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Revisiting the Green Ameiva, *Ameiva ameiva* (Linnaeus, 1758), corpse bride: dead bodies tell stories

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The mechanisms underlying the reception of chemical signals and the recognition of conspecific individuals are pivotal in shaping the social behaviours of lizards, including courtship and mating (Halpern, 1992; Martín and López, 2014). During the breeding season, pheromone-based communication plays a vital role, enabling males to locate potential mates or entice them to their territories (Martín and López, 2014). However, these same chemical cues can lead males to exhibit atypical behaviors, such as necrophilia or Davian behaviour, when attracted to recently deceased reproductive females (Costa et al., 2010; Sazima, 2015; Siqueira et al., 2015). This phenomenon has been documented in various lizard families such as Varanidae (Lambiris, 1966), Scincidae (Sharrad, King, and Cairney, 1995; How and Bull, 1998), Crotaphytidae (Fallahpour, 2005), Phrynosomatidae (Brinker and Bucklin, 2006; Valdez-Villavicencio and Peralta-García, 2020) and Teiidae, including *Salvator merianae* (Duméril and Bibron, 1839) and *Ameiva ameiva* (Linnaeus, 1758) (Vitt, 2003; Costa et al., 2010, Sazima, 2015).

Ameiva ameiva is an oviparous, medium-sized, diurnal, and terrestrial lizard widely distributed in Brazil (Vitt, 1982; Magnusson, 1987; Colli, 1991; Rocha, 2008, Vitt et al., 2008; Benício et al., 2019). They are active foragers in open areas with high solar incidence, where their body temperature can vary between 33–40 ºC during the activity period (Vitt et al., 2008). Its reproductive cycle can vary according to the geographical distribution of the species, tending to be extended in areas where the rainy season is less defined and seasonal in areas where the rainy season is more defined (Colli, 1991; Vitt and Colli, 1994; Costa et al., 2010). Despite this, the mating season of the species seems to occur markedly during the spring and summer (Ramalho et al., 2021).

On 12 March 2009, during the summer, in an urban area at the Brazilian Atlantic Forest domain, Costa et al. (2010) recorded a male *Ameiva ameiva* repeatedly attempting to copulate with a conspecific female that appeared to be deceased, presumably due to a vehicular collision. These observations also included confrontations between the initial male and a rival male who subsequently approached the female. The present study aims to revisit the findings reported by Costa et al. (2010) to address several lingering questions related to the observed events: 1) Were the mating attempts by the initial male indeed successful? 2) Did the deceased female carry "fertilised eggs" and "developing follicles"? 3) Could the reproductive status of the deceased female be linked to the production and dispersal of pheromones that attracted the male and triggered mating behaviour? Answering these questions is important because, even though this behaviour has been reported several times (Vitt, 2003; Costa et al., 2010; Sazima, 2015; Valdez-Villavicencio and Peralta-García, 2020), these generally concern only the in-situ observational records. However, these require more in-depth investigations as done by Siqueira et al. (2015) to provide novel insights that could be related to other aspects of the species' reproductive biology (e.g., reproductive cycles, endocrine regulation of reproduction, mating season and number of copulations).

Material and methods

The female specimen collected by Costa et al. (2010) was fixed in 10% formalin and preserved in 70% ethanol and deposited in the herpetological collection

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of the Museu João Moojen, Universidade Federal de Viçosa, Minas Gerais, Brazil (MZUFV 766). We examined the specimen as part of a broader study on the species' reproduction. It had previously been partially dissected, but it was necessary to make a ventral incision through the pelvic girdle to the scapular girdle, exposing the organs for macroscopic, microscopic, and photo-documentation analyses of the reproductive tract, following the methodology proposed by Almeida-Santos et al. (2014).

Samples from the right oviduct were collected, and its contralateral counterpart was preserved. These samples included the infundibulum, glandular and non-glandular uterus, following the nomenclature proposed by Siegel et al. (2014). Additionally, samples from the femoral glands were also collected. Subsequently, the removed tissue fragments were dehydrated in a series of increasing ethanol concentrations (70–100%) and processed using the standard paraffin embedding method for optical microscopy (Junqueira and Carneiro, 1995). Longitudinal sections were cut at an average thickness of 5 µm and stained with haematoxylin and eosin (HE). Histochemical techniques such as Periodic acid-Schiff's (PAS) and Alcian Blue (AB) at pH 2.5 were also performed to detect neutral and acidic mucopolysaccharides, respectively, to identify the chemical nature of some cellular constituents in portions of the oviduct and relate them to the reproductive status of the deceased female. We analysed the histological slides using an Olympus BX51 optical microscope coupled with an Olympus DP73 digital camera. The cellSens software (version 5) was used for image capture.

Results

The macroscopic analysis of the female revealed a pair of intact oviducts, each consisting of the infundibulum and glandular and non-glandular uteri (Fig. 1). Both ovaries had been collected, and there were no signs that eggs had been recently allocated to the oviducts. Microscopic analysis of the oviduct showed that the posterior infundibulum had a ciliated epithelium filled with glandular invaginations where no stored spermatozoa were observed (Fig. 2A). In the glandular uterus there was the epithelium and lamina propria, which was densely packed with hypertrophied uterine glands (Fig. 2B). The non-glandular uterus had a ciliated epithelium with the formation of crypts that were filled with stored spermatozoa in the most caudal portion, near the cloaca (Figs. 2C, D). Periodic acid-Schiff's (PAS) and Alcian Blue (AB) reacted positively in the

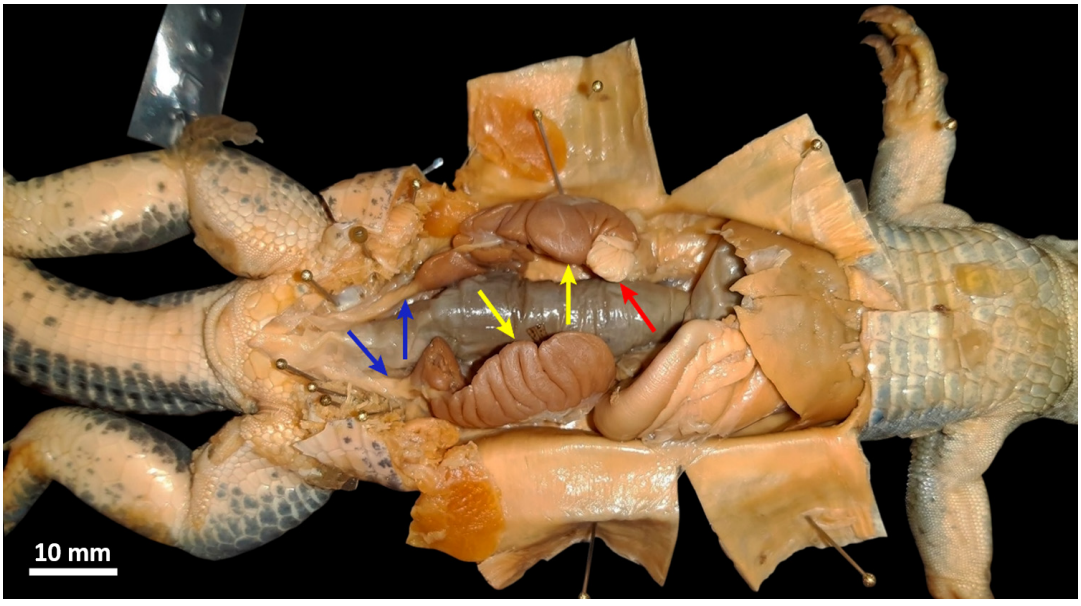


Figure 1. Macroscopic analysis of the dead female *Ameiva ameiva* (MZUFV 766) showing the right infundibulum (red arrow), the glandular uteri (yellow arrows), and the aglandular uteri (blue arrows). The left infundibulum was folded behind the glandular uterus.

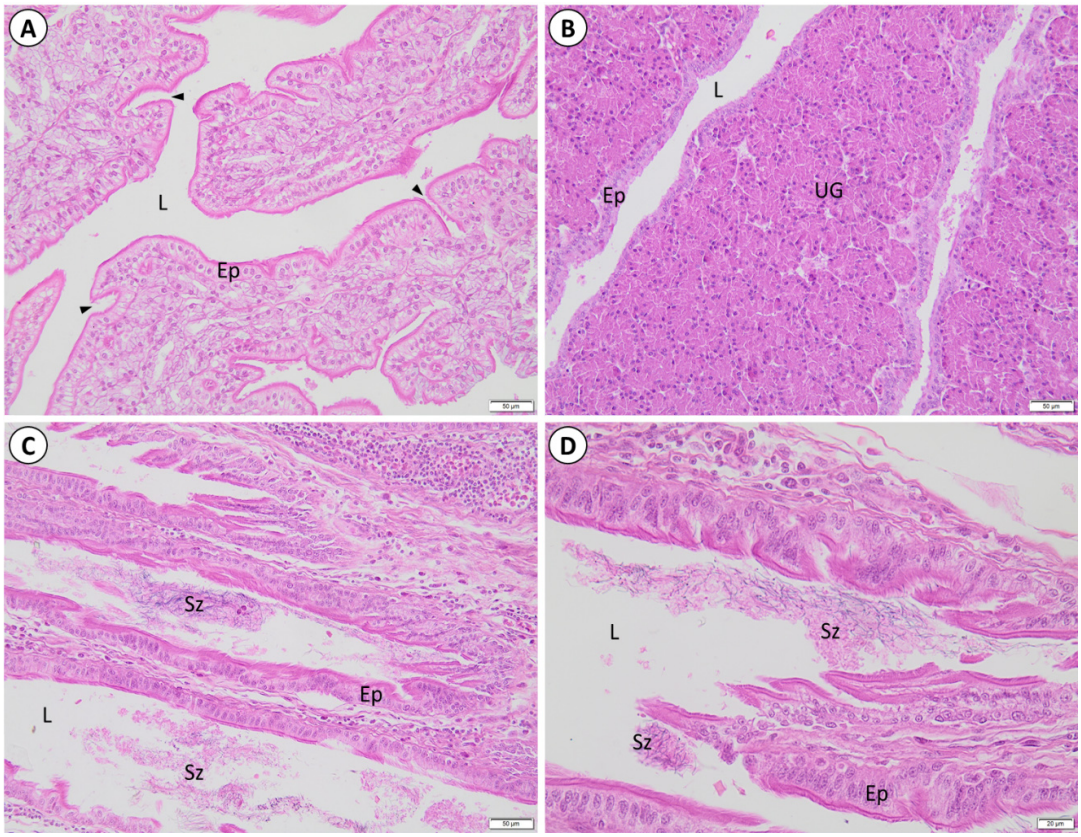
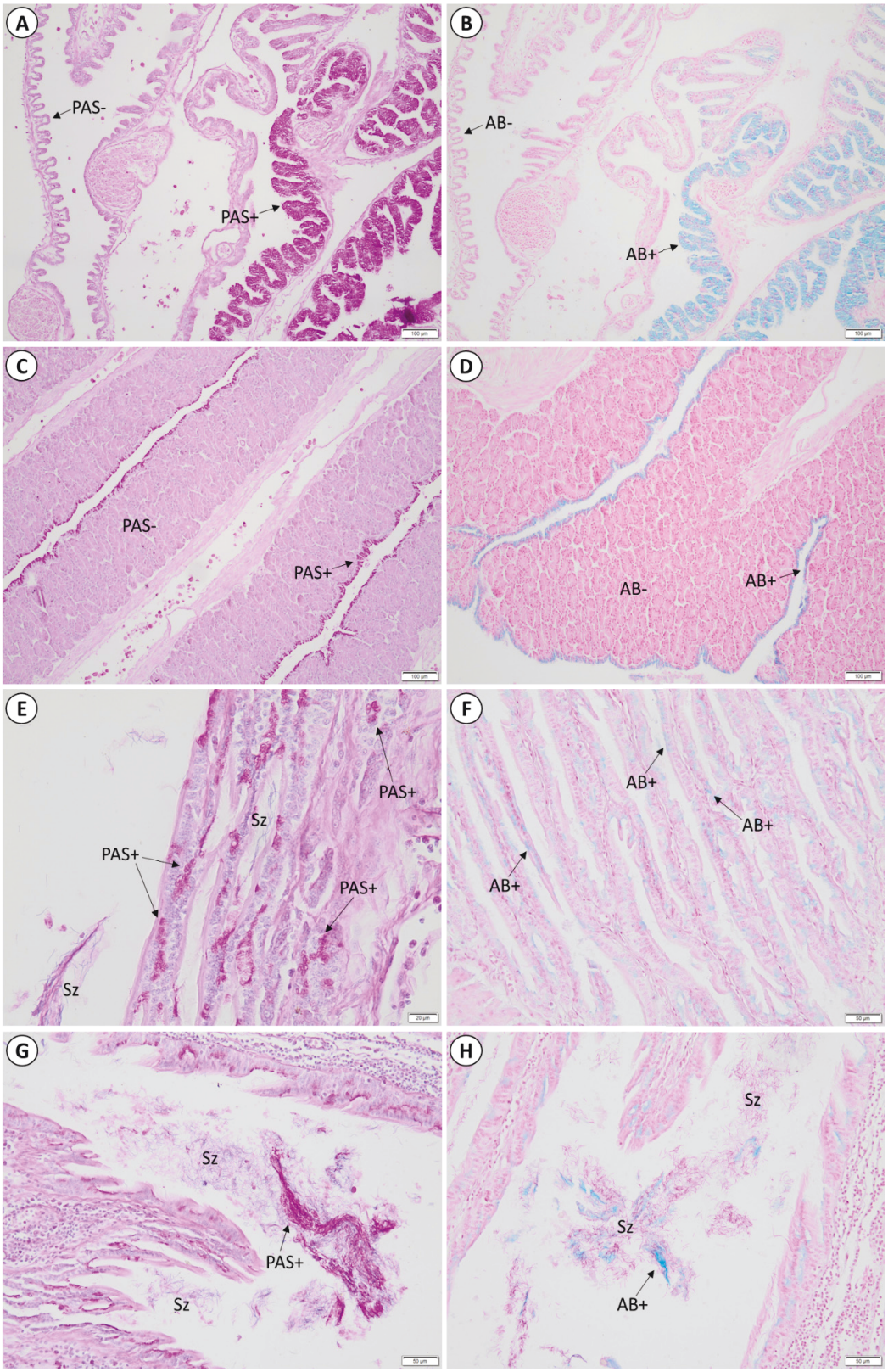


Figure 2. Histology of the oviduct of the dead female *Ameiva ameiva* (MZUFV 766). A) infundibulum showing glandular invaginations (arrowheads). B) glandular uterus showing the uterine glands (UG). C, D) aglandular uterus with sperm (Sz). L: lumen; Ep: epithelium. Haematoxylin and eosin.

epithelium of the posterior region of the infundibulum (Figs. 3A, B), in the epithelium of the glandular uterus (Figs. 3C, D), in isolated portions of the epithelium of the non-glandular uterus (Figs. 3E, F), and in the secretion associated with spermatozoa (Figs. 3G, H).

Microscopic analysis of the femoral glands showed various glandular bodies consisting of secretory cells at different stages of differentiation, filled with granules and with a high affinity for eosin (Fig. 4A). The main duct appeared quite robust, lined with stratified epithelium, and filled with a secretion plug also with a high affinity for eosin (Fig. 4B). PAS reacted positively in the granules of the secretory cells and in the plug of the main duct (Figs. 4C, D). AB did not show a positive reaction in any of the glandular structures (Figs. 4E, F).

Figure 3. Histochemistry of the oviduct of the dead female *Ameiva ameiva* (MZUFV 766). A, B) infundibulum with a positive reaction only in the epithelium of the posterior region. C, D) glandular uterus with positive reaction only in the epithelium. E, F) aglandular uterus with a positive reaction in isolated portions of the epithelium. G, H) Positive reaction in secretion associated with spermatozoa (Sz). PAS+: positive for neutral mucopolysaccharides; PAS-: negative for neutral mucopolysaccharides; AB+: positive for acid mucopolysaccharides; AB-: negative for acid mucopolysaccharides. Left column: Periodic Acid-Schiff's (PAS); Right column: Alcian Blue (AB).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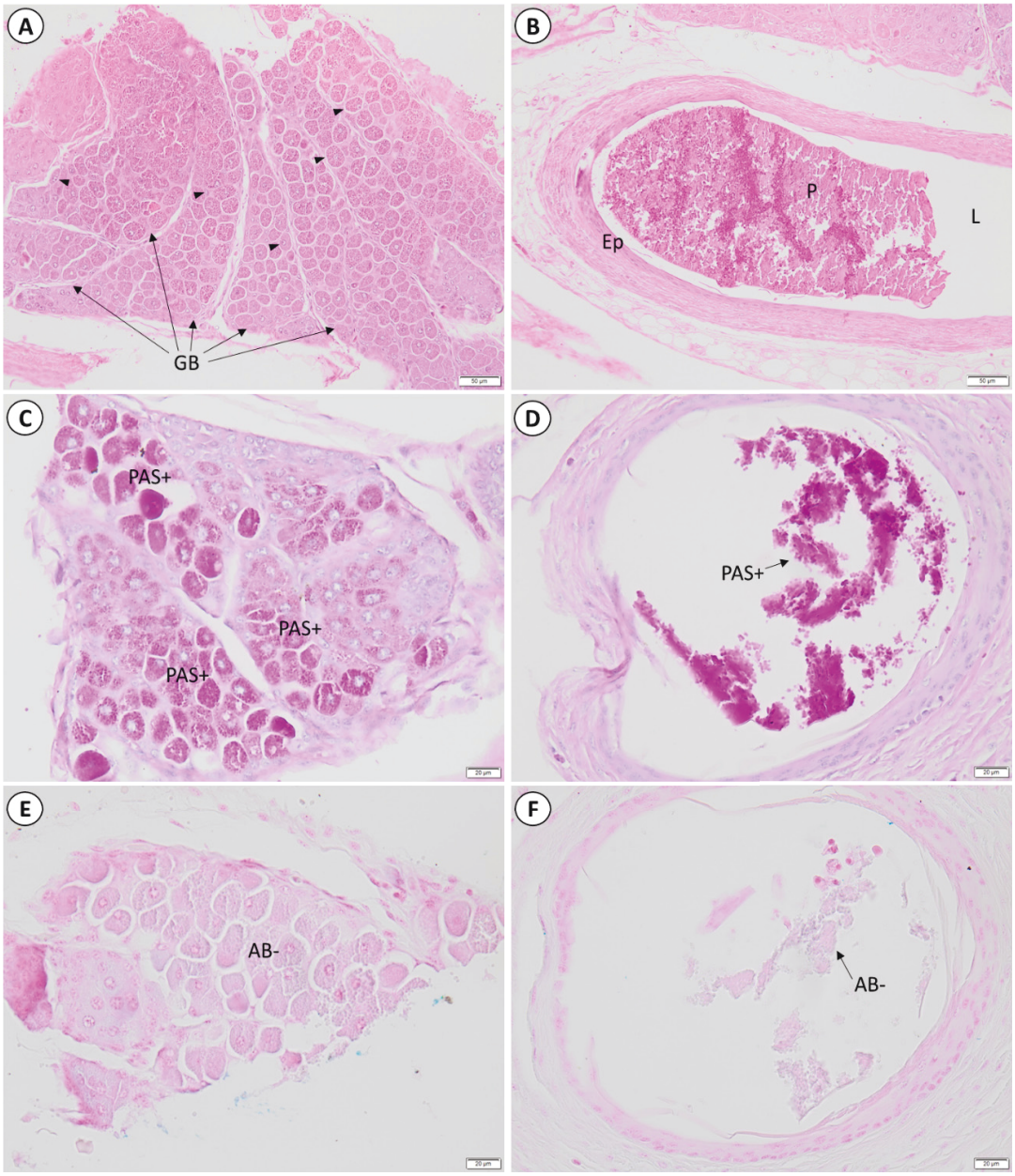

Figure 4. Histology and histochemistry of the femoral glands of the dead female *Ameiva ameiva* (MZUFV 766). A) glandular bodies (GB) made up of secretory cells (arrowheads). B) main duct filled by the secretory plug (P). C) glandular body with positive reaction in the secretory granules. D) main duct with positive reaction in the secretion plug. E) glandular body with negative reaction. F) main duct with negative reaction. L: lumen; Ep: epithelium; PAS+: positive for neutral mucopolysaccharides; AB-: negative for acid mucopolysaccharides. A, B: Haematoxylin and eosin; C, D: Periodic Acid-Schiff's (PAS); E, F: Alcian Blue (AB).

Discussion

The copulation period of female lizards may be associated with vitellogenesis (Van Dyke, 2014). The estradiol hormone mobilises energy reserves and regulates the process of yolk deposition in the ovarian follicles, which can stimulate the female's receptivity (Hahn, 1967; Bonnet et al., 2001; Almeida-Santos et al., 2004; Van Dyke, 2014). On the contrary, the period in which females are carrying eggs does not seem to be the most favourable time for copulation due to the decrease in estradiol and increase in progesterone, inhibiting vitellogenesis and helping to maintain pregnancy (Bonnet et al., 2001; Almeida-Santos et al., 2004). Costa et al. (2010) reported that the dead female contained six fertilised eggs, and although the simultaneity between pregnancy and vitellogenesis does occur in *A. ameiva* (Vitt, 1982; Vitt and Colli, 1994) and this facilitates new periods of copulation (R.A. Ramalho, unpublished data), this was not the case. Since both oviducts were intact and highly pleated, it excludes any possibility of recent pregnancy. Furthermore, the size described for the so-called 'eggs' (14.48 ± 0.38 mm, range = 14–15 mm, n = 6) more closely resembles that of follicles in secondary vitellogenesis for the species (Vitt and Colli, 1994).

During vitellogenesis, females can signal their estrus to males through the dispersion of pheromones in the environment (Cooper and Vitt, 1984; Mason, 1992; Cooper and Pèrez-Mellado, 2002; Martín and López, 2014). In *A. ameiva*, this dispersion occurs through femoral pores in the centre of modified scales called rosettes (Imparato et al., 2007). These pores are connected to glandular bodies (Imparato et al., 2007) that secrete a plug of chemical compounds that include neutral mucopolysaccharides (Imparato et al., 2007; this study). Assessment of the femoral glands showed that they were filled with a secretion plug. This indicates that at the time of death the female was releasing fragments of this plug into the environment, leaving chemical clues that attracted the attention of both males, transmitting their reproductive status, and even leading one of the males to an attempt copulation. The evolution of this communication tactic plays a very important role in the social behaviour of non-territorial species such as *A. ameiva*, as they actively move in search of food and sexual partners (Pianka and Vitt, 2003; Imparato et al., 2007).

In addition to the macroscopic analysis, the entire microscopic analysis revealed that the female was morphologically prepared for copulation. Variations in the epithelial height of the posterior region of the infundibulum during the reproductive period as well as the positive reaction for PAS and AB have already been reported in several species (Guillette et al., 1989; Girling et al., 1998; Nogueira et al., 2011). In relation to the uterus, the presence of hypertrophied glands has also been associated with the reproductive period of female lizards (Siegel et al., 2014) and the positive reaction to PAS and AB in the epithelium is an indication of its secretory function (Corso et al., 2000; Hosie et al., 2003; Ramírez-Pinilla et al., 2012). The aglandular uterus shows little variation between the reproductive and non-reproductive periods, however, the constitution of the epithelium by ciliated cells and secretory cells explains the positive reaction to PAS and AB in isolated portions, highlighting the secretory cells (Girling et al., 1998; Sever and Hopkins, 2004; Nogueira et al., 2011). Finally, secretions associated with sperm may have come from the male and transferred to the female during copulation, produced by the renal sexual segment and promoting sperm survival and quality (Sever and Hopkins, 2004; Rheubert et al., 2014).

Although Costa et al. (2010) were unable to observe whether the first male managed to insert any of the hemipenes into the female's cloaca, our observations indicate sperm presence in the most caudal portion of the aglandular uterus, confirming copulation occurred. Although interpretation of the chemical signals led the male to mistakenly copulate with a dead female, wasting energy and resources, Davian behaviour may have occurred under environmental conditions that kept the female's body temperature high enough for the male to believe she was still alive (Costa et al., 2010). Furthermore, necrophilia may result from the strong action of pheromones on the carcass of the recently deceased reproductive female (Sazima, 2015; Siqueira et al., 2015), or even from the passivity of females during courtship and copulation behaviours (Ramalho et al., 2021), which may have confused the male.

In conclusion, our findings underscore the invaluable contribution of preserved specimens within scientific collections to advance of our knowledge on the reproductive biology and behavioural patterns of species. This holds particularly true for taxa in which field-based behavioural observations can be particularly challenging, as is often the case with many Neotropical lizard species. Additionally, we emphasise the role of regional collections, such as the Museu de Zoologia João Moojen. Despite often grappling with limited financial and human resources, these smaller collections possess a wealth of information to offer the scientific

community, which become even more apparent in large countries, like Brazil.

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