Article

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Two new species of freshwater flatworms (Platyhelminthes: Tricladida: Continenticola) from South American caves

STELLA SOUZA[1], ANA LAURA MORAIS[1], MARIA ELINA BICHUETTE[2] & ANA LEAL-ZANCHET[1,3]

[1]*Instituto de Pesquisas de Planárias and Programa de Pós-Graduação em Biologia, Universidade do Vale do Rio dos Sinos – UNISI-NOS, 93022-000 São Leopoldo, RS, Brazil*
[2]*Departamento de Ecologia e Biologia Evolutiva, Universidade Federal de São Carlos – UFSCAR, 13565-905 São Carlos, SP, Brazil*
[3]*Corresponding author. E-mail: zanchet@unisinos.br*

Abstract

The diversity of freshwater triclads in the Neotropical region is considered to be low, but extensive areas of South America remain almost unexplored. Herein we describe two cave-dwelling, new species of *Girardia*, one from a transition zone of the Cerrado and Caatinga phytophysiognomies and the other from the Cerrado phytophysiognomy. The species from the Cerrado-Caatinga transition is a troglobite, eyeless and whitish; the species from the Cerrado area is possibly a troglophile, since it shows heavily pigmented body and eyes. Each species is easily recognized by a unique combination of features in its external morphology and copulatory apparatus. The two new species of *Girardia* show a restricted distribution, even the troglophile, and occur in caves without legal protection. Therefore, they must be considered as vulnerable organisms in a conservation context.

Key words: Platyhelminthes, Tricladida, *Girardia*, new cave-dwelling species, subterranean fauna, Brazilian savannah, Caatinga

Introduction

The diversity of freshwater triclads in the Neotropical region is considered to be low (Sluys *et al.,* 2005). However, extensive areas of South America remain almost unexplored, such as the Caatinga (broad mosaic of vegetation in a semi-arid climatic zone) and the Cerrado (savannah-like vegetation) ecosystems.

Two triclad suborders have representatives in Brazilian freshwater environments, namely Continenticola and Cavernicola. Knowledge of triclad diversity in South American subterranean environments is limited to a few records in north and central Brazil, corresponding to the occurrence of a troglophilic and a troglobitic species of the suborder Continenticola (Kawakatsu & Froehlich 1992; Souza *et al.* 2015), besides a troglobitic species of the suborder Cavernicola (Leal-Zanchet *et al.* 2014).

Other, unidentified species of Continenticola were recorded from caves at three different locations (Trajano & Bichuette 2010). One of these is a troglobite, occurring in the southeast portion of the state of Bahia, in a transition area of Cerrado-Caatinga. More recently, another species of Continenticola, which represents a possible troglophile, has been sampled from caves in the state of Minas Gerais. Both of these freshwater flatworms are herein described as new species, one of them representing the first troglobitic Continenticola for such an ecotone and for the state of Bahia.

Material and methods

Specimens of *Girardia desiderensis* Souza & Leal-Zanchet **sp. nov.** were collected from "Gruta da Baixa Fria" cave (Figs. 1–3), located at 598 m a.s.l. in the karst area of São Desidério, Bambuí Geomorphological Unit (S 12°22'55.6"; W 44°56'12.0"), São Desidério, Bahia, Brazil. This locality belongs to the João Rodrigues System,

which is one of the largest hydrogeological systems in South America (Rubbioli, 2004) and is situated in a transition zone of the Cerrado and Caatinga phytophysiognomies.

Specimens of *Girardia pierremartini* Souza & Leal-Zanchet **sp. nov.** were sampled in "Toca do Charco" cave (S 18º11'05.6"; W 46º09'39.31") and "Lapa da Fazenda São Bernardo" cave (S 18°16'36.83"; W 46°06'45.52"), located at 764 m and 802 m a.s.l., respectively, in the karst area of Presidente Olegário, Bambuí Geomorphological Unit, Presidente Olegário, Minas Gerais, Brazil (Figs. 1, 4–5). Both sampling locations are situated in the Cerrado phytophysiognomy.

The Brazilian savannah is mainly dominated by a tropical climate with a dry winter (type Aw of Köppen's classification; see Peel *et al.* 2007). The mean annual rainfall is approximately 1,400 mm, and the mean annual temperature is about 22°C to 24°C in Cerrado (Sallun *et al.* 2010). The Caatinga ecosystem is dominated by a semi-arid climate (type BSw'h' of Köppen's classification). The mean annual rainfall ranges from 240 mm to 1,500 mm, and the mean annual temperature is about 26°C to 28°C in the Caatinga (Nimer 1989; Prado 2003).

During field work, most specimens were directly fixed in ethanol 70%. One specimen of *G. pierremartini* sampled in "Toca do Charco" was also analysed alive in the laboratory and fixed in neutral formalin. Preserved specimens were analysed and photographed under a stereomicroscope. They were subsequently dehydrated and embedded in Paraplast. This material was sectioned at 5−7 µm and stained with hematoxyline/eosin or Goldner's Masson (Romeis, 1989). Colour descriptors, based on the uptake of dyes of particular colours, were used for classifying secretions with trichrome methods.

Type-material was deposited in the Museu de Zoologia da Universidade do Vale do Rio dos Sinos, São Leopoldo, Rio Grande do Sul, Brazil (MZU), and the Helminthological Collection of Museu de Zoologia da Universidade de São Paulo, São Paulo, São Paulo State, Brazil (MZUSP).

Abbreviations used in the figures. a: anterior tip; bc: bulbar cavity; br: brain; cb: copulatory bursa; cbc: bursal canal; cg: cyanophil glands; ceg: cement glands; ci: cilia; cm: circular cutaneous musculature; de: dorsal epidermis; eg: erythrophil glands; ej: ejaculatory duct; em: external pharyngeal musculature; es: esophagus; fc: female canal; go: gonoduct; i: intestine; im: internal pharyngeal musculature; in: insunk nuclei; lm: longitudinal cutaneous musculature; m: mouth; ma: male atrium; mg: glands with mixed secretion; o: ovary; om: oblique cutaneous musculature; ov: oviducts; pb: penis bulb; pg: penial glands; ph: pharynx; php: pharyngeal pouch; pp: penis papilla; r: rhabdites; sd: sperm duct; sg: shell glands; t: testes; ve: ventral epidermis; vi: vitellaria.

Taxonomic part

Order Tricladida Lang, 1884

Suborder Continenticola Carranza *et al.*, 1998

Family Dugesiidae Ball, 1974

Genus *Girardia* Ball, 1974

***Girardia desiderensis* Souza & Leal-Zanchet sp. nov.**

Etymology. The species name refers to its type-locality, the region of São Desidério, with one of the largest hydrogeological systems in South America.

Material examined. Holotype: MZUSP PL.1644: "Gruta da Baixa Fria" cave, São Desidério, state of Bahia (BA), Brazil, 4. November 2008, *coll*. M.E. Bichuette, sagittal sections on 19 slides.

Paratypes: "Gruta da Baixa Fria" cave, São Desidério, BA, Brazil, 4. November 2008, *coll*. M.E. Bichuette. MZU PL. 00208: sagittal sections on 19 slides; MZU PL. 00209: sagittal sections on 9 slides.

Type-locality. "Gruta da Baixa Fria" cave, São Desidério, BA, Brazil.

Distribution. Known only from the type-locality, São Desidério, BA, Brazil.

Diagnosis. Troglobitic *Girardia* species characterized by ventral testes, an ovoid, branched bulbar cavity; small, blunt penis papilla; broad bursal canal of the angled type.
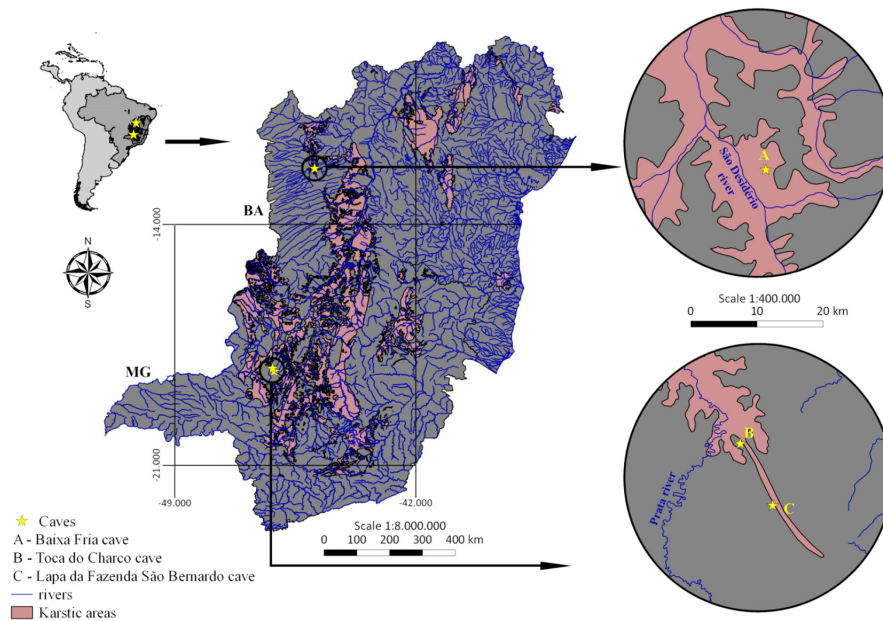
FIGURES 1–5. Sampling areas of *Girardia desiderensis* and *Girardia pierremartini* in the karst areas of São Desidério, Bahia state, and Presidente Olegário, Minas Gerais state, Brazil: (1) location of the studied areas; (2) outcrops of Baixa Fria cave, São Desidério karst area (Photograph: J. E. Gallão); (3) sampling site of *G. desiderensis* in the aphotic zone of Baixa Fria cave, São Desidério karst area (Photograph: J. E. Gallão); (4) sampling location of *G. pierremartini* in the photic zone of Lapa da Fazenda São Bernardo cave, Presidente Olegário karst area (Photograph: Ericson C. Igual); (5) sampling site of *G. pierremartini* in the twilight zone of Toca do Charco cave, Presidente Olegário karst area (Photograph: Tamires Zepon).
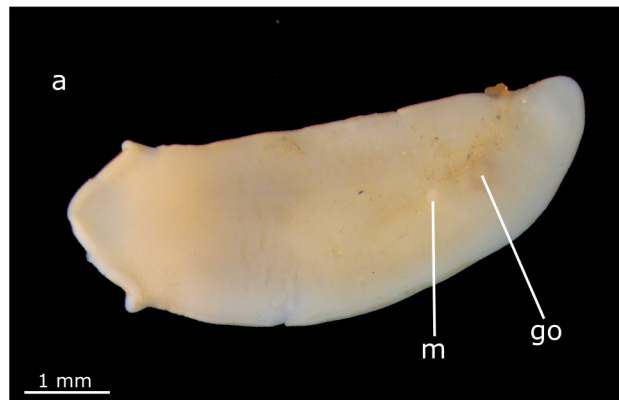
FIGURES 6–7. *Girardia desiderensis*. Photograph of a preserved specimen (paratype MZU PL. 00209) in dorsal (6) and ventral (7) view.

Description. *External morphology.* Live specimens are eyeless and whitish. After fixation, pale yellow dorsal surface with scattered, fine pigmentation (Fig. 6). Ventral surface whitish (Fig. 7). Triangular head with moderately sized auricles; posterior tip rounded (Figs. 6–7). Body up to 6.5 mm long and 3 mm wide (Table 1). Mouth and gonopore located in the posterior half of the body (Table 1; Fig. 7).

TABLE 1. Measurements, in mm, of preserved specimens of *Girardia desiderensis*. DG: distance of gonopore from anterior end; DM: distance of mouth from anterior end. The numbers given in parentheses represent the position relative to body length.

|	Holotype MZUSP PL. 1644	Paratype MZU PL.00208	Paratype MZU PL.00209
Length	6.5	6	5.5
Width	3	3	2
DM	5 (77%)	4 (67%)	4 (73%)
DG	5.5 (85%)	5 (83%)	4.5 (82%)

Epidermis, cutaneous musculature and sensory organs. Epidermis constituted of a columnar epithelium (Figs. 8–9), ciliated on the ventral body surface. The whole epidermis receives xanthophil, rhabditogen secretion (rhammites), as well as sparse secretions of three types of glands: (1) xanthophil, coarse granular secretion; (2) erythrophil, fine granular secretion; (3) cyanophil amorphous secretion. Rhammites are more densely distributed at the dorsal surface (Fig. 8). The xanthophil and erythrophil glands concentrate their openings medially at the anterior and posterior tips of the body.
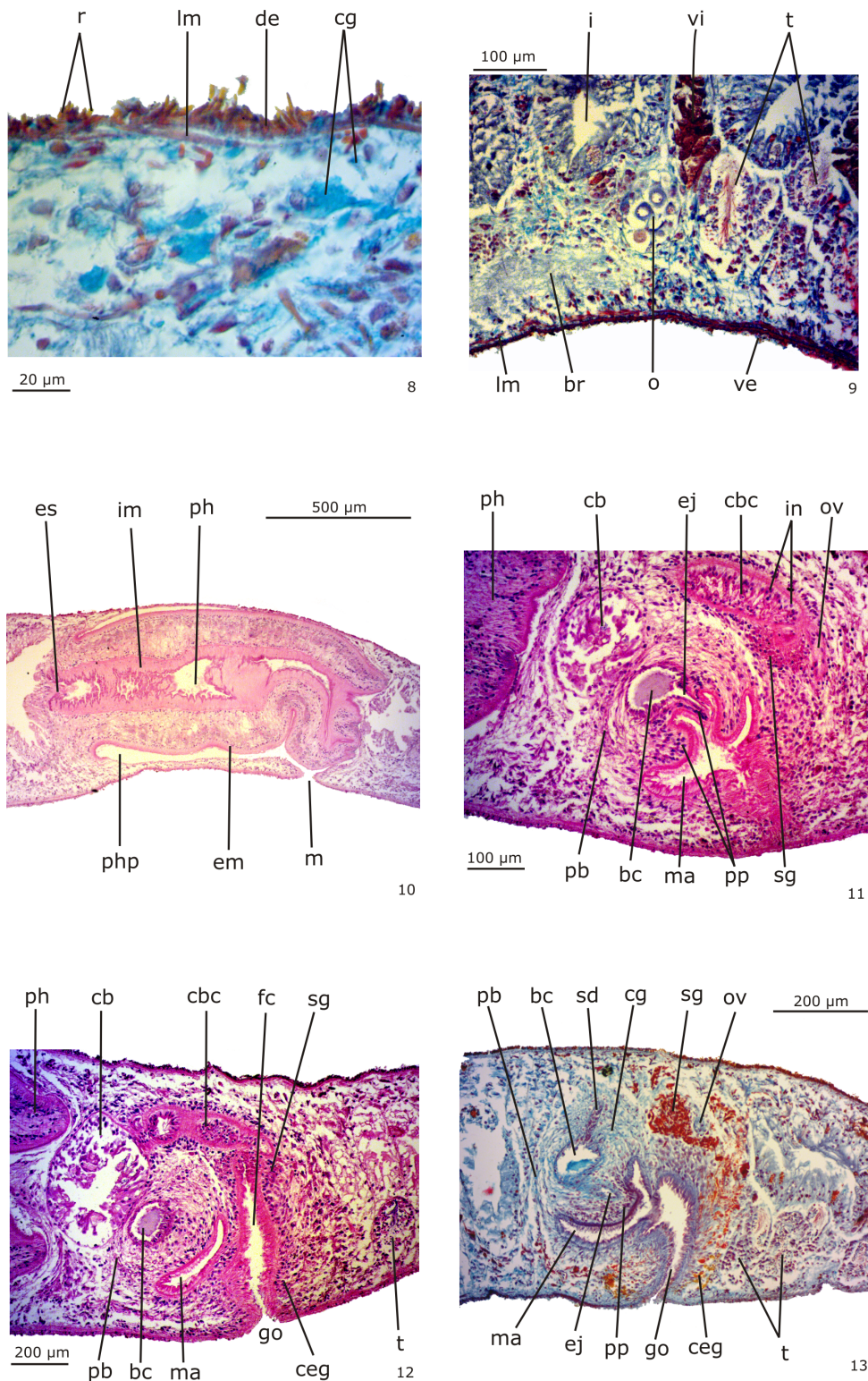
FIGURES 8–13. *Girardia desiderensis*, in sagittal sections: (8) dorsal surface of the body of paratype MZU PL. 00209; (9) ovary and anterior testes of paratype MZU PL. 00209; (10) pharynx of the holotype; (11–13) copulatory apparatus of the holotype (11–12) and paratype MZU PL. 00209 (13).

Cutaneous musculature of three layers, viz. a thin subepithelial circular layer, followed by an oblique layer with decussate fibers and a thicker layer of longitudinal muscle. The cutaneous musculature is weakly developed; the ventral (13–16 µm thick) is slightly higher than the dorsal musculature (5–10 µm thick) in the pre-pharyngeal region.
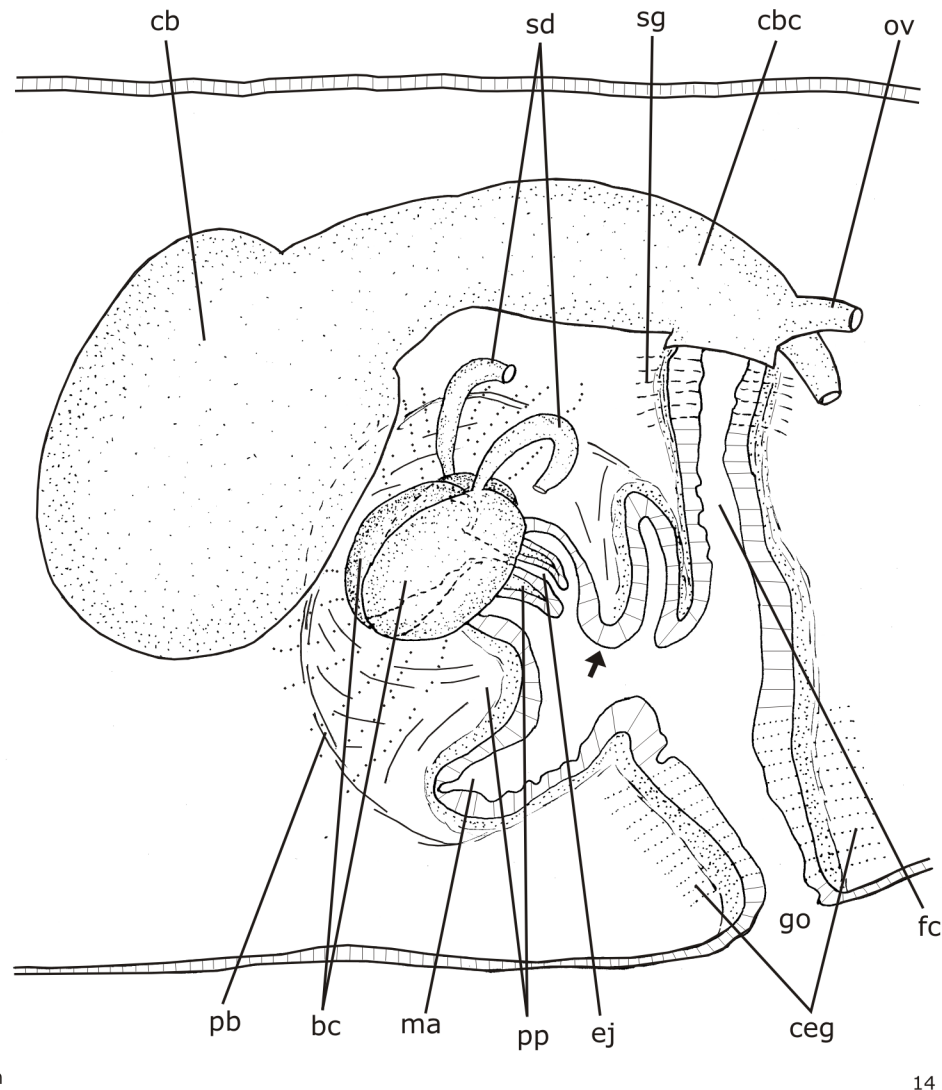
FIGURE 14. *Girardia desiderensis*. Sagittal composite reconstruction of the copulatory apparatus of the holotype. The arrow indicates the contracted penis papilla.

The auricular sensory organs are lined with densely ciliated, columnar epithelium, with insunk nuclei. Few cyanophil secretory cells open through this epithelium. The cutaneous musculature is very thin at the level of the sensory organs.

Digestive system. Pharynx cylindrical, non-pigmented; about 1/4 of the body length. It is located in the median third of the body. Mouth at the posterior end of the pharyngeal pouch (Fig. 10). An esophagus, between 1/8 to 1/9 of the pharyngeal length, connects the pharynx with the intestine (Fig. 10). Intestine with the usual tricladid form, with the anterior intestinal trunk extending onto the brain.

Male reproductive system (Figs. 9–14). Numerous testicular follicles, about 70–100 μm in diameter, arranged in various irregular ventral rows on either side of the body (Figs. 9, 12–13). Testes extend from about 1 mm from the anterior tip (equal to 20–25% of body length in sagittal sections), just behind the ovaries (Fig. 9), to the posterior end of the body. Sperm ducts dorsal to the nerve cords. They ascend laterally to the copulatory apparatus, in an almost straight vertical course, and separately penetrate the penis bulb. Close to their opening, the sperm ducts diminish in diameter, form a short loop, and turn anteriad. Each duct opens into lateral expansions of the bulbar cavity. This cavity is large, with an ovoid aspect in the sagittal plane, and shows two lateral ovoid expansions (Figs. 12–14). A narrow and short ejaculatory duct traverses the blunt penis papilla (about 140–190 μm long and 110–125 μm wide at its basis), which may form a circular fold at the basis of the penis papilla (holotype and paratype MZU PL. 00208). The penis papilla is obliquely oriented in the male atrium (Figs. 11, 13–14).

Sperm ducts lined with a ciliated, cuboidal epithelium, becoming flattened in the spermiducal vesicles; they are coated mainly with circular muscles (about 2 μm thick). The penis bulb consists of a loose connective tissue

with necks of cyanophil penial glands and scarce interwoven muscle fibers (Figs. 11–13). Bulbar cavity lined with a non-ciliated, cuboidal to columnar epithelium, which is underlain by a weak layer of interwoven circular and longitudinal muscles (about 2 μm thick). Abundant cyanophil penial glands, with coarse granular secretion and extrabulbar cell bodies, open into the bulbar cavity. This secretion is usually visible in the bulbar cavity as an amorphous mass (Figs. 11–13). Ejaculatory duct lined with non-ciliated, flat to cuboidal epithelium; it is surrounded by a thin muscularis composed of interwoven circular and longitudinal muscles (about 1–2 μm thick). Cyanophil glands with coarse granular secretion and extrabulbar cell bodies and scarce xanthophil/ erythrophil glands with subepithelial cell bodies open into this duct. The penis papilla is covered with a non-ciliated, cuboidal to columnar epithelium containing some insunk nuclei. Muscularis of the penis papilla composed of a subepithelial layer of circular fibres, followed by a layer of longitudinal fibres (about 6–7 μm thick), becoming thinner and consisting of interwoven circular and longitudinal muscles towards the tip of the papilla. Penial glands with amorphous, slightly cyanophil secretion and xanthophil glands with granular secretion open through the epithelium of the penis papilla. Cyanophil glands with extrabulbar cell bodies; xanthophil/erythrophil glands with subepithelial cell bodies. Male atrium lined with a non-ciliated, columnar epithelium with some insunk nuclei (Figs. 11–13). The male atrial muscularis (3–4 μm thick) is constituted of a subepithelial layer of circular fibres, followed by a thin layer of longitudinal fibres. Glands with cyanophil amorphous secretion and subepithelial cell bodies open into the male atrium (Fig. 13).

Female reproductive system (Figs. 9, 11–14).Vitellaria well developed, located dorsally and between intestinal branches. Ovaries ovoid (Fig. 9), about 90 μm in diameter. They are situated dorsally to the ventral nerve cords, in close proximity to the brain (between 0.1–0.7 mm behind it) and about 0.9–1.2 mm behind the anterior tip (equal to 18% to 23% of body length in sagittal sections). Ovovitelline ducts arising from the lateral surface of the ovaries, sometimes displaced to the dorsal surface, and running backwards dorsally to the sperm ducts and nerve cords. Just behind the gonoduct, the ovovitelline ducts turn dorsally to open separately into the most distal part of the bursal canal, in close proximity to each other. Copulatory bursa ovoid, located in close proximity to the pharyngeal pocket (Figs. 11–12). Bursal canal short and broad, mainly horizontal disposed, showing a sharp bend just before communicating with the female canal (Figs. 12, 14). Gonoduct almost straight (Figs. 12–14), slightly inclining forward to communicate with the atria.

Ovovitelline ducts lined with ciliated, cuboidal nucleated epithelium with some insunk nuclei. They are covered mainly by circular muscle fibres (about 2 μm thick). Copulatory bursa lined with non-ciliated, columnar epithelium with the usual morphology. The bursa of the holotype contains some spermatozoids absorbed by its epithelial cells (Figs. 11–12). Bursal canal lined with a ciliated, columnar nucleated epithelium with irregular height (Figs. 11–12) and some insunk nuclei, receiving cyanophil amorphous secretion. Glands with fine granular, strong xanthophil secretion (shell glands) open into the bursal canal distally to the ovovitelline duct openings (Fig. 12). The muscularis of the bursal canal (about 5–10 μm thick) is constituted of interwoven circular and longitudinal fibres. Female canal lined with a tall columnar epithelium (Fig. 12), the cells of which show irregular height and some insunk nuclei. The muscularis of the female canal (about 10 μm thick) is constituted of interwoven circular and longitudinal fibres. Few cyanophil glands with subepithelial cell bodies open into the female canal. Gonoduct lined by tall columnar epithelium with irregular height, ciliated in its ectal portion, and coated with a subepithelial layer of circular muscle, followed by a layer of longitudinal muscle (about 8–12 μm thick). Abundant cement glands with coarse granular, xanthophil secretion of two types (one coarser than the other, the coarser one with cromophobous secretion in the holotype) and glands with heavily stained, cyanophil amorphous secretion discharge into the gonoduct. These glands have long cell necks and their cell bodies are scattered in the mesenchyme, some of them adjacent to the muscle layers of the gonoduct (Figs. 12–13).

Variability. All analysed specimens show well developed vitellaria, indicating that they are mature specimens. The occurrence of a circular fold at the basis of the penis papilla of the holotype (Figs. 11, 14) and paratype MZU PL. 00208 may be due to contraction of the specimens during fixation, since this characteristic was not observed in paratype MZU PL. 00209 (Fig. 13).

Comparative discussion. Considering the lack of eyes and whitish body, in live specimens, the troglobitic *Girardia desiderensis* differs from the majority of its congeners, which are heavily pigmented, epigean organisms. After fixation, it resembles the hypogean *Girardia mckenziei* (Mitchell & Kawakatsu, 1973), from Mexico, regarding the slight pigmentation, but this species has two minute eyes (Mitchell & Kawakatsu 1973a). Eyes and pigmentation are also absent in other troglobitic congeners, such as *G. typhlomexicana* (Mitchell & Kawakatsu, 1973) and *G. barbarae* (Mitchell & Kawakatsu, 1973), both from Mexico, and *G. multidiverticulata* Souza *et al.*, 2015, from the Brazilian Cerrado ecosystem (Mitchell & Kawakatsu 1973b; Souza *et al.* 2015). However, the preserved specimens of these three species have unpigmented body, differing from the pale-yellow body, with scattered pigmentation, of preserved specimens of *G. desiderensis*. Regarding body length, mature

specimens of *G. desiderensis*, with a maximal length of 6.5 mm, are smaller than those of *G. multidiverticulata*, with 12 mm and 20 mm lengths after fixation (Souza *et al.* 2015). *Girardia desiderensis* also differs from the hypogean *Girardia guatemalensis* (Mitchell & Kawakatsu, 1973), from Mexico, as well as from the troglophilous specimens of *G. paramensis* (Fuhrmann, 1914), reported for the state of Pará, in northern Brazil, which show heavily pigmented body and a pair of eyes (Mitchell & Kawakatsu 1973b; Kawakatsu & Mitchell 1981; Kawakatsu & Froehlich 1992).

Regarding the reproductive system, *G. desiderensis* can be differentiated from other troglobitic species, such as *G. barbarae*, *G. mckenziei* and *G. typhlomexicana*, which have dorsal and/or dorsoventral testes and do not show the distinctly angled bursal canal (Mitchell & Kawakatsu 1973a, b) as in *G. desiderensis*. Similar to *Girardia desiderensis*, the troglobitic *G. guatemalensis* and *G. multidiverticula* show ventral testes. However, both species differ from *G. desiderensis* by the form of the bulbar cavity and the position of the opening of the spermioducts. In *G. guatemalensis*, the single bulbar cavity receives the opening of the spermioducts in its very distal part, whereas in *G. multidiverticulata* the large bulbar cavity, with multiple diverticula, receives the opening of each sperm duct in one of its lateral diverticula.

In relation to the epigean species, the reproductive system of *G. desiderensis* has a similar gross morphology with that of some specimens of *G. festae* (Borelli, 1898), by having ventral testes, a forked bulbar cavity and an angled bursal canal (Kawakatsu & Mitchell 1984, Sluys 1992, Sluys *et al.* 2005). *Girardia festae* is a polymorphic species, originally described from Bahía Blanca, Argentina, which was later reported for other localities in South America (Kawakatsu & Mitchell 1984, Sluys 1992, Sluys *et al.* 2005), showing great variation in the anatomy of the bulbar cavity, penis papilla, ejaculatory duct and bursal canal. According to Sluys *et al.* (2005), the shape of the penis papilla is a well distinguishable feature of *G. festae*, whereas Kawakatsu & Mitchell (1984) considered that its papilla is highly variable. *Girardia desiderensis* shows a small and blunt penis papilla, which only sometimes contracts and forms a circular fold or annular zone, superficially resembling or not resembling the penis papilla of *G. festae*, depending on the interpretation. However, the bulbar cavity of *G. desiderensis* shows a characteristic feature, which is its ovoid aspect at the sagittal plane and also ovoid lateral portions maintaining diameter to receive the openings of the spermioducts. In contrast, the single or forked, elongated or ovoid bulbar cavity of *G. festae* gradually diminishes in diameter in order to receive the openings of the spermioducts (Kawakatsu & Mitchell 1984, Sluys 1992, Sluys *et al.* 2005).

Other species with large and forked bulbar cavity, such as *G. anderlani* (Kawakatsu & Hauser, 1983) and *G. capacivasa* Sluys, 2005, have dorsal or dorsoventral testes, besides other differential characters (Kawakatsu *et al.* 1983, Sluys *et al.* 2005).

In conclusion, the troglobitic *G. desiderensis* shows a unique combination of characters of its external morphology, such as absence of eyes and body pigmentation, and of its copulatory apparatus, such as ventral testes, a small and blunt penis papilla, a broad bursal canal of the angled type, and, especially, an ovoid, branched bulbar cavity, which distinguish the new species from its congeners.

Girardia pierremartini Souza & Leal-Zanchet sp. nov.

Etymology. The species name is dedicated to the Grupo Pierre Martin de Espeleologia as acknowledgement of numerous discoveries concerning caves in Brazil and especially in the Presidente Olegário region.

Material examined. Holotype: MZUSP PL.1645: "Lapa Fazenda São Bernardo" cave, Presidente Olegário, state of Minas Gerais (MG), Brazil, 30. September 2013, *coll*. M.E. Bichuette, sagittal sections on 9 slides.

Other specimens: Paratype MZU PL. 00210: "Lapa Fazenda São Bernardo" cave, Presidente Olegário, MG, Brazil, 30. September 2013, *coll*. M.E. Bichuette, sagittal sections on 11 slides, MZU PL. 00211: "Toca do Charco" cave, Presidente Olegário, MG, Brazil, 29. September 2013, *coll*. M.E. Bichuette, sagittal sections on 22 slides; MZU PL. 00212: "Toca do Charco" cave, Presidente Olegário, MG, Brazil, 29. September 2013, *coll*. M.E. Bichuette, sagittal sections on 13 slides.

Type-locality. "Lapa Fazenda São Bernardo" cave, Presidente Olegário, MG, Brazil.

Distribution. Known only from the Presidente Olegário karst area, MG, Brazil.

Diagnosis. This troglophilic *Girardia* species is characterized by ventral testes, a branched bulbar cavity with lateral funnel-shaped portions, obliquely oriented penis showing two distinct portions and a bursal canal smoothly inclining ventrally to communicate with the female canal.
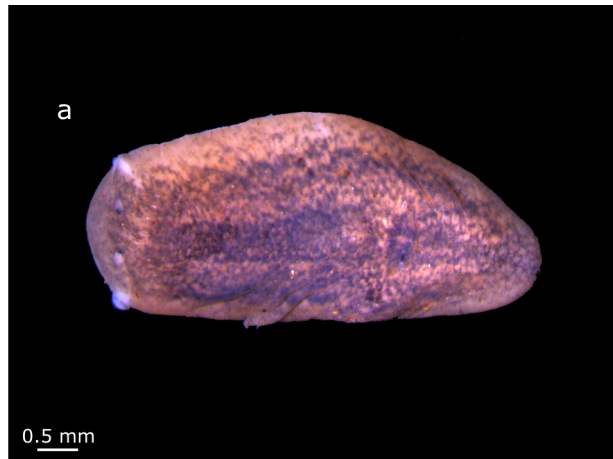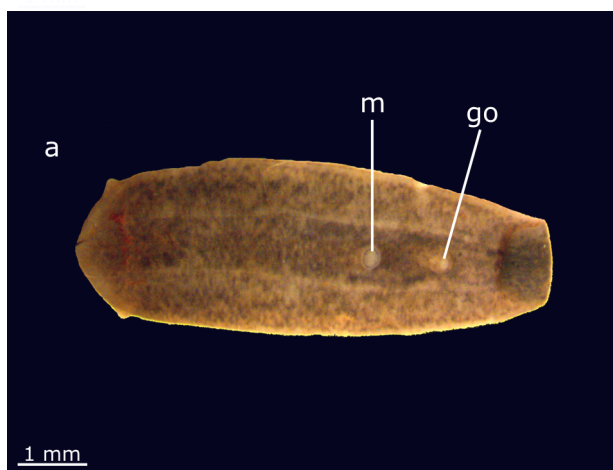
FIGURES 15–17. *Girardia pierremartini*. Photographs of preserved specimens: (15) paratype MZU PL. 00210 in dorsal view; (16–17) specimen MZU PL. 00212 in dorsal (16) and ventral (17) view. The posterior tip of the specimens is bent.

Description. *External morphology.* Live specimens are brown and provided with two eyes. After fixation, the light-brown ground colour is covered by numerous dark-brown flecks that are homogeneously distributed over the dorsal surface and more sparsely distributed on the ventral surface. Head broadly triangular with moderately sized auricles after fixation (Figs. 15–17); posterior tip pointed. Preserved specimens up to 8.5 mm long and 3 mm wide (Table 2). Mouth located in the median third of the body and gonopore located in the posterior half of the body (Table 2, Figs. 15, 17).

TABLE 2. Measurements, in mm, of preserved specimens of *Girardia pierremartini*. DG: distance of gonopore from anterior end; DM: distance of mouth from anterior end. The numbers given in parentheses represent the position relative to body length.

	Holotype MZUSP PL. 1645	Paratype MZU PL.00210	Specimen MZU PL. 00211	Specimen MZU PL. 00212
Length	6	6	8	8.5
Width	2	2.5	3	2.5
DM	3 (50%)	3 (50%)	4 (50%)	4.5 (53%)
DG	4 (67%)	4 (67%)	5 (62%)	5 (59%)

Epidermis, cutaneous musculature and sensory organs. Epidermis constituted of a columnar epithelium (Figs. 18–19), ciliated on the ventral body surface. The whole epidermis receives secretions of four types of glands: (1) xanthophil, rhabditogen secretion (rhammites), more densely distributed at the dorsal surface; (2) erythrophil, fine granular secretion; (3) xanthophil, fine granular secretion; and (4) cyanophil amorphous secretion. The erythrophil glands and a xanthophil, coarse granular secretion concentrate their openings medially at the anterior and posterior tips of the body. Numerous pigment cells are located under the dorsal and ventral epidermis.

Cutaneous musculature consisting of three layers, viz. a thin subepithelial circular layer, followed by an oblique layer with decussate fibers and a thicker layer of longitudinal muscle. Ventral musculature thicker than the dorsal musculature in the pre-pharyngeal region (20–25 μm thick and 6–15 μm thick, respectively).

The auricular sensory organs are lined with a densely ciliated, low cuboidal epithelium without rhammites, with scarce glands and with some insunk nuclei. The cutaneous musculature is very thin at the level of the sensory organs.

Digestive system. Pharynx cylindrical, pigmented; between about 1/5 and 1/4 of the body length. It is located approximately in the median third of the body. Mouth at the posterior end of the pharyngeal pouch. An esophagus, about 1/10 of the pharyngeal length, connects the pharynx with the intestine. Intestine with the usual tricladid form, with the anterior intestinal trunk extending onto the posterior half of the brain.

Male reproductive system (Figs. 19–24). Abundant testicular follicles, 50–90 μm in diameter, arranged in various irregular rows on each side of the body. They are situated mainly ventrally (Figs. 19–20), but may occupy the entire dorso-ventral space. Testes extend from about 0.8 mm to 1 mm from the anterior tip (equal to 13% or 14% of body length in sagittal sections), just behind the brain, to the posterior end of the body. Sperm ducts run posteriorly dorsally to the nerve cords and form spermiducal vesicles laterally to the pharynx. Laterally to the copulatory apparatus, the spermiducal vesicles ascend, in an almost straight vertical course, and penetrate the penis bulb. They diminish in diameter and bend to open dorsally into the large, branched and ample bulbar cavity (Figs. 20–24). Each lateral branch of the bulbar cavity has the form of a funnel, narrowing to receive the opening of the sperm ducts. The long ejaculatory duct widens towards its opening at the tip of the blunt penis papilla. At the basis of the bulbar cavity, proximally to the transition to the ejaculatory duct, there is a diverticulum. The penis papilla (about 250–310 μm long and 310–400 μm wide at its basis), obliquely oriented in the male atrium, shows two parts, a basal portion constituted of a circular fold and a distal truncated portion (Figs. 20, 23–24). The basal portion of the papilla contains the most distal part of the bulbar cavity and the distal portion is traversed by the ejaculatory duct.

Sperm ducts lined with a ciliated, cuboidal epithelium, becoming flattened in the spermiducal vesicles; they are coated with a circular muscle layer (1–2 μm thick). The penis bulb, occupied by the large and branched bulbar cavity (Figs. 20–24), consists of a loose connective tissue containing abundant gland necks of penial glands and interwoven muscle fibers. Bulbar cavity lined with a non-ciliated, columnar epithelium. The muscle fibres of the penis bulb may surround this cavity. Numerous penial glands with coarse granules containing a mixed secretion open into the bulbar cavity (Figs. 20–23). This secretion has a cyanophil external part and an erythrophil internal core and it is usually seen in the bulbar cavity. In addition, erythrophil penial glands with coarse granules, densely disposed in the cytoplasm, open into the bulbar cavity. Both types of penial glands have extrabulbar cell bodies. Ejaculatory duct lined with cuboidal to columnar epithelium (Fig. 20) which may contain sparse cilia. The muscularis of the ejaculatory duct is thin (about 2 μm thick), sometimes inconspicuous. Numerous erythrophil glands with fine granules and scarce glands with amorphous, slightly cyanophil secretion have their openings into
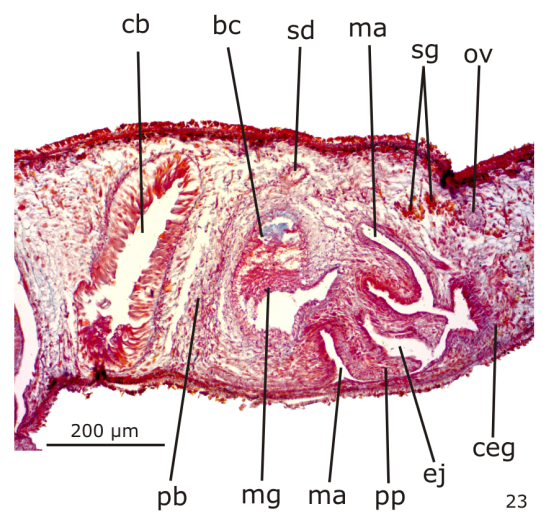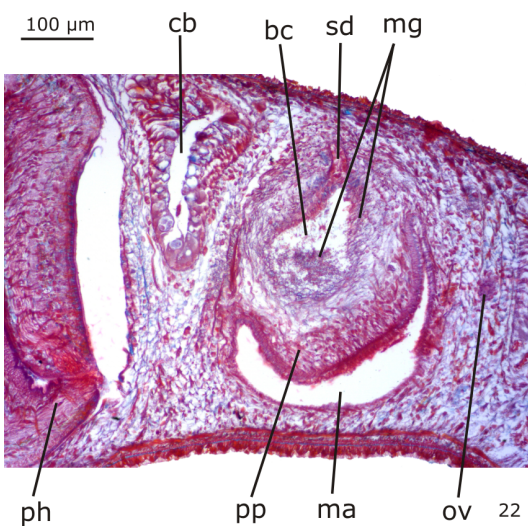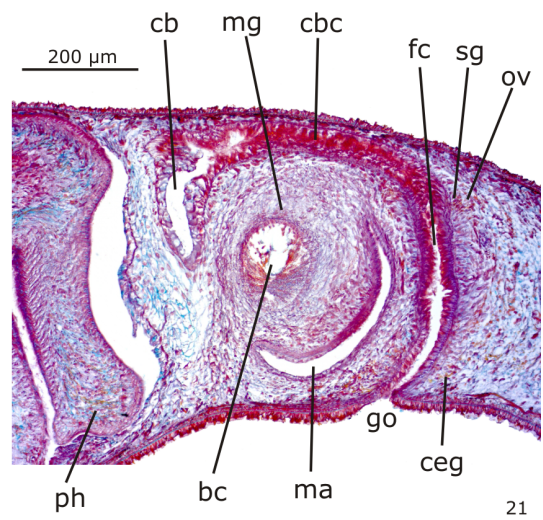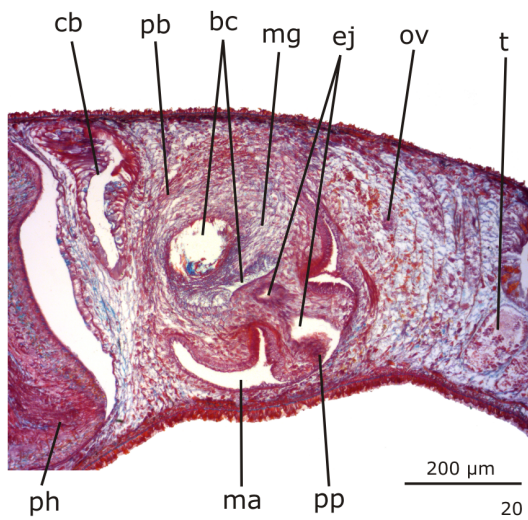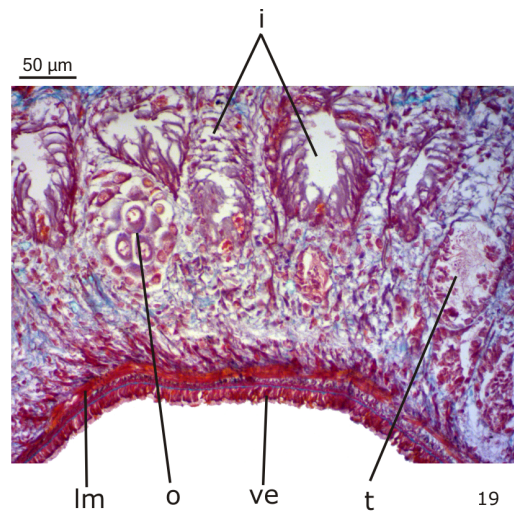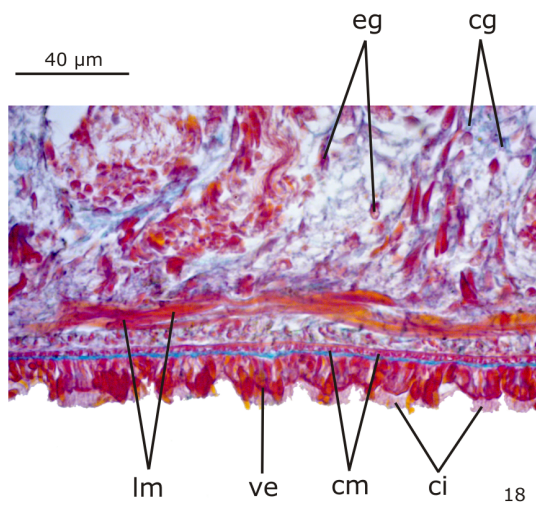
FIGURES 18–23. *Girardia pierremartini*, in sagittal sections: (18) ventral surface of the body of the holotype; (19) ovary and anterior testes of the holotype; (20–23) copulatory apparatus of the holotype (20–22) and of the specimen MZU PL. 00212 (23).
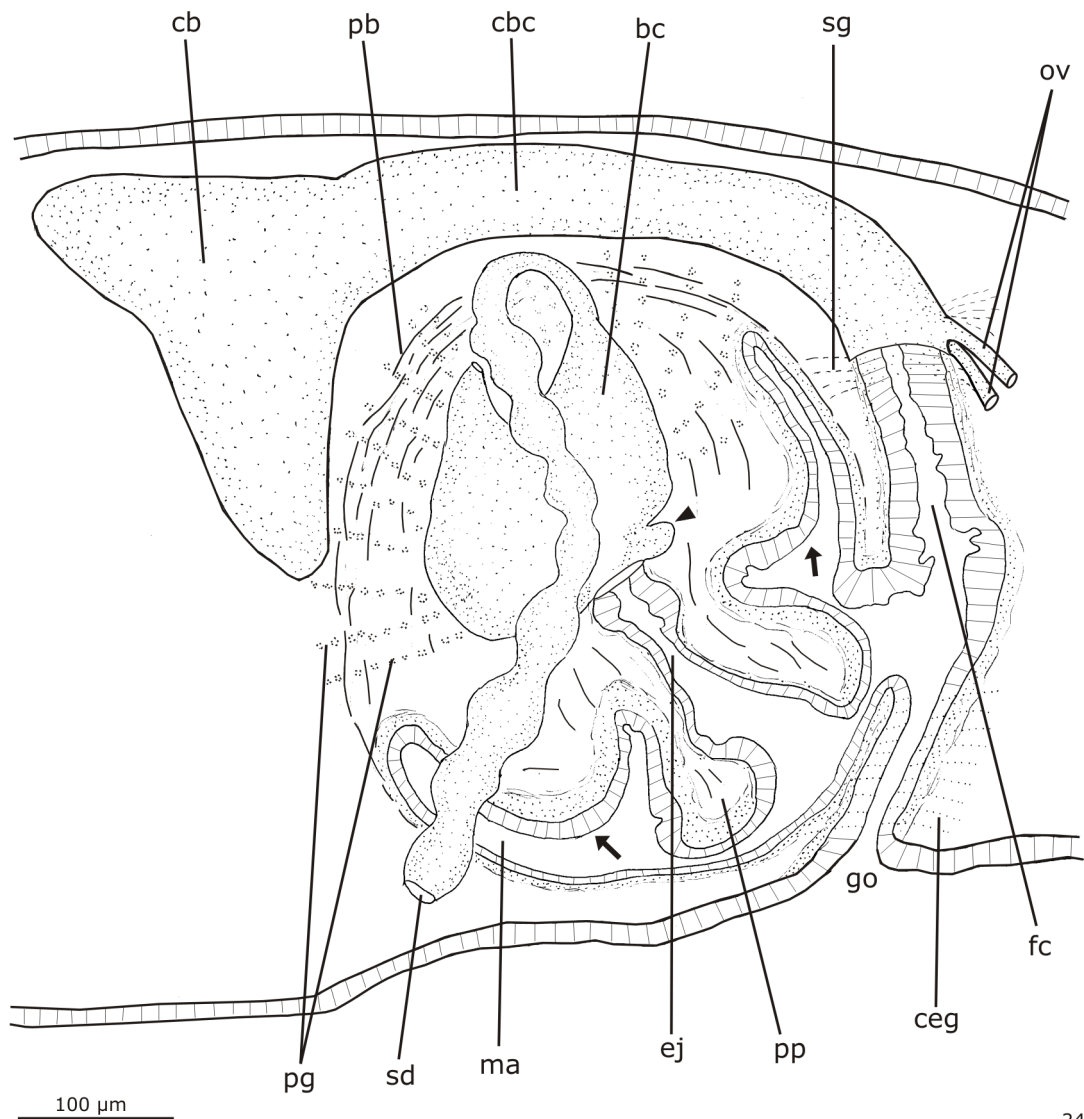
the ejaculatory duct. Penis papilla covered with a non-ciliated, cuboidal to columnar epithelium with some insunk nuclei. Muscularis of penis papilla composed of a thick subepithelial layer of circular fibres (5–10 μm thick), followed by a layer of longitudinal fibres. The longitudinal layer is thicker in the distal portion of the penis papilla (10–12 μm thick) than in its basal portion (2–5 μm thick). Two types of penial glands, one with fine granular, erythrophil secretion and the other with amorphous, cyanophil secretion, open through the epithelium of the penis papilla. Both glands have intrapenial cell bodies. Male atrium lined with a non-ciliated, cuboidal to columnar epithelium with some insunk nuclei. The male atrial muscularis is constituted of a thin subepithelial layer of circular fibres (1–3 μm thick), followed by a thicker layer of longitudinal fibres (2–6 μm thick). Glands with slightly cyanophil amorphous secretion and erythrophil glands with fine granular secretion open sparsely into the male atrium. Cell bodies of these glands are located subepithelially or around fibers of the atrial musculature.

Female reproductive system (Figs. 19–24). Vitellaria inconspicuous in the holotype and most other examined specimens. Ovaries ovoid (Fig. 19), 120–170 μm in diameter. They are situated medially to the ventral nerve cords, with a small part dorsally to them, at about the same transversal level as the anteriormost testes and in close proximity to the brain, about 0.7 mm to 1 mm behind the anterior tip (equal to 13% or 14% of body length in sagittal sections). Ovovitelline ducts arising from the dorsal surface of the ovaries, but laterally or medially displaced, and running backwards dorsally to the nerve cords, following a sinuous path. Behind the gonoduct, the ovovitelline ducts turn towards the dorsal surface of the body and separately open into the most distal part of the bursal canal, in close proximity to each other. Copulatory bursa large and ovoid in most examined specimens, occupying the space between the penis bulb and the pharyngeal pouch (Figs. 20–24). Bursal canal long, with its proximal half parallel to the dorsal surface of the body and its distal half smoothly inclining ventrally to opening into the large, vertically disposed female canal. Gonoduct almost straight (Figs. 21, 24).

Ovovitelline ducts lined with ciliated, cuboidal and nucleated epithelium with some insunk nuclei; it is covered mainly by circular muscle fibres (about 2 μm thick). Copulatory bursa lined with non-ciliated, columnar epithelium composed of cells with erythrophil secretion and cells with heavily stained, cyanophil secretion, receiving xanthophil, amorphous secretion as well as erythrophil, coarse granular secretion from glands with subepithelially located bodies. The bursa is covered by a thin muscle coat constituted of interwoven longitudinal and circular muscle fibres (3–8 μm thick). Bursal canal lined with a ciliated, columnar epithelium, the cells of which show irregular height (Fig. 21). There are some insunk nuclei and cell bodies of xanthophil glands with amorphous secretion, as well as erythrophil glands with coarse granular secretion around the bursal canal. The latter concentrate their openings into the most distal part of the canal, around the openings of the ovovitelline ducts, and constitute the shell glands (Figs. 21, 23–24). The muscularis of the bursal canal is constituted of interwoven circular and longitudinal muscle fibres (4–8 μm thick). In the holotype, there is sperm in the bursal canal and a few in the copulatory bursa. Female canal lined with a tall columnar epithelium and some insunk nuclei, the cells of which show irregular height and apocrine secretion. The muscularis of the female canal is constituted of interwoven circular and longitudinal fibres (8–12 μm thick). Abundant glands of two types open into the female canal: cyanophil glands with amorphous secretion and erythrophil glands with coarse granular secretion. Gonoduct lined with tall columnar epithelium, with some insunk nuclei, becoming ciliated close to the ventral surface of the body. The gonoduct is coated with a subepithelial layer of circular muscle (about 4–6 μm thick), followed by a layer of longitudinal muscle (about 5–10 μm thick). Abundant cement glands of two types discharge into the gonoduct, one xanthophil and the other erythrophil (Figs. 21, 23). Both glands show coarse granular secretion. In addition, cyanophil glands with amorphous secretion and erythrophil glands with fine granular secretion also open into the gonoduct. These glands have long cell necks and cell bodies scattered in the mesenchyme, some of them adjacent to the muscle layers of the gonoduct (Fig. 23).

Variability. Specimen MZU PL. 00211 seems to be the only fully mature specimen, showing well developed vitellaria. All examined specimens contain a large, massive penis papilla constituted of the basal and distal portions described above, but the specimen MZU PL. 00211, which is very much contracted, shows an everted pharynx and an extended penis papilla. Due to this extension, the papilla is more elongated, with twice the length than when in the resting stage, and the distal part of the bulbar cavity, as well as the ejaculatory duct, are longer than in other specimens. Both portions of the penis papilla are still recognized in this specimen, with the distal portion projected through the gonopore. It also shows signs of recent copula, since the bursa contains parts of a spermatophore with sperm mixed with erythrophil and cyanophil secretions in its lumen.

FIGURE 24. *Girardia pierremartini*. Sagittal composite reconstruction of the copulatory apparatus of the holotype. Arrows indicate the basal part of the penis papilla; the arrowhead indicates a diverticulum of the ejaculatory duct.

Comparative discussion. The trogophilic *Girardia pierremartini* differs from the majority of its hypogean congeners by having a heavily pigmented body and a pair of eyes, superficially resembling *G. guatemalensis*, as well as the troglophilic specimens of *G. paramensis* recorded in north Brazil (Mitchell & Kawakatsu 1973b; Kawakatsu & Froehlich 1992). It also differs from the hypogean *G. mckenziei* which only presents slight, minute pigmentation and two minute eyes (Mitchell & Kawakatsu 1973a).

Regarding the reproductive system, comparing *G. pierremartini* to other troglobitic species with ventral testes and a forked bulbar cavity, it has only superficial similarities with *G. desiderensis*. However, they differ mainly by the form of the bulbar cavity and the form and histological constitution of the bursal canal. The bulbar cavity has a characteristic form in *G. desiderensis*, as already commented, showing an ovoid aspect at the sagittal plane and ovoid lateral portions that maintain their diameter to receive the openings of the spermioducts. In contrast, *G. pierremartini* has funnel-shaped forked portions, gradually diminishing their diameter so as to receive the openings of the spermioducts. Furthermore, *G. desiderensis* has an angled bursal canal, whereas in *G. pierremartini* it smoothly inclines ventrally to communicate with the female canal. In addition, the relative abundance of the epidermal glands, the pigmentation of the pharynx, the musculature of the bursal canal, and penial glands distinguish both species one from another.

Considering the epigean species, similarly to *G. desiderensis*, the reproductive system of *G. pierremartini* has overall similarities with those of some specimens of *G. festae*, by having ventral testes, a penis papilla containing a

circular fold, and a forked and ample bulbar cavity (Kawakatsu & Mitchell, 1984, Sluys 1992, Sluys *et al.* 2005). However, in *G. pierremartini*, the spermioducts ascend in an almost straight vertical course laterally to the copulatory apparatus, whereas in *G. festae* they have a characteristic recurving course (Kawakatsu & Mitchell 1984, Sluys 1992). In addition, in *G. festae*, the bulbar cavity gradually narrows to form the ejaculatory duct, but, in *G. pierremartini*, the transition of the bulbar cavity to the ejaculatory duct is sharp and there is a diverticulum at the basis of the bulbar cavity. The anatomy of the bursal canal can also be used to distinguish both species, since *G. festae* has an angled bursal canal (Kawakatsu & Mitchell, 1984, Sluys 1992, Sluys *et al.* 2005), whereas the bursal canal of *G. pierremartini* smoothly inclines ventrally towards the female canal.

In conclusion, the troglophilic *G. pierremartini* shows a combination of features of its copulatory apparatus, namely ventral testes, a large, branched and ample bulbar cavity with a lateral funnel-shaped portion, a large and truncate papilla showing two different portions, and a bursal canal smoothly inclining ventrally to communicate with the female canal, among others, which distinguish it from its congeners.

Notes on ecology and distribution. In the karst area of São Desidério, specimens of *G. desiderensis* were directly sampled from small pools inside Baixa Fria cave, ca. 200 m from the cave entrance in the aphotic zone (Fig. 3). Its abundance was low in comparison to other freshwater flatworms. The water body has a maximum depth of 0.2 m and shows slow water current and a bottom formed by silt and clay.

In the karst area of Presidente Olegário, specimens of *G. pierremartini* were always abundant and co-occurred with amphipod crustaceans in both sampling areas. The pools showed a silt and clay bottom, with slow moving waters and depths of ca. 0.2 m, located in the twilight and photic zones, at variable distances from the caves' entrance (Figs. 4−5).

General discussion. The two new species herein described enhance the knowledge on triclad diversity in South American subterranean environments. One of them (*G. desiderensis*) represents the first Continenticola described for an area of Cerrado-Caatinga transition. *Girardia desiderensis* is also the first aquatic troglobitic species described for São Desidério karst area and Bahia state. This species occurs in the aphotic zone of a single cave (Baixa Fria) located at the boundary of a huge hydrogeological system (João Rodrigues), in a peripheral water body formed by phreatic water. Thus, *G. desiderensis* is restricted not only to a cave environment, but also to specific microhabitats. Regions with high diversity of troglobites in Brazil have been recognized, based not only on species richness, but also on a high proportion of these species in relation to the total subterranean richness, including also troglophiles and trogloxenes, as observed by Gallão & Bichuette (2015) for caves from Chapada Diamantina in the state of Bahia. Such areas should be considered to have conservation priority, since they represent unique elements of subterranean evolution.

Our knowledge of the aquatic cave fauna of the Presidente Olegário karst area is still incipient. One species of amphipod (*Hyalella veredae* Cardoso & Bueno, 2014) was described for the Vereda da Palha cave, close to the sampling localities of *G. pierremartini* (Cardoso *et al.* 2014). Thus, this flatworm represents the second freshwater species described for Presidente Olegário caves. Due to their heavily pigmented body, the presence of eyes, and a possible connection between the surface streams and the subterranean pools where the planarians occur, we consider the species as a troglophile.

It is noteworthy that the regions of São Desidério and Presidente Olegário are not part of any legally protected conservation area. In São Desidério, the agriculture is the most threatening pressure imposed on its cave fauna. The surroundings have very large plantations of soybean and cotton, while contamination of phreatic waters is documented for at least the urban area of the municipality. In Presidente Olegário there are at least 200 caves documented up to now (GPME, pers. comm.) and the main threats are gas extraction and hydro-electrical projects, which strongly impact the water streams and phreatic oscillation. In the latter case, the drainage can be entirely modified. According to Brazilian law, caves should be protected if they constitute habitats for rare or endemic troglobites and possible endangered species (BRASIL, 2008). We suggest that these two regions should be protected through the creation of Conservation Units (e.g., Natural Monuments or Parks), and that these two flatworm species should be taken up in the Brazilian Red List as vulnerable organisms.

Acknowledgements

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