 (18)	–
Marginal hooklet 1 length	30.8–33.6 (31.2)	23–27 (25)	–	–	–	–

Species	*P. lopezromani*	*P. guevarai*	*P. naevius*	*P. napoensis*	*P. praecox*	*P. stellai*	*P. touzeti*
Source	Combes & Laurent (1979)	Combes & Laurent (1979)	Caballero & Cerocero (1941)	Vaucher (1987)	Combes & Laurent (1978)	Pérez-Vigueras (1955)	Vaucher (1987)
Body length (BL)	6,990–8,160	6,790–7,880	3,864–5,876	3,120–3,470	3,000–6,400 (4,600)	7,100	4,180
Body maximum width	2,220–2,730	2,050–2,390	1,225–1,625	1,290–1,490	700–1,900 (1,200)	2,100–2,600	755
Haptor length (HL)	1,160–1,430	960–1,330	805–982	1,000–1,220	900–1,200 (1,000)	1,400	815
Haptor width	1,600–2,110	1,940–2,180	1,062–1,685	1,200–1,410	1,000–1,900 (1,300)	2,100	1,020
HL/BL ratio	0.18	0.10	0.18	0.34	0.22–0.27	0.20	0.20
Sucker diameter	316–401	316–401	273–370	286–403	260–410 (330)	350–380	270–311

Table 1 continued

Species	*P. lopezromani*	*P. guevarai*	*P. naevius*	*P. napoensis*	*P. praecox*	*P. stellai*	*P. touzeti*
Source	Combes & Laurent (1979)	Combes & Laurent (1979)	Caballero & Cerocero (1941)	Vaucher (1987)	Combes & Laurent (1978)	Pérez-Vigueras (1955)	Vaucher (1987)
Hamulus length	544–606	298–348	–	286–368	350–377	480	315–319
Hamulus hook length	–	–	–	–	–	–	–
Hamulus shape	Deep cut	Solid	–	–	Deep cut	–	Moderate cut
Pharynx length	292–330	286–342	161–402	186–209	110–220 (200)	–	213
Pharynx width	201–241	230–274	128–300	139–153	140–210 (170)	–	176
Anastomoses	Network	1–2	Network	Network	0	Network	0
Ovary length	–	–	–	–	–	–	–
Ovary width	–	–	–	–	320–620 (425)	530	–
Egg length	–	–	–	82–102	170–195 (180)	–	–
Egg width	–	–	–	180–210	–	–	–
No. of genital spines	–	–	–	–	–	–	–
Genital spine length	–	–	–	–	–	21	–

(Y) 360–457 (387); handle longer than guard: X/Y ratio 1.08–1.16 (1.12); hamulus hook length 75–98 (84). Marginal hooklet pairs 1 and 2 marginal, along periphery of haptor between posterior-most pair of suckers; marginal hooklet pairs 6–8 anterior in haptor (between sucker pair 3); posterior-most marginal hooklet 1 (Fig. 3D) 33 long.

Mouth subterminal, ventral. False oral sucker 190–350 (259) wide; pharynx length 280–325 (303); pharynx width 212–270 (237). Intestine forms reticulated network of anastomoses (Fig. 3A).

Testes and vas deferens obscured by intestinal caeca; seminal vesicle a dilatation of vas deferens, sigmoid, crossing midline, dorsal to oötype and uterus. Genital pore opening mid-ventral, posterior to intestinal caeca bifurcation; genital atrium muscular; genital bulb 90–210 (128), armed with 8 genital spines, each 41–45 (44) long (Fig. 3B). Ovary small, elongated and doubled over, dextral, submedian, anterior in body, constricted at level of middle portion, distal portion subovate, ascendant, proximal portion elongate, ovary length 710–1,360 (915), ovary width 310–580 (396). Vagina comprising double vaginal aperture with marginal opening; vaginal vestibule cup-shaped, with soft tissue. Vitelline collecting ducts join vaginal canal close to vaginae. Vitello-vaginal canal descends from both, confluent in middle of body and posterior to ovary. Mehlis' glands bilateral to oötype. Genito-intestinal canal prominent, joining intestinal caeca posterior to and on same side as ovary. Uterus tubiform, convoluted (Fig. 3a). No eggs observed *in utero*. Vitellarium distributed throughout body excluding around mouth, ovary, uterus and haptoral suckers (Fig. 3A).

Remarks

Polystoma knoffi n. sp. and *P. travassosi* n. sp. differ from each other and the other named and accepted species of *Polystoma* from the Neotropical Realm by a combination of characters (Table 1): *Polystoma knoffi* n. sp. and *P. travassosi* n. sp. have an intestine comprising a reticulated network. All known species of *Polystoma* from the Neotropical region have

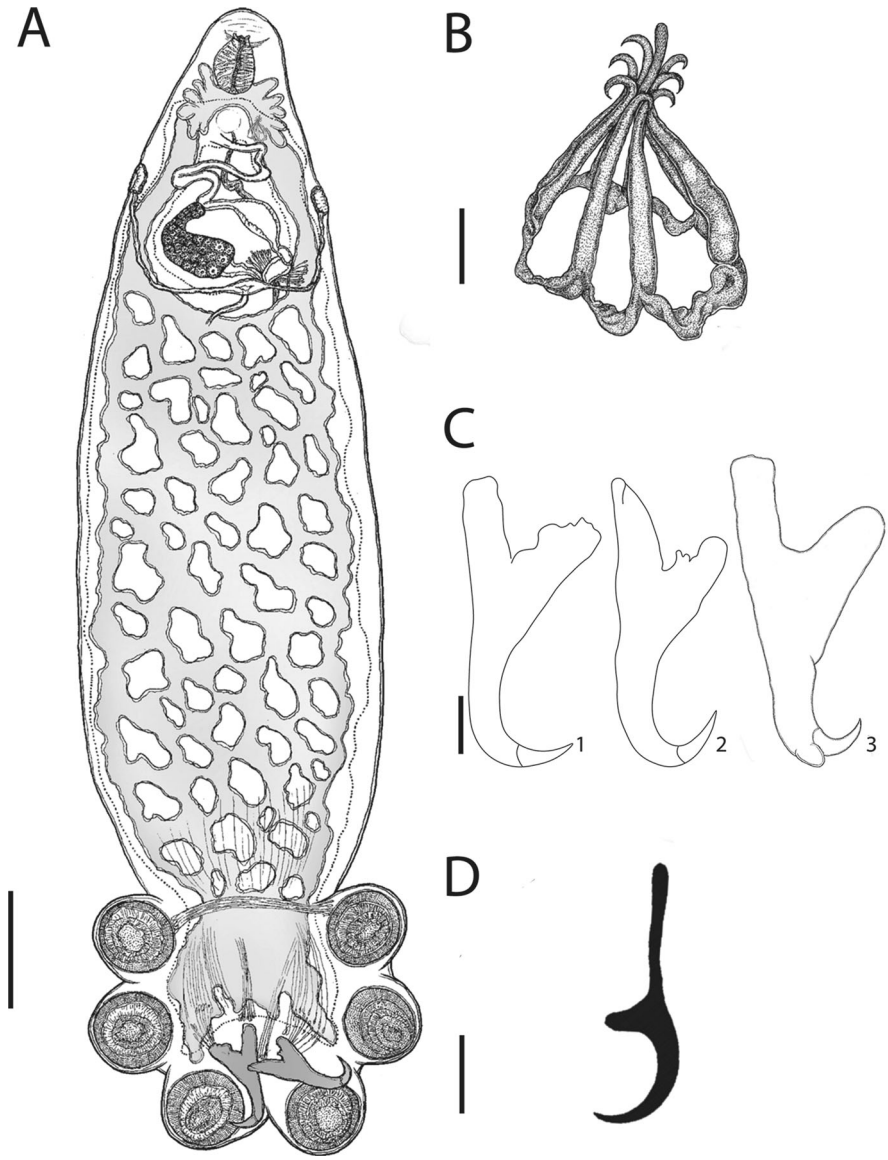

Fig. 3 *Polystoma travassosi* n. sp. A, Ventral view of the holotype; B, Genital spines, C, Hamulus; D, Marginal hooklet C1. *Scale-bars*: A, 1,000 μm; B, 50 μm; C, 100 μm; D, 10 μm

intestinal caeca forming a network with multiple anastomoses between the two intestinal caeca, with the exception of *P. guevarai* containing a single or at most two anastomoses, and *P. praecox* and *P. touzeti* lacking anastomoses.

Based on body length of mature specimens, the smallest specimen for both species is larger than the largest reported specimen for *P. cuvieri*, *P. napoensis* and *P. touzeti*. The haptor length/body length ratio of *P. knoffi* n. sp. is in the same range as reported for *P. andinum*, *P. borelli* and *P. praecox*; larger than that reported for *P. guevarai* Combes & Laurent, 1979, *P. lopezromani*, *P. naevius*, *P. stellai* Pérez-Vigueras, 1955 and *P. touzeti*, yet smaller than that reported for *P. cuvieri*, *P. diptychi* and *P. napoensis* (Table 1). The haptor length or body length ratio of *P. knoffi* n. sp. is similar to *P. andinum*, *P. praecox*, *P. stellae* and *P. touzeti*, smaller than that of *P. borelli*, *P. cuvieri*, *P. diptychi* and *P. napoensis*, and larger than the remainder of the known species. For *P. travassosi* n.

sp. the haptor length/body length ratio is in the same order as for *P. guevarai*, *P. lopezromani* and *P. naevius* and smaller than the remainder of the known species (Table 1).

Discussion

Global distribution patterns of parasite groups do often not reflect the true range of species but rather research effort towards certain hosts or areas. Polystomatid flatworms follow the same trend: the known distribution for species represented by clusters of species in parallel with research efforts. Examples are the polystome diversity reported for Argentina (5 species), Cameroun (7 species), Ivory Coast (7 species), South Africa (11 species), and Togo (6 species); which is a direct result of research efforts. A survey in the Vernon Crookes Nature Reserve, which is a relatively small reserve on the east coast of South Africa, revealed no less than six polystome species within a mere 20 km^2. The current known distribution of polystomes in the Neotropical Realm is no exception. Of polystomes known from anuran hosts of the Neotropical Realm, 63% were described by two research teams. Combes and Laurent described five species from Argentina (Combes & Laurent, 1974, 1978, 1979) while Vaucher described two from Equador (Vaucher, 1986, 1990), two from Paraguay (Vaucher, 1986, 1990), and one from Peru (Vaucher, 1981).

Tropical forests cover a mere 7% of the global continental surface but support over half of the Earth's species (http://www.wri.org/publication/content/8190). It is estimated that the Neotropics harbour almost 50% (Young et al., 2004) of the world's 8,010 amphibians (Frost, 2019) and around 32% of the reptiles (Urbina-Cardona, 2008). Based on these figures and a high degree of host specificity for amphibian polystomes (Du Preez & Kok, 1997; Tinsley, 2004), we may infer that a vast number of undescribed polystomes await discovery in the Neotropical Realm.

Among *Polystoma* spp., the intestinal caeca may vary from two caeca with hardly any diverticula, e.g. *P. chiromantis* (see Dupouy & Knoepffler, 1978) and *P. touzeti* (see Vaucher, 1987) on the one extreme to that of multiple intestinal diverticula and many anastomoses forming a reticulated network as in most species of *Polystoma* from the Neotropical Realm. Where a reticulated network is found in all but three of the known Neotropical species of *Polystoma*, it has been reported in only three of the 50 species of *Polystoma* from the Ethiopean Realm.

Prudhoe & Bray (1982) suggested that the lineage comprising species of *Polystoma* may have originated some 140 Myr ago during the Early Cretaceous period and that isolation as a result of continental drift played a major role in the evolution of *Polystoma*. Unpublished studies (Sinnapah N.D., Phylogeography of Monogenean Polystomatidae; A molecular approach to infer the evolutionary history of this group of parasites, thesis 1998, University of Perpignan) suggest that the lineage comprising species of *Polystoma* originated in South America and subsequently colonised North America, Europe and Africa. Verneau et al. (2002) stated that phylogenetic relationships within the Polystomatidae are linked with key events in host evolution. According to Badets et al. (2011), the divergence between species of *Polystoma* from Africa and South America is estimated around 156 Mya; close to the reported separation of the two continents (Macdonald et al., 2003). Detailed studies into the evolution and phylogeny of polystomes on the South American continent will only be possible when tissue from the various known species becomes available.

The value of documenting the shape and size of sclerotised structures (e.g. genital spines, hamuli, marginal hooklets) are well documented reagarding polystomes (Murith et al., 1978; Murith, 1981; Kok & Van Wyk, 1986; Kok & Seaman, 1987; Du Preez & Kok, 1992, 1993, 1995; Van Niekerk, Kok & Seaman, 1992; Du Preez & Lim, 2000; Lim & Du Preez, 2001; Du Preez et al., 2002, 2003; Du Preez & Maritz, 2006). Hamulus length is known for most of the 11 known species of *Polystoma* from the Neotropical Realm, whereas genital spine number and length are known for only three species from this region. Marginal hooklet length was not reported for any of the known species of *Polystoma* from the Neotropical Realm.

Traditionally, polystomes are fixed under coverslip pressure such that the genital spines, hamuli, and marginal hooklets are pressed flat to the slide; thereby allowing the observer to see them in lateral profile. Platt et al. (2011) argued that polystomes should not be flat-fixed as the coverslip pressure distorts the soft bodied parasites and that body measurements are as a result not accurate. This may be true but when

specimens are not flat fixed two major problems arise. In the first instance, hamuli are not flat orientated making it difficult to study the shape and obtain measurements, and secondly the preparation is quite thick which implies that the specimen cannot be studied using a 100× objective and in some instances not even the 40× objective. As a result, it is often impossible to locate and measure marginal hooklets in those unflattened specimens. Genital spines are densely packed in a crown and high magnification is also required to count and measure them. Documenting the body measurements of unflattened specimens does have value and therefore we propose a protocol whereby some specimens are fixated flat under coverslip pressure while others are killed by placing the live specimen in a drop of water or amphibian saline on a specimen glass and heating it from underneath with a butane flame until the parasite stops moving and then fix it in 10% buffered formalin. These unflattened specimens should be used for reporting soft body measurements. We further suggest to take time to study the live specimens while under coverslip pressure. Flattened specimens viewed alive under DIC can reveal subtle, delicate connections of the genital ducts as well as glands associated with the various ducts that may not be revealed by routine staining and clearing. We furthermore suggest that one specimen be fixed in molecular grade ethanol for molecular studies and stored in a freezer.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable institutional, national and international guidelines for the care and use of animals were followed. This study received the relevant ethical approval from North-West University's AnimCare ethics committee (Ethics Number: NWU-00380-16-A5).

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