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ANIMAL SCIENCE

Locomotion and morphological adaptations in the glass lizard *Ophiodes* cf. *fragilis* (Raddi, 1820) (Squamata: Anguidae)

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Abstract: There are few studies related to the biological and ecological aspects of the glass snake, a limbless lizard and with a wide geographic distribution. The aim of this study was to analyze the locomotion mode of specimens of *Ophiodes* cf. *fragilis* in different substrates and to investigate the morphological adaptations associated with this type of behavior. We observed that the analyzed specimens presented slide-push locomotion modes and lateral undulation in different substrates, using their hind limbs to aid locomotion in three of the four substrates analyzed. The bones of the hind limbs (proximal - femur - and distal - tibia and fibula) were present and highly reduced and the femur is connected to a thin pelvic girdle. Our data support that hind limbs observed in species of this genus are reduced rather than vestigial. The costocutaneous musculature was macroscopically absent. This is the first study of locomotor behavior and morphology associated with locomotion in *Ophiodes*, providing important information for studies on morphological evolution in the genus.

Key words: diaphanization, functional morphology, histology, limbless lizard, locomotion mode.

INTRODUCTION

Terrestrial limbless vertebrates have traditionally intrigued humans (Gans 1962, Barros-Filho et al. 2008, Hohl et al. 2014, 2017), particularly for their morphology, raising questions about their locomotion. Limbless animals can move using different methods (Gans & Gasc 1990); four tradicional methods of locomotion are known in terrestrial limbless vertebrates: lateral undulation (or locomotion in "S", the whole body moves simultaneously passing over the same point); rectilinear (displacement in a straight line), concertina (posterior portion of the body fixed and anterior portion is extended, with modification of the body-tail distance), sidewinding (= by lateral loops or "J" movement, lateral displacement lateral flexion to the side that is later propagated throughout the body). Recently, new forms of locomotion for limbless vertebrates are being described, such as lasso locomotion, highlighting the possibility that forms of locomotion are much larger. (Rocha-Barbosa et al. 2015, Jayne 2020, Savidge et al. 2021). Some species can move using more than one type of movement, on occasion at the same time (Gans 1962, Gans & Gasc 1990).

Locomotion in each line of vertebrates is related to the structural organization/anatomy of the organism and mainly to an association between stability, maneuverability, energy capacity and resistance (Rocha-Barbosa et al. 2015). The way an animal moves is directly related to specific biological and ecological factors. Locomotor capacity is directly associated with the ecology of animals as is related to accessible habitat parts (Irschick & Garland 2001); it can be directly influenced by factors such as body size and shape, proportion of limbs, feet, and fingers, in addition to environmental conditions such as temperature, substrate, inclination and diameter of supports (Rocha-Barbosa et al. 2015).

In reptiles, the absence or reduction of limbs evolved independently in certain groups, and, in most cases, it is associated with animals that dig or move through the grass (Pianka & Vitt 2003, Camaiti et al. 2021). One of the great ecomorphological enigmas is to identify how much a morphological characteristic determines the performance of an individual, such as foraging (search for food), predation (escaping predators) and reproduction (courtship and copulation) (Sathe & Husak 2015). To analyze a morphological characteristic, it is essential to know how it works (Losos 1990) and how it influences the animal's environmental performance.

Gans (1962) highlighted the need for studies of locomotion with limbless vertebrates. Most of these studies, when it comes to reptiles, were carried out with snakes and amphisbaenians (Gans 1962). Locomotion in limbless lizards was initially described for two species: *Pseudopus apodus* (Pallas, 1775) and *Anguis fragilis* Linnaeus, 1758, both from the Anguidae family (Gans & Gasc 1990, Gasc & Gans 1990). For the anguids of the genus *Ophiodes*, Rocha-Barbosa et al. (1991) described movements related to parturition of a female specimen of *Ophiodes striatus* (Spix, 1824) in captivity. Given the diversity of species of existing limbless lizards and considering the different habitats they occupy, the gap still remains regarding the shape and functional morphology of locomotion in these animals.

The genus *Ophiodes* is composed of lizards with serpentine body, absent or highly reduced forelimbs, popularly known as glass snakes or glass lizards (Pizzato 2005, Borges-Martins 1998, Oliveira et al. 2016). This genus is exclusively neotropical and can be found in Argentina, Paraguay, Uruguai and Brazil (Uetz et al. 2022). Several biological and ecological aspects of *Ophiodes* have not yet been elucidated, this is mainly related to the difficult capture and collection of specimens, such as the cryptozoic / semifossorial life habit (Vitt & Caldwell 2014). The rarity of studies with species of the genus is also reflected in the lack of taxonomic analyses, making some researchers believe that *Ophiodes* includes more species than those already described (Borges-Martins 1998, Cacciali & Scott 2015). The specimens analyzed in this study have characteristics attributed to the candidate species *Ophiodes* sp. 2 (Borges-Martins 1998), as has not yet been officially described and the specimens in this study have morphological characteristics closer to *Ophiodes fragilis*, we chose to name them as *Ophiodes* cf. *fragilis*.

In view of the importance of knowing the methods of locomotion and the structures related to this behavior, in addition to the need for studies on locomotion in lizards without limbs, we aim to investigate the locomotion mode in *Ophiodes* cf. *fragilis* by analyzing: (1) the types of locomotion presented; (2) the possible use of hind limbs for locomotion; and (3) morphological characteristics associated with its locomotion.

MATERIALS AND METHODS

Analyzed specimens

In total, ten specimens of *Ophiodes* cf. *fragilis* were used in this study; two specimens (both adults, without apparent autotomy) used for locomotor behavior analysis, three specimens (two adults and one juvenile) for diaphanization analysis and five specimens (adults, totaling ten hind limbs) for histological analysis. Juvenile and adult indentification was performed by size (snouth-cloacal length) and sexing of adult specimens was performed by gonad analysis (Oliveira et al. 2021). Sex differences related to locomotion were not the objective of analysis in our study; therefore, no specific analyzes were performed on sex differences in locomotion and their associated morphology. The collection and handling followed the determinations valid in the authorizations of SISBIO n° 59680/3 and the Animal Ethics Committee of the Universidade Federal de Juiz de Fora protocol 040/2017.

The specimens processed according to diaphanization, and histology methods belong to the Herpetological Collection of the Universidade Federal de Juiz de Fora - Reptiles (CHUFJF - Reptiles). For the clearing technique, the specimens of interest were necropsied, and the viscera were removed. As for histological analysis, only the piece of interest was sectioned.

The specimens used for locomotor behavior analysis were euthanized after the experiments following Gomides et al. (2013). All specimens used in this study are deposited at the CHUFJF-Reptiles with the deposit numbers: 205, 1423, 1473, 1643, 1646, 1649, 1650, 1731, 1866 and 1867. Body size measurement of the specimens were performed using a tape measure and a precision digital pachymeter, since they are fixed in a standard way for specimens with serpentiform bodies - “coiled”; due to the occurrence of caudal autotomy in some analyzed specimens, only the snouth-cloacal length (SVL) was measured. The macroscopic analysis of the anterolateral musculature, for observation of the costocutaneous musculature, was performed according to the model by Hohl et al. (2020), with macroscopic analysis of the region and morphology of the musculature in the studied specimens of *Ophiodes*.

Locomotor behavior

To investigate locomotor behavior, the two adult specimens of *Ophiodes* cf. *fragilis* were tested on four different surfaces (adapted from Gans & Gasc 1990, Gasc & Gans 1990, Gans et al. 1992): sand (small and light grains), sandpaper (rough surface), glass plate (smooth surface) and smooth wood with nails (derivation), (Supplementary Material – Video S1); in order to analyze how the specimens moved and their hind limbs were used.

The different surfaces were arranged in a wooden box of 100 cm x 100 cm x 15 cm (length x width x height); being divided into four parts, 100 cm x 25 cm (length x width), with a barrier between the different surfaces so that the animal did not have contact with more than one surface simultaneously. The nails were arranged in four straight lines, separated by 5 cm. The specimens were filmed with the Kodak PixPro AZ522 camera with 1920 x 1080 resolution (Full HD) and 60 fps frame rate. The film was made for 5 minutes on each substrate, with repetition within one week. In this way, each specimen was filmed 2 to 3 times per substrate. The filming was done with each specimen separately and alternately (that is, specimen 1 was filmed and then specimen 2 was filmed for substrate 1 - this was the case for each substrate), with an interval of around 10 minutes between substrates, to avoid/reduce stress through manipulation and stimulation. The specimens were stimulated to move by touching the tip of the tail, whenever they remained immobile on the different surfaces.

The hind limb was analyzed through frame-by-frame observation of the produced video on each substrate. Through the video, a still-image was taken whenever the specimens moved the hind limbs (regardless if the use was only on one side or both) for later measurement of the opening angle. The opening angle was measured whenever the specimen moved the hind limb away from the side of the body; corresponding, therefore, to the calculated distance of separation of the posterior limb from the side of the body, in angles (°). The opening angle was calculated for each substrate separately using ImageJ v. 1.45. The gross numbers of the angles measured on each substrate are shown in Table I. To ascertain the existence of significant variation between the angles measured in the different substrates, the Kruskal-Wallis statistical test was performed in the program R v. 3.5.3. The test was jointly applied between specimens 01 and 02, analyzing the total number obtained in each substrate.

Table I. Angle of the hind limb measured on each substrate in the specimens analysed.

Specimen	Substrate	Angulation (°)
S 01	Sand (n = 5)	59.74
		71.57
		42.60
		23.90
		53.43
	Sandpaper (n = 3)	79.48
		34.99
		36.03
	Glass plate (n = 5)	86.47
		62.76
		52.70
		60.02
		35.54
	Wood with nails (n = 1)	23.40
S 02	Sand (n = 5)	64.34
		86.93
		36.49
		76.81
		23.04
	Sandpaper (n = 5)	61.74
		77.91
		80.54
		32.66
		32.70
	Glass plate (n = 3)	46.65
		82.30
		49.18
	Wood with nails (n = 1)	80.54

Specimen: (S 01) – collected in Juiz de Fora and (S 02) – collected in Ritápolis.

Diaphanization

The clearing method used was according to Silveira et al. (2013). The samples were placed in transparent flasks with a 2% potassium hydroxide solution (2% KOH) to clarify the animal's external structures. This solution was changed daily, until the samples were clarified. This procedure took about 40 days in adult specimens and 16 days in juveniles. After the clarification procedure, the samples were submitted in a period of 24h to the staining process of bone structures, with 1% alizarin (1 g of alizarin Red S in 90 ml of ethanol). At the end of this process, the samples were washed with a 2% KOH solution and 3: 1 glycerin for 24 h. Every 24 h, the proportion of 2% KOH and glycerin was changed in the following order: (2: 1), (1: 1), (1: 3) until they were conditioned in pure glycerol in clear glass jars.

The analysis of the material and the respective photographic documentation were performed in an Olympus SZX16 stereomicroscope with Canon A3100S digital photographic equipment and a coupled micrometric eyepiece.

Histology

For studies of light microscopy material, hind limbs, and ventral scales from specimens of *Ophiodes* cf. *fragilis* were dehydrated in increasing concentrations of alcohol, clarified with xylol and infused / embedded in paraffin. Subsequently, serial cuts with a thickness of 4 µm were made. The slides were stained with hematoxylin-eosin (HE) (Tolosa et al. 2003).

The analysis of the material and the respective photographic documentation were performed under an Olympus BX41 microscope with Canon A3100S digital photographic equipment and an attached micrometer eyepiece.

RESULTS

Locomotion

There about 64 (sixty-four) cycles were observed in which the analyzed specimens used some of the locomotor modes. Each video was analyzed separately, and details were noted whenever the animal moved (Video S1). Our observations suggest that there is no interference of substrate type (sand, sandpaper, glass plate and wood with nails) with the form of locomotion. Our preliminary analysis indicate that it did not influence or favor the observed method of locomotion. The summary of the results obtained was presented in Table II. The methods of locomotion observed were, predominantly, slide-pushing followed by lateral undulation verified in the four different types of substrates. The details of each of these two types of locomotion are described below.

Slide-pushing

The slide-pushing (Figure 1a, b) was observed when the animal moved in a straight line, even when the surface was composed of obstacles (nails) (Figure 2a-c). The type of slide pushing locomotion was described when the specimen moved without body without any evident undulation.This type of movement started, for the most part, in the anterior portion of the animal's body (head / beginning of the trunk) and was disseminated throughout the body, up to the tail (Figure 1a). This type of movement was also observed, starting in the median region of the body (cloacal) and extending to the tip of the tail.

A variation of the slide-pushing was considered: starting in a portion of the body, usually in the head, and "pulling" the rest of the body in a straight line (Figure 1b). As the name implies, the animal moved its body forming a half arc, but it did not characterize a serpentine movement. The movement is generally associated with a change in direction of the animal, resulting in a large amplitude curve, similar to the letter "U" (= "U" movement).

Table II. Summary of the results obtained in the present study of locomotion and associated morphology of *Ophiodes* cf. *fragilis*.

Analyzed Variable	Results	
Types of locomotion	Lateral undulation and slide-pushing	
Opening angle	52° - 55° (sand, sandpaper and wood with nails)	60° (glass plate)
Age	Juvenile (n = 1)	Adults (n = 9)
Sex	Female (n = 4)	Male (n = 5)
Biomes	Cerrado (Ritápolis, Ribeirão Vermelho)	Atlantic Forest (Juiz de Fora)

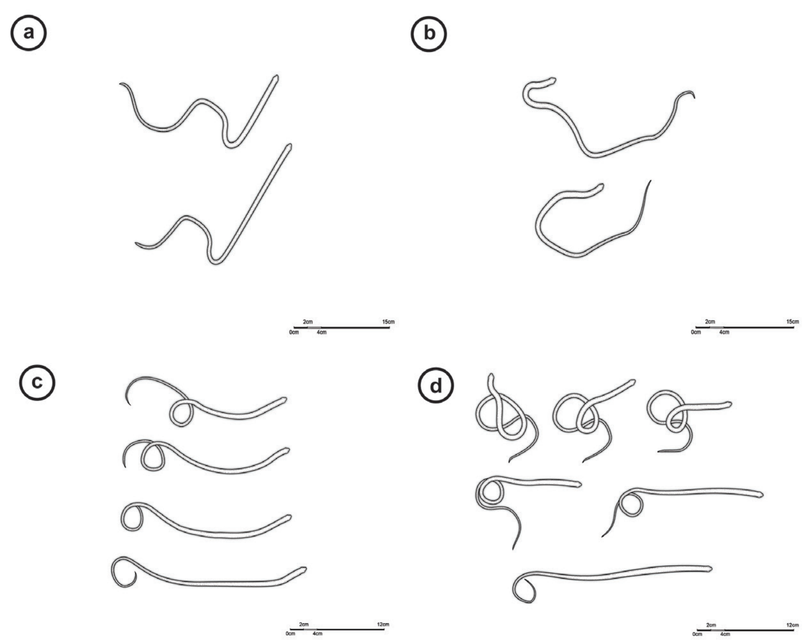

Figure 1. **Schematic drawings of the locomotor movements observed in *Ophiodes* cf. *fragilis*. (a, b) Slide pushing movement; highlighted in (b) for the "U" movement. (c, d) Loop sequence, emphasis on the complexity of the loop in (d). Scale: (a, b) 0cm to 15cm with each fraction equivalent to 2cm; (c, d) 0cm to 12cm with each fraction equivalent to 2cm. Schematic drawings developed from the joint analysis of the filming of this study.**

Lateral undulation

The lateral undulation movement (Figure 3) was observed at different times and was associated with variations in the undulations. It was described that the specimen moved by lateral undulation when it, when moving the body, presented some type of evident undulation. The starting point of the lateral undulation movement varied in the same specimen, starting in the anterior, median, and posterior regions (before the cloaca). Regardless of the starting point, upon receiving stimulus, the region moved forming an "S". More closely spaced ripples were associated with a higher velocity of substrate space coverage (velocity not calculated). On the other hand, less closely spaced ripples were observed while the animal was motionless and/or at a lower velocity of substrate space coverage (velocity not calculated).

Looped disposition

The looped disposition movement was noticed when the animal was immobile on the different surfaces (Figure 1c, d). The point of origin could not be determined. We also could not clearly establish the stimulus for this arrangement. However, when placing them on the substrate or during the experiment (recordings), the looped arrangement was observed with some frequency in the specimens. Different loops were observed, from a more complex form (with two or more nodes) (Figure 1d) as well as in a simpler form (a single node) (Figure 1c). This seems to be a common behavior of the species since it was observed in these specimens even when they were not being tested.

Hind limbs

Both specimens removed the hind limb from the side of the body and moved it frequently on three of the four surfaces tested. Movement of the hind limb was more frequent, respectively, on the sand, sandpaper, and glass plate surfaces. It is absent or imperceptible in wooden surface with nails.

Upon receiving stimulus (sensorimotor), the hind limb detaches from the lateral region of the animal's body, making rhythmic and consecutive forwards and backwards movements, resembling an oar. Apparently, the

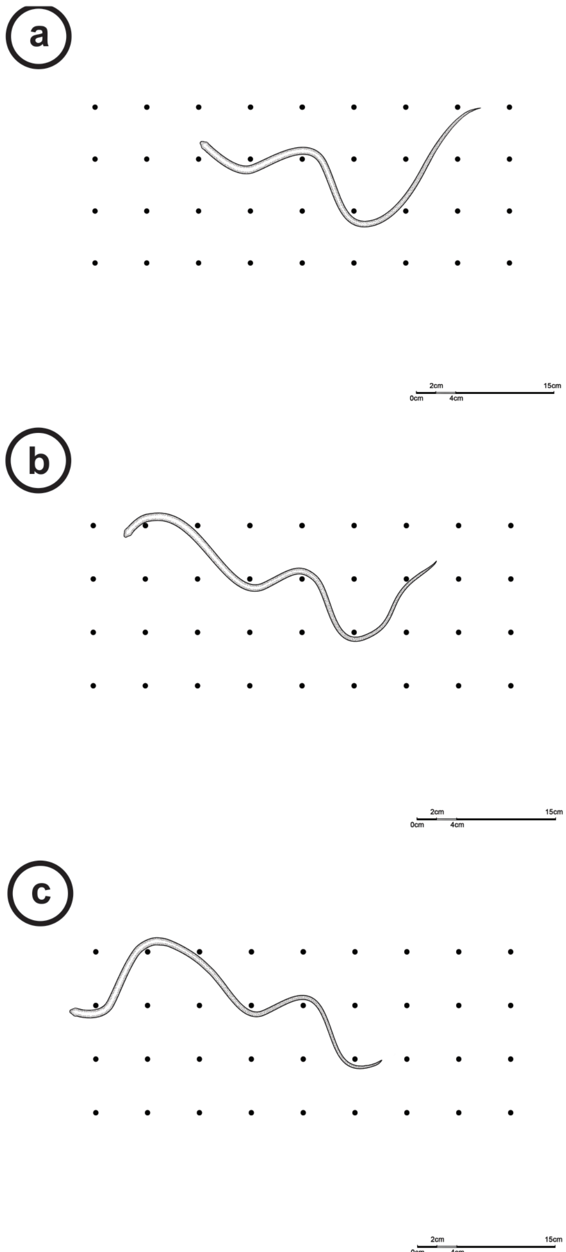

Figure 2. Schematic drawing of locomotion between the nails of *Ophiodes* cf. *fragilis* (a-c). Scale: 0cm to 15cm with each fraction equivalent to 2cm. Schematic drawings developed from the joint analysis of the filming of this study.

limb does not touch the substrate (no evident markings were observed on the sand substrate); however, future specific analyses will be carried out. The animals indicated that they had control over the use of the limb on only one side or on both sides, probably being associated with the animal's locomotor need.

The hind limbs presented different opening angles (details in Table I), with an average of approximately 54 ° (± 22 °, n = 10) in the sand substrate, 55 ° (± 22.6 °, n = 8) in the sandpaper, 60 ° (± 17.5 °, n = 8) in the glass plate and 52 ° in the wood with nails (± 40.4 °, n = 2), the variation in angulation between the substrates not significant (Kruskal-Wallis chi-squared = 0.52339, p = 0.9137).

Morphology associated with locomotion

The macroscopic analysis of the unstained adult specimens indicated the absence of costocutaneous musculature. The coloring of the bone structure of *Ophiodes* cf. *fragilis* by alizarin through the clearing technique confirmed structures already known and revealed new data, which are corroborated by histological analysis.

In adult specimens, the skeletal structure was not stained. While the external muscular part and scales of the animal was stained in purple. The presence of osteoderms on the body and tail of the specimens may have interfered with the skeletal staining of the adult specimens. With the calcium composition in the osteoderms, the dye accumulated in the muscular portion and scales, making this structure a “barrier” for the passage of dye to the skeleton in the analyzed specimens. This layer of osteoderms on the scales was confirmed through histological processing. A different result was observed in the analyzed juvenile, with alizarin strongly staining the bone structure.

With the coloring of the skeleton, a thin, longitudinal bone structure was identified in the upper portion of the cloaca, indicating that it was a pelvic girdle. This structure has direct contact with the hind limb. Each hind limbs have three ossicles: one in the proximal portion (femur)

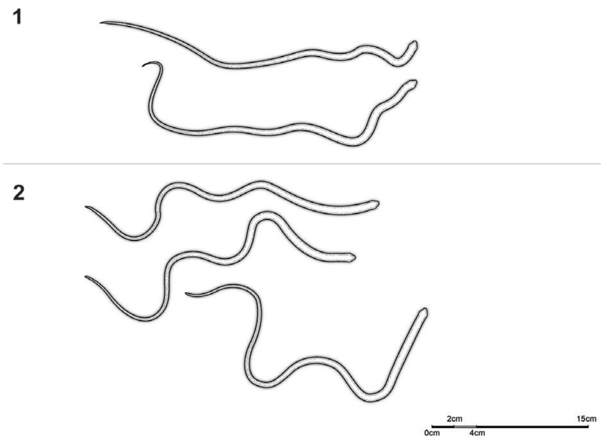

Figure 3. Schematic drawings of the locomotor movements observed in *Ophiodes* cf. *fragilis*. (1 and 2) Lateral undulation; emphasis on the different amplitudes of the "S". Scale: 0cm to 15cm with each fraction equivalent to 2cm. Schematic drawings developed from the joint analysis of the filming of this study.

and two in the distal portion (tibia and fibula, arranged side by side). The presence of the three ossicles in the hind limbs was confirmed by histology (Figure 4a-f). Between the femur, tibia and fibula there is a layer of cartilaginous tissue with high cellular activityinside them, being related to the production of bone cells and vascularization (Figure 4e, f).

DISCUSSION

Locomotion

This study offers new reports on locomotion and the morphology associated with this behavior in *Ophiodes* cf. *fragilis*, thus contributing to the study of adaptations for locomotion in limbless vertebrates. *Ophiodes* cf. *fragilis* presents as predominant methods of locomotion the slide-pushing, and the lateral undulation. The locomotion of non-anguid limbless lizards was in the form of lateral undulation, in which the lateral undulation occurred through portions of the animal's body that were pushed against the substrate (Pianka & Vitt 2003). The lateral undulation stands out due to it's sliding form of propulsion, in which the animal pushes its body in a lateral and irregular way (Gans et al. 1992). However, we also observed a form of slide-pushing. Slide-pushing locomotion was described by Gans et al. (1992) for *Anniella pulchra* (Annielidae) and by Gans & Fusari (1994) for *Lerista* sp. It is characterized by the animal pushing its body against the substrate, promoting the "sliding" of the individual. In *Ophiodes* cf. *fragilis* this type of locomotion can be easily confused with the rectilinear movement in the first analysis. The costocutaneous muscles was not observed in the analyzed specimens. Apparently, they are absent in lizards without limbs and with a serpentiform body (Jayne 2020). The absence of costocutaneous musculature and the analysis of the film reinforce that this would be the type of movement observed.

The movement of the hind limb during locomotion stands out. Our hypothesis is that it is still functional for the locomotion of this species. However, specific complementary analyses are needed to test this hypothesis. The usefulness of posterior vestigial limbs of booid snakes was observed by Carpenter et al. (1978), in which the so-called spurs are functional, assisting males during courtship (combat between males), and copulation (female dominance). A similar function was defended by Montechiaro et al. (2011) when verifying that male of *Ophiodes* cf. *striatus* presented hind limbs significantly larger than females. The movement of the hind limb at different angles on the different substrates was observed. Although the opening angle of the hind limb does not vary significantly between the different tested substrates, our hypothesis is that the distance from the side of the body and its movement may be related to greater stability of the animal, which could imply greater agility, optimizing locomotion on different substrates.

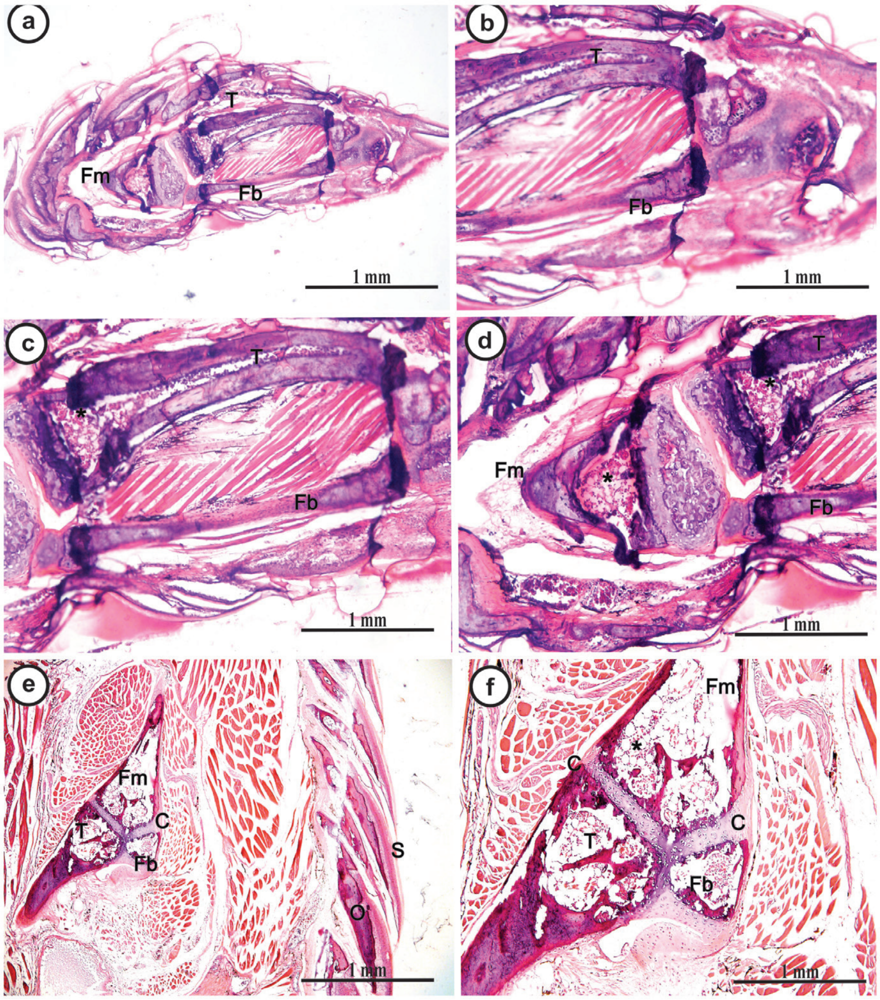

Figure 4. **(a, b, c, d, e, f) Photomicrographs of hind limbs of *Ophiodes* cf. *fragilis* stained with hematoxylin and eosin. (T) tibia; (Fb) fibula; (Fm) femur; (C) cartilage; (S) scales; (O) osteoderms; (*) blood cells indicating metabolic activity. Magnification: 4x - a, e; 10x - b, c, d, f.**

It is important to analyze the response to different types of surfaces due to the diversity of habitat composition that these species can occupy (Irschick & Garland 2001); in addition, different substrates can decrease or increase an individual's performance (Herrel et al. 2008, Sathe & Husak 2015) such as territory protection, escaping from predators, search for prey, among others. Locomotion is of extreme ecological importance for species, affecting their performance in different vital functions such as fleeing predators, finding food and partners and defending the territory (Aerts et al. 2000). Studies such as those by Sathe & Husak (2015) and Zamora-Camacho (2018) test the performanceof locomotion in lizards with limbs, which is a field that has not yet been clarified for limbless lizards. This fact may be directly related to the difficulty of capturing and collecting these species, mainly related to the morphological and physiological characteristics of these animals, which interferes with the knowledge of basic behavioral characteristics, including locomotion. We did not analyze the locomotor performance of *Ophiodes* cf. *fragilis*, being a promising area for future studies.

Morphology and locomotion

Osteoderms have a large intraspecific variation in respect to format and composition, being widely used in the Anguidae system (Zylberberg & Castanet 1985, Buffrenil et al. 2010, Bochaton et al. 2015). Bochaton et al. (2015) highlights that the osteoderms found in Diploglossinae and *Anguis* sp. are rounded, not bevelled, allowing for flexible armor, which is supposedly a synapomorphy of Anguidae. *Ophiodes* osteoderms have been described by Strahm & Schwartz (1977). These authors pointed out that in *Ophiodes* osteoderms would be derived, in terms of morphology and composition, from the genus *Diploglossus*, presenting as a main characteristic the presence of an extensive radix system (channels inside the osteoderms) in the form of a cloud. Such architecture presented by Strahm & Schwartz (1977) was confirmed in our study with the clearing technique. We analyzed only the morphology of ventral osteoderms in the cloacal region and found that there is a difference between juveniles and adults. The difference observed in the coloring in the clearing technique may be related to the variation in scale composition between juvenile and adult specimens. Possibly because a juvenile, with little time to live outside the maternal body, has an accumulation of calcium in the scales that is lower than that found in the studied adult specimens. Despite being considered an important taxonomic character in Anguidae, osteoderms can vary in position (head, trunk, tail, ventral and dorsal), age (juvenile and adult) and condition (regenerated or unregenerated) (Bochaton et al. 2015). Thus, analyses of *O.* cf. *fragilis* are still preliminary, as well as in other species belonging to this genus and family, requiring a more in-depth description to enable the use of this structure in the group's taxonomy.

The morphology of the appendicular skeleton is poorly known in several species with reduced or absent limbs (Andrade et al. 2016). Borges-Martins (1998) described the pelvic girdle and hind limb bones of three species of *Ophiodes* (*O. fragilis*, *O. striatus* and *O.* sp "1"). As described by that author for *O.* sp "1", the metatarsal, calcaneal and astragalus bones are absent in *O.* cf *fragilis*. Cellular activity within and between the bones of the limb may be related to growth. It is also noteworthy that the hind limb has different sizes in juvenile and adult specimens; therefore, hind limb growth may occur concurrently with body growth (Rocha-Barbosa et al. 2015).

Vestigial biological structures provide an important line of evidence for macroevolution (Andrade et al. 2016), since they can be considered evolutionary reflections of the historical effects of microhabitats and the biotic environment (Pianka & Vitt 2003). The reduction of limbs in anguids occurred at about 70 Mya, with 26 to 32 different origins of reduction forms being estimated. In *Ophiodes* it is mainly associated with tail elongation (surface ecomorph) (Wiens et al. 2006, Brandley et al. 2008). In *Ophiodes,* our data shows the existence of bones in the reduced hind limbs as well as cellular activity, making us question its evolutionary functionality for the species and even for the group. The vestigial hind limbs of *Ophiodes* cf. *fragilis* were described for the first time as well as their morphology and function. The types of locomotion in different substrates observed were slide-pushing and lateral undulation. Functional traits play an important role in the structure of the community and in the functioning of the ecosystem (Cianciaruso et al. 2009); and these, in turn, are highly variable due to local adaptation or phenotypic plasticity in response to different environmental characteristics or biotic interactions (Albert et al. 2012). Much remains to be clarified about the morphological and physiological adaptations that this species and others of limbless vertebrates underwent to adapt to their environment. Despite this, our data fills in some gaps and create new questions to be answered about the modes and adaptations of the morphology associated with locomotion in limbless lizards or with reduced limbs.

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SUPPLEMENTARY MATERIAL

Video S1.

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PRO, ORB and BMS designed the study; PRO, VGA and TTS applied the methodologies and performed the analyses; JAD and BMS guided and coordinated the study; all authors reviewed and approved the final manuscript.