



# Novel type of egg-clustering in threadsnakes (Serpentes: Leptotyphlopidae)

Mariana Chuliver<sup>1</sup>, Agustín Scanferla<sup>1</sup>

<sup>1</sup> CONICET – Fundación de Historia Natural “Félix de Azara”, Hidalgo 775, CABA C1405, Argentina

<https://zoobank.org/97AFCC7B-7922-44FE-A5B5-AC60F85428FB>

Corresponding author: Mariana Chuliver (marianachp@yahoo.com.ar)

Academic editor Uwe Fritz

Received 21 June 2023

Accepted 18 August 2023

Published 12 September 2023

**Citation:** Chuliver M, Scanferla A (2023) Novel type of egg-clustering in threadsnakes (Serpentes: Leptotyphlopidae). *Vertebrate Zoology* 73 691–696. <https://doi.org/10.3897/vz.73.e108402>

## Abstract

Snakes lay their eggs in clutches of different size, which are usually attached to each other forming a cluster. Egg-clustering is a widespread phenomenon across alethinophidian snakes, mostly recorded in Pythonoidea and caenophidian clades. Here we report a new type of egg-clustering for threadsnakes (Leptotyphlopidae) that departs from the alethinophidian type. We found that females of *Epictia australis* and *Leptotyphlops sylvicolus* lay their eggs connected to each other through a filament, and we dubbed it ‘string-egg clustering’. The histomorphology of the filament linking the eggs in *E. australis* showed an outer calcareous layer underlain by a thick layer of collagen fibers, demonstrating that it is an integral part of the eggshell formed during its deposition process in the oviduct. String egg-clustering seems to be present only among species belonging to both subfamilies of threadsnakes, Epictinae and Leptotyphlopinae. Egg-clustering in alethinophidians has been demonstrated to have several advantages for embryo development and post-hatching survival, including fixing the position of the embryo within the egg, protection against predators, and embryo-to-embryo communication. The presence of a filament connecting the eggs in leptotyphlopoid species might be relevant for maintaining the position of the embryo in the egg, to avoid the dispersion of the egg in the nesting site, and potentially for the transmission of physical cues. Thus, we hypothesize that the string-egg clustering constitutes an advantageous reproductive trait among threadsnakes.

## Keywords

Blindsnakes, egg clutch, eggshell histology, *Epictia australis*, *Leptotyphlops sylvicolus*, string egg-clustering

## Introduction

Oviparous squamate reptiles lay shelled eggs in clutches of different size, which develop together in nests of variable complexity (Vitt and Caldwell 2009). Notably, snake eggs are usually clustered, wherein eggs are firmly attached to each other soon after laying and are held together until hatching. The egg attachment seems to be a by-product of the drying of the secretions released by the mother during egg-laying, although the type of secretion

and the secretory glands involved in this process are unknown (Aldridge and Sever 2011). Moreover, despite the distinctiveness of egg-clustering as a reproductive trait of snakes, its relevance for incubation success has only recently been addressed (Aubret et al. 2015, 2016a).

Thus far, oviposition and/or structure of the eggshell have been documented across alethinophidian snakes, mostly in Pythonoidea and caenophidian clades (Schle-

ich and Kästle 1988; Packard and De Marco 1991; Hedges 2008 and literature cited there). Despite recent efforts, several reproductive aspects of the species belonging to the blindsnake clades (Typhlopoidea, Leptotyphlopidae and Anomalepididae) still remain unknown. Furthermore, due to their secretive habits, traits such as egg-laying are usually anecdotal (Erasmus and Branch 1983; Webb et al. 2000, 2001).

Herein, we documented the presence of a novel type of egg-clustering for snakes in two species of the family Leptotyphlopidae: *Epictia australis* (Freiberg & Orejas-Miranda, 1968) and *Leptotyphlops sylvicolus* Broadley & Wallach, 1997. We also characterized the histomorphology of the eggshell of *E. australis*, and conducted an exhaustive bibliographic search on blindsnake reproduction. This allowed us (1) to determine the nature of the structure connecting the eggs, (2) to explore whether different types of egg-clustering were previously reported for other species, and (3) to test the hypothesis of the exclusiveness of the novel type of egg-clustering for the family Leptotyphlopidae.

## Methods

A gravid female of the austral threadsnake *Epictia australis* was collected in Sierra de la Ventana region (38°12'21"S; 61°28'59"W) on 20 November 2022 (BA permit EX-2022-20466509-GDEBA-DSTAMDAGP). It was housed individually in a plastic container and kept in captivity in the laboratory. Soon after its capture, it laid five eggs, which were incubated in a plastic container at 27–30°C temperature using perlite substrate. Both the female and embryos were euthanized, preserved in an ethanol 70% solution, and deposited in the herpetological collection of Fundación Azara (CFA-RE 663). Additionally, a female of *E. australis* (CFA-RE 666) was dissected in order to observe oviductal eggs. Longitudinal (sagittal) serial sections of 6 µm thickness of a paraffin-embedded egg of *E. australis* were stained with hematoxylin–eosin (Gamble and Wilson 2002).

A second gravid female of the forest threadsnake *Leptotyphlops sylvicolus*, was found below a stone in the Midmar Nature Reserve in KwaZulu-Natal (South Africa). The specimen was housed individually in a plastic container and then laid three eggs, which were photographed and measured before being left in the same place where the adult was found. All measurements were taken using a dial caliper (accuracy: 0.05 mm) with measurements given in millimeters.

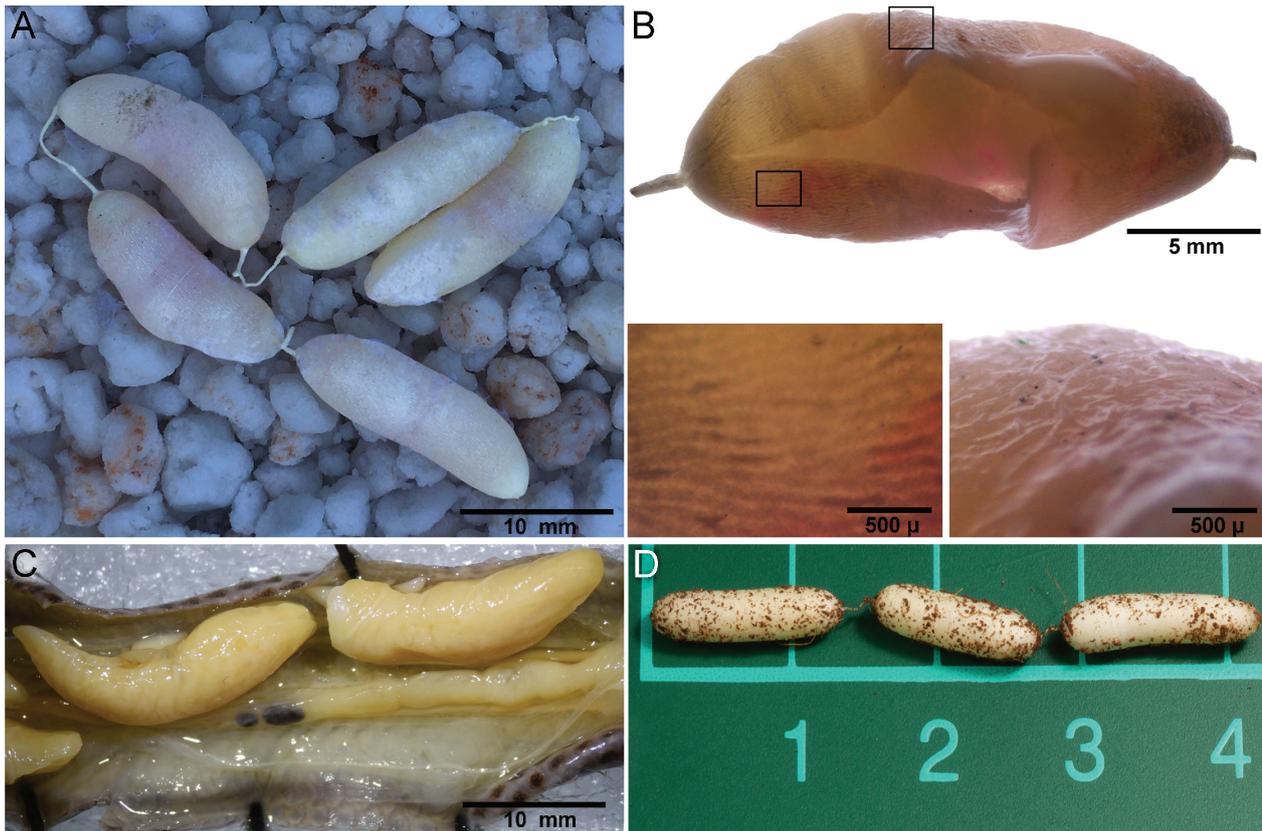
Finally, we performed an exhaustive bibliographic survey on blindsnake reproduction (Table 1). We gathered data from text books, field guides, and scientific articles. The full text of the selected articles was reviewed to check for reports of clutches found in nature or laid in captivity, therefore, we excluded those reports where gravid females were dissected and only oviductal eggs were described.

## Results

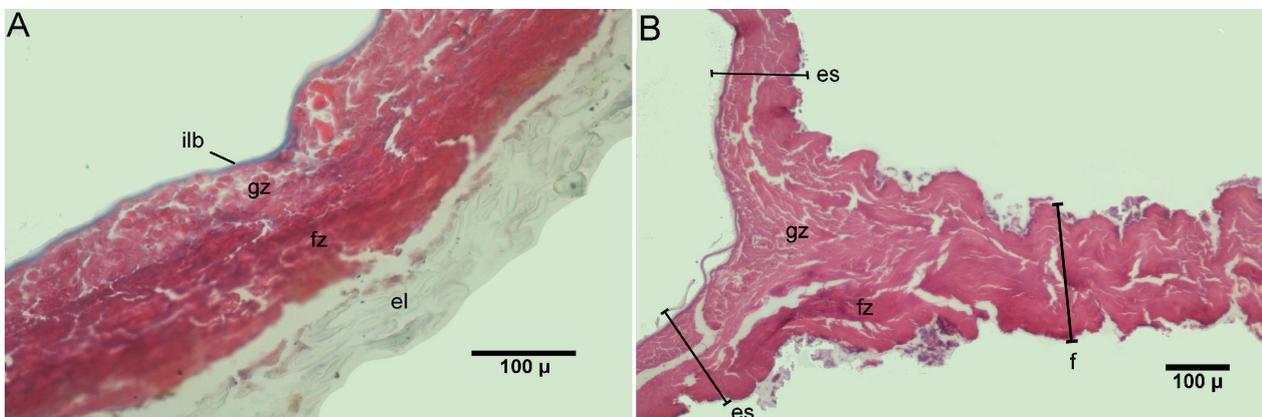
The snout-vent length (SVL) of the gravid female of *Epictia australis* (CFA-RE 663) was 159 mm. The clutch had five eggs, each of which measured 15 mm (Fig. 1A, B). The flexible eggshell exhibits a leathery surface finely striated, forming longitudinal ridges and covering its entire surface (Fig. 1B). The surface gross morphology resembles that described for the pythonoid *Python sebae* (Gmelin, 1789), and the colubroids *Zamenis longissimus* (Laurenti, 1768) and *Philodryas patagoniensis* (Girard, 1858) (Schleich and Kästle 1988). Notably, the eggs were connected to each other through their poles by a slender filament of approximately 6 mm in length (Fig. 1A), which coils after oviposition. The filament linking the eggs seems to be an extension of the eggshell, but unlike the eggshell it does not show a markedly striated surface. The dissected gravid female (CFA-RE 665; SVL = 161 mm) also yielded five eggs in the oviduct, thus it seems to be the average clutch size for this species (Fig. 1C). The gravid female of *Leptotyphlops sylvicolus* had a snout-vent length of 130 mm. This individual laid three elongated eggs, ranging from 13 to 16 mm in length. These were also connected to each other through an apical filament (Fig. 1D) in the same fashion as the *E. australis* eggs, although the filaments appear to be shorter than in the latter species.

The histomorphology of the eggshell of *E. australis* (Fig. 2A) is similar to that described for other squamates. It comprises an outer calcareous layer underlain by an organic membrane composed of multiple layers. Starting at the interior, the organic membrane of the eggshell of *E. australis* consists of an inner limiting boundary (ILB), which is a thin layer immediately adjacent to the albumen, followed by two distinct zones of collagen fibers exhibiting different orientations (Fig. 2A). The inner layer next to the ILB is the globular zone, formed by transverse fibers, observed as minute pink globules with large cavities among them. The second layer comprises a thick sheet of longitudinal collagen fibers highly parallel and tightly packed (Fig. 2A), which is the fibrillar zone. The calcareous layer constitutes the outermost structure of the eggshell. This layer is not obviously attached to the surface of the organic membrane through fibers; it is formed by both nodular structures and thin, flat plaques (Fig. 2A). Despite a general fenestrated appearance due to the discrete nature of its components, the calcareous layer is continuous and covers the entire surface of the underlying organic membrane. The nature of the filament uniting the eggs in *E. australis* is easily discernible in the histological sections. The filament is composed of both the fibrillar zone and the calcareous layer of the eggshell (Fig. 2B), although the orientation of the collagen fibers is not well defined. Thus, it constitutes an extension of the eggshell, being an integral part of it.

The compiled information on blindsnake reproduction showed that, in addition to the two species documented here, the filament was only mentioned for *Leptotyphlops scutifrons* (Peters, 1854) (Table 1). Moreover, the available descriptions of egg clutches of other species, including



**Figure 1.** **A** egg clutch of the leptotyphlopoid *Epictia australis*, **B** egg of *E. australis*, black squares depict details of the surface of the eggshell, **C** dissected female of *E. australis* showing two oviductal eggs, **D** egg clutch of *Leptotyphlops sylvicolus* (photo: Tyrone Ping).



**Figure 2.** Histological sections of the **A** eggshell and **B** apical filament of *Epictia australis*. Abbreviations: **el** external layer, **es** eggshell, **f** filament, **fz** fibrillar zone, **gz** globular zone, **ilb** inner limiting boundary.

species of the clades Anomalepididae and Typhlopoidea, do not report in any case the presence of egg clusters or a filament-like structure linking the eggs (Table 1).

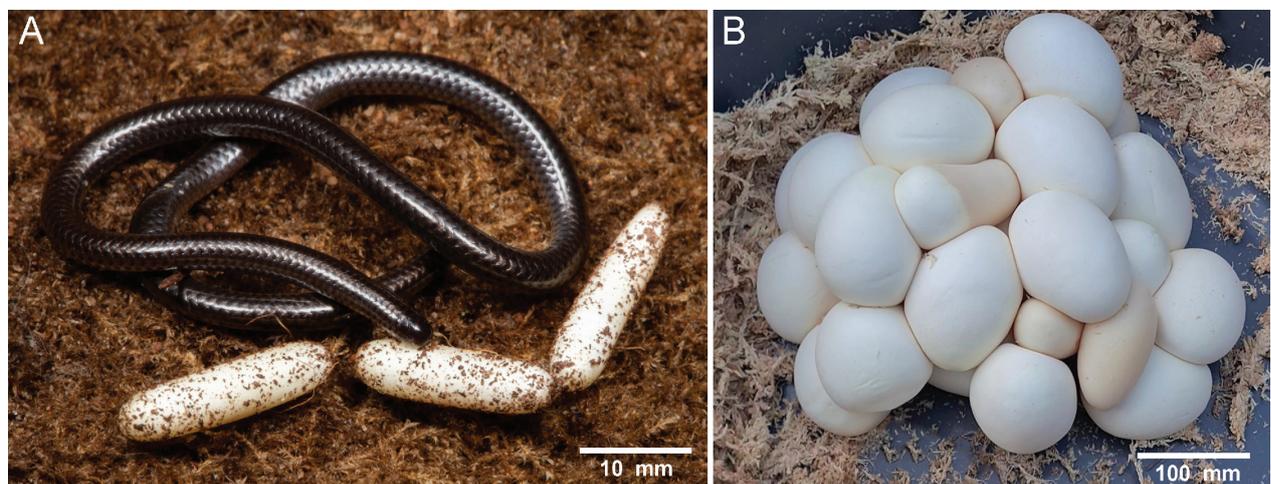
## Discussion

We documented a novel type of egg-clustering for two blindsnake species, dubbed here ‘string egg-clustering’. The results of this study agree with an anecdotal account, as the string egg-clustering type was mentioned by the

African herpetologist William Roy Branch in his renowned field guide of southern African reptiles (Branch 1998). This author observed that the eggs of *Leptotyphlops scutifrons* “... remain attached to each other like a string of sausages ...” (Branch 1998). Later, Webb and collaborators (2000) quoted this observation in their analysis of the life-history strategies of the same threadsnake species, addressing that this attachment appears to be a unique feature of leptotyphlopids. Accordingly, the species analyzed in the present work represent both Epictinae and Leptotyphlopinae, the two subfamilies currently recognized for Leptotyphlopidae (Adalsteinsson et al. 2009). Thus, we support the aforementioned conjecture

**Table 1.** Available information on clutch size and presence of egg clustering in blindsnake species.

Family	Species	Clutch size	Egg clustering	Source
Typhlopidae	<i>Afrotiphlops bibronii</i>	5 to 12	No	Jacobsen (1989), Yeadon (1991), Webb et al. (2001)
Typhlopidae	<i>Amerotyphlops brongersmianus</i>	4	No	Sandoval et al. (2020)
Typhlopidae	<i>Amerotyphlops paucisquamus</i>	3	No	da Silva Amaral et al. (2020)
Typhlopidae	<i>Anilios nigrescens</i>	4 to 20	No	Shine and Webb (1990)
Typhlopidae	<i>Indotyphlops braminus</i>	3	No	Cagle (1946), Kamosawa and Ota (1996)
Typhlopidae	<i>Letheobia acutirostrata</i>	6	No	Bogert (1940)
Leptotyphlopidae	<i>Epicitia australis</i>	5	Yes	This work
Leptotyphlopidae	<i>Leptotyphlops sylvicolus</i>	3	Yes	This work
Leptotyphlopidae	<i>Leptotyphlops scutifrons</i>	3 to 7	Yes	Branch (1998)
Leptotyphlopidae	<i>Leptotyphlops conjunctus</i>	3	No	Branch and Haagner (1996)
Leptotyphlopidae	<i>Rena humilis</i>	4	No	Klauber (1940), <a href="https://www.sciencephoto.com/media/379876/view/western-blind-snake-with-eggs">https://www.sciencephoto.com/media/379876/view/western-blind-snake-with-eggs</a>
Leptotyphlopidae	<i>Rena dulcis</i>	4	No	Gehlbach and Baldrige (1987)
Leptotyphlopidae	<i>Myriopholis longicauda</i>	2	No	Jacobsen (1989)
Anomalepididae	<i>Liotyphlops albirostris</i>	3	No	Bruner et al. (2012)

**Figure 3.** Different types of egg-clustering in snakes. **A** string egg-clustering of leptotyphlopids represented by *Leptotyphlops sylvicolus* and its egg clutch (photo taken by Tyrone Ping), **B** contact egg-clustering of alethinophidian snakes represented by the egg clutch of the pythonid *Morelia spilota* (photo taken from <https://www.bromiespythons.com/post/eggs-are-here>).

about the presence of the string egg-clustering exclusively in species of Leptotyphlopidae, and probably absent in Typhlopoidea or Anomalepididae.

Based on its general structure, the eggshell of *Epicitia australis* can be classified into the group of flexible-shelled eggs with little calcareous layer, as in most squamates (Packard et al. 1982a). However, little is known about the histomorphology of the eggshell of squamate reptiles (Packard et al. 1982b, Trauth and Fagerberg 1984, Trauth et al. 1994), making it difficult to generalize about phylogenetic patterns. Indeed, to our knowledge, the present description of the eggshell of *E. australis* represents the first histological characterization for a snake. Specifically, the eggshell of this threadsnake species is similar to that of the lizard species *Anolis limifrons* Cope, 1862 and *A. sagrei* Duméril & Bibron, 1837 (Sexton et al. 1979), *Callisaurus draconoides* Blainville, 1835 (Packard et al. 1982b) and *Aspidoscelis sexlineatus* (Linnaeus, 1766) (Trauth and Fagerberg 1984). It shares with these lizards the presence of a thin ILB separating the outer

layers from the underlying tissues. Furthermore, the presence of zones distinguishable by their fiber arrangement also resembles the eggshell of the aforementioned lizards. The different orientation of fibers between zones in the eggshell of lizards is produced by the rotation of the egg in the oviduct (Packard and DeMarco 1991, Palmer et al. 1993). Thus, the difference observed between the globular and fibrillar zones in the eggshell of *E. australis* may indicate that egg rotation is also present during the secretion of the complex organic membrane in this blindsnake species.

The histological analysis demonstrates that the filament linking the eggs constitutes an extension of the fibrillar and calcareous layers of the eggshell, indicating that both the eggshell and the filament are formed by the same eggshelling process. In squamates, the deposition of the organic membrane of the eggshell begins once fertilization has occurred, from the shell glands located in the middle oviduct (Fox 1977, Girling 2002). In lizards, fibers secreted by shell glands become wrapped around the egg,

probably induced by extraembryonic structures, and peristaltic movements of the oviductal musculature seem to play a role in deposition as well (Palmer et al. 1993). Notably, the process of shell formation in snake eggs remains largely unexplored. Thus, the question arises how the filament is formed in the oviduct of leptotyphlopids. The middle oviduct constitutes the largest portion of the oviduct in snakes (Siegel et al. 2011), and some regional differences between threadsnakes and alethinophidians have been described (Fox and Dessauer 1962, Fox 1977), suggesting that oviductal structures might be involved in the filament formation. However, further studies are necessary to provide new clues about the mechanisms involved in the formation of the filament.

Historically, it has been assumed that rolling of the eggs of squamate reptiles during early phases of incubation may result in the death of the embryo, suggesting that eggs must be incubated in the position in which the female laid them. This hypothesis has recently been tested in snakes, demonstrating that egg turning did not significantly influence egg development, hatching success or hatchling phenotypes but decreases survival of postnatal individuals (Aubret et al. 2015). The presence of a filament connecting the eggs in both leptotyphlopids species, therefore, highlights the relevance of maintaining the position of the embryo within the egg. The benefits of string-egg clustering might further be related to size, as leptotyphlopids eggs are minute, no more than 15 mm in length. Thus, we hypothesize that string-egg clustering might have evolved to prevent the dispersion of individual minute eggs in the nesting site, whether this is a place below a log/stone or in a nest of social insects.

The type of egg-clustering reported here for threadsnakes contrasts with the egg-clustering described for alethinophidian snakes, where eggs are directly adhered to each other through extensive areas of their eggshells (Fig. 3). Recent studies have described intraclutch embryo-to-embryo communication in alethinophidian snakes, with benefits for developing embryos and postnatal individuals (Aubret et al. 2016a, 2016b). For example, heart frequency synchronization between neighboring embryos influences metabolic rates, synchronizes development and hatching, and also influences postnatal social behavior (Aubret et al. 2016a, 2016b). As such, the transmission through the filament of physical cues and, therefore, some kind of communication between embryos seems to be plausible in the string-egg clustering of leptotyphlopids snakes. Consequently, communication enabled by egg-clustering, either through a filament-like structure or through direct contact between the eggshell, emerges as an advantageous reproductive trait among snakes. Nonetheless, further studies are needed to explore if the filament acts as an intraclutch communication route.

## Acknowledgements

Both authors were funded by Agencia Nacional de Promoción Científica y Técnica (PICT Serie A 2020-2443). We thank our South African colleague Tyrone Ping who generously provided valuable data and im-

ages of *Leptotyphlops sylvicolus*. We are also grateful to Sergio Bogan (Fundación Azara) for giving access to specimens in collections. Verónica Dorfman (Universidad Maimónides) kindly provided technical assistance with histological procedures. Appropriate protocols for the collection and handling of the specimens used in this study were followed. The manuscript also benefitted from reviews by two reviewers, and Krister Smith generously provided suggestions and help with English grammar.

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