



Research paper

Reproductive modes of the South American water snakes: A study system for the evolution of viviparity in squamate reptiles

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ABSTRACT

Viviparity has evolved at least 115 times in squamates and these multiple origins have raised a series of functional and evolutionary questions. Ideally, testing evolutionary hypotheses on squamate viviparity requires focusing on closely related taxa that exhibit both reproductive modes. South American water snakes of the tribe Hydrosini (genera *Hydrops*, *Pseudoeryx*, and *Helicops*) are a potential model system for studying the evolution of viviparity. However, available information about reproductive modes in this group is often confusing and contradictory. Herein, we identify the reproductive modes of the Hydrosini species by combining original data taken from both museum and live specimens with a critical review of the published literature. In addition, we mapped the reproductive modes on different phylogenetic hypotheses to identify origins of viviparity. The genus *Hydrops* is exclusively oviparous. Contrary to previous suggestions, we found *Pseudoeryx plicatilis* to be oviparous, and available evidence suggests that females of the species remain with eggs throughout incubation. Our results show that reproductive mode varies only in the genus *Helicops*. Two *Helicops* species are oviparous and eight species are viviparous. Interestingly, we also found that one species (*Helicops angulatus*) exhibits both reproductive modes. Intraspecific variation in reproductive mode is a rare phenomenon in squamates, and *H. angulatus* is the only snake species in which reproductive bimodality has been confirmed. *H. angulatus* is oviparous from northern to mid-eastern and north-eastern South America, and viviparous from north-western to mid-western South America. The allopatric distribution of oviparous and viviparous forms of *H. angulatus* does not support the hypothesis of facultative changes in the reproductive mode. Geographic variation in the embryonic stage at oviposition is likely to occur in *H. angulatus*. Ancestral state reconstructions suggest that oviparity is plesiomorphic in Hydrosini, as well as in the bimodal genus *Helicops*, and that viviparity has evolved independently at least three times in *Helicops*. We argue that the water snakes of the tribe Hydrosini (and more importantly, the genus *Helicops* and the bimodal *H. angulatus*) are an excellent model to test hypotheses on the evolution of squamate viviparity.

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1. Introduction

The term “reproductive mode” refers to the type of reproductive product deposited by the mothers (Blackburn, 1993). Accordingly, two modes of reproduction are recognized in amniotes. Oviparity is characterized by the deposition of shelled eggs that undergo or just complete their development outside the uterus, whereas viviparity

consists of the retention of fertilized eggs in the uterus throughout development and the subsequent parturition of fully developed young, which may or may not be surrounded by transparent shell membranes (Blackburn, 1993). Oviparity is the ancestral and most common reproductive mode in reptiles. All turtles, archosaurs, the tuatara, and a majority of squamates (lizards, snakes, and amphisbaenians) reproduce by laying eggs (Packard et al., 1977; Shine, 1985). Nevertheless, embryonic stage at oviposition varies substantially among reptile lineages. Whereas turtles, archosaurs, and the tuatara lay eggs at very early stages (Bellairs, 1991; Ewert, 1985; Ferguson, 1985; Moffat, 1985), most oviparous squamates lay eggs with embryos between the late organogenesis and early growth stages (Andrews and Mathies, 2000; Blackburn, 1995; Shine, 1983). Viviparity occurs in nearly 20% of the squamate

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species (Shine, 1985), but despite this low proportion, it has evolved independently at least 115 times (Blackburn, 2015a). These multiple origins of viviparity in various squamate lineages have raised a series of functional and evolutionary questions of broad interest, and consequently, researchers have put effort into understanding how and why viviparity evolved so often in squamate reptiles (see reviews in Andrews and Mathies, 2000; Blackburn, 2015a, 2000; Guillette, 1993; Shine, 2014, 1985; Stewart and Thompson, 2000; Thompson et al., 2002). Nevertheless, a major challenge in studying historical events is that they involve past processes that are not directly observed (Mayr, 2004). In the case of the evolution of viviparity, the difficulties arise because many features of current taxa may actually be specializations developed after viviparity has evolved (Blackburn, 2000; Guillette, 1993). A robust approach has been to focus on closely related taxa that exhibit both reproductive modes and show minor differences in other features (Blackburn, 2000; Guillette, 1993; Shine, 1985; Tinkle and Gibbons, 1977). In these cases, modifications observed in viviparous taxa are more likely to be related to the evolution of viviparity.

Many genera and some species of Squamata are reported to contain both oviparous and viviparous representatives, and consequently are ideal models of closely related taxa varying in reproductive modes (Shine, 1985; Tinkle and Gibbons, 1977). However, many of the cases of reproductive bimodality have been challenged and suggested to be the result of taxonomic misidentification or incorrect interpretation of reproductive modes (Blackburn, 1993; Shine, 1985; Tinkle and Gibbons, 1977). The genera and the few species in which reproductive bimodality is confirmed have been extensively used as model systems for studying the evolution of viviparity in a range of biological disciplines (e.g., Adams et al., 2007; Heulin et al., 2005; Stewart et al., 2010; Watson et al., 2014; Whittington et al., 2015). Comparative studies at such taxonomic levels have clarified the sequence of events during the evolution of viviparity, as well as the selective pressures that favour it, and its subsequent adaptations and specializations (Blackburn, 2000; Shine, 1985). Most of these studies have generally focused on a few lizard groups (e.g., scincids and phrynosomatids), but they have revealed considerable diversity of ways that viviparity has evolved (Blackburn, 2000; Stewart and Thompson, 2000; Thompson et al., 2002; Whittington et al., 2015). This likely reflects the multiple origins of viviparity and the several evolutionary pathways used to solve a physiological problem (Blackburn, 2006, 2000). Thus, studies involving a few species are not enough to explain the evolution of reproductive modes, and additional groups are useful to capture the range of ways viviparity can evolve in squamates (Blackburn, 2006, 2000).

The water snakes of the tribe Hydropsini are a potential model system for studying the evolution of viviparity. Hydropsini contains 21 species allocated to three genera (*Hydrops*, *Helicops*, and *Pseudoeryx*) widely distributed in South America (Uetz and Hošek, 2015; Zaher et al., 2009). The tribe contains both oviparous and viviparous species, and at least one genus (*Helicops*) is certainly reproductively bimodal. Well-supported instances of both oviparity [e.g., *Helicops angulatus* (Linnaeus, 1758); Ford and Ford, 2002; *Hydrops caesurus* (Scrocchi et al., 2005); Etchepare et al., 2012] and viviparity [e.g., *H. leopardinus* (Schlegel, 1837); Scartozzoni and Almeida-Santos, 2006] are available for some species. Indeed, early studies have suggested at least two origins of viviparity in the genus *Helicops* (Blackburn, 1985; Shine, 1985). However, there is considerable uncertainty about the reproductive mode of many Hydropsini. As occurs with many squamates (Blackburn, 1993), statements about the reproductive modes of Hydropsini are often presented with no empirical evidence and explicit criteria to support them (e.g., Albuquerque and Camargo, 2004; Chippaux, 1986; Whitworth and Beirne, 2011), and therefore confirmation is required. Moreover, published information is often confusing and

contradictory, and interpretations are fairly hampered by the recurrent use of the term “ovoviviparous” (e.g., Amaral, 1978; Chippaux, 1986; Cunha and Nascimento, 1993), an ambiguous and obsolete term that was largely used to refer to a wide variety of reproductive patterns, some mutually exclusive (see Blackburn, 1994, 1993). For example, the genus *Helicops* has been described as exclusively oviparous (e.g., Abuys, 1983), viviparous (Fitch, 1970) or “ovoviviparous” (e.g., Pérez-Santos and Moreno, 1991). *Helicops gomesi* (Amaral, 1921) and *H. hagmanni* (Roux, 1910) were first described as oviparous (Amaral, 1921; Cunha and Nascimento, 1981), but subsequently reported as “ovoviviparous” by the same authors (Amaral, 1978, 1927; Cunha and Nascimento, 1993). Intraspecific variation in reproductive mode is suggested in at least two species of Hydropsini, but confirmation is still required. *Pseudoeryx plicatilis* (Linnaeus, 1758) has been reported to lay eggs (Chippaux, 1986; Hoge, 1980) and give birth to young (Abuys, 1986). Moreover, Cunha and Nascimento (1981) reported a preserved female of *P. plicatilis* that contained 33 oviductal eggs, of which four had small developing embryos surrounded by thin membranes, and 29 were non-embryonated eggs surrounded by thick and leathery membranes. The authors interpreted this finding as evidence that such female could be simultaneously oviparous and viviparous. However, this assumption has never been confirmed. The other species suggested to be reproductively bimodal is *H. angulatus*, with some well-documented reports of egg-laying (e.g., Ford and Ford, 2002; Gorzula and Señaris, 1998; Rossman, 1973), but at least one record of viviparity (Rossman, 1984). A preserved female collected in Peru contained seven apparently fully developed young in the uterus without eggshell and residual yolk (Rossman, 1984). Nevertheless, there are no other records of viviparity for *H. angulatus*. In addition to all these issues, the reproductive mode of several Hydropsini species is unknown and, therefore, a clear overview on how reproductive mode varies within the tribe remains to be developed.

Our objectives here are twofold. Firstly, we aim to clarify how the reproductive modes vary within the Hydropsini. For that, we identify the reproductive modes of the Hydropsini species by combining original data collected from both museum and live specimens with a critical review of the published literature. Secondly, we mapped the reproductive modes on different phylogenetic hypotheses available for the group to identify origins of viviparity, and thereby to explore the potential for the group to act as a model system to test hypotheses on the evolution of squamate viviparity.

2. Material and methods

2.1. Original data

We collected original data from observations of both oviductal contents of museum specimens and the reproductive product at parition (i.e., young or eggs; Blackburn, 1993) in live specimens. Information about preserved specimens was collected from individuals housed in 24 scientific collections throughout Brazil (see Appendix A for a full list of museums). Whenever available, we also re-examined the specimens reported in two previous studies (Albuquerque and Camargo, 2004; Cunha and Nascimento, 1981). Specimens were identified using diagnostic characters provided in several taxonomic studies on the tribe members (Albuquerque and Lema, 2008; Amaral, 1921; Frota, 2005; Hofstadler-Deiques and Cechin, 1991; Kawashita-Ribeiro et al., 2013; Rossman, 2010, 1975, 1973, 1970). Efforts were made to gather information from different locations along the geographical distribution of each species.

A mid-ventral incision was made in 1424 adult females of 12 species to expose the reproductive tract. When a female was gravid/pregnant, we opened longitudinally one uterine incubation chamber and collected one egg. Then, we recorded whether a

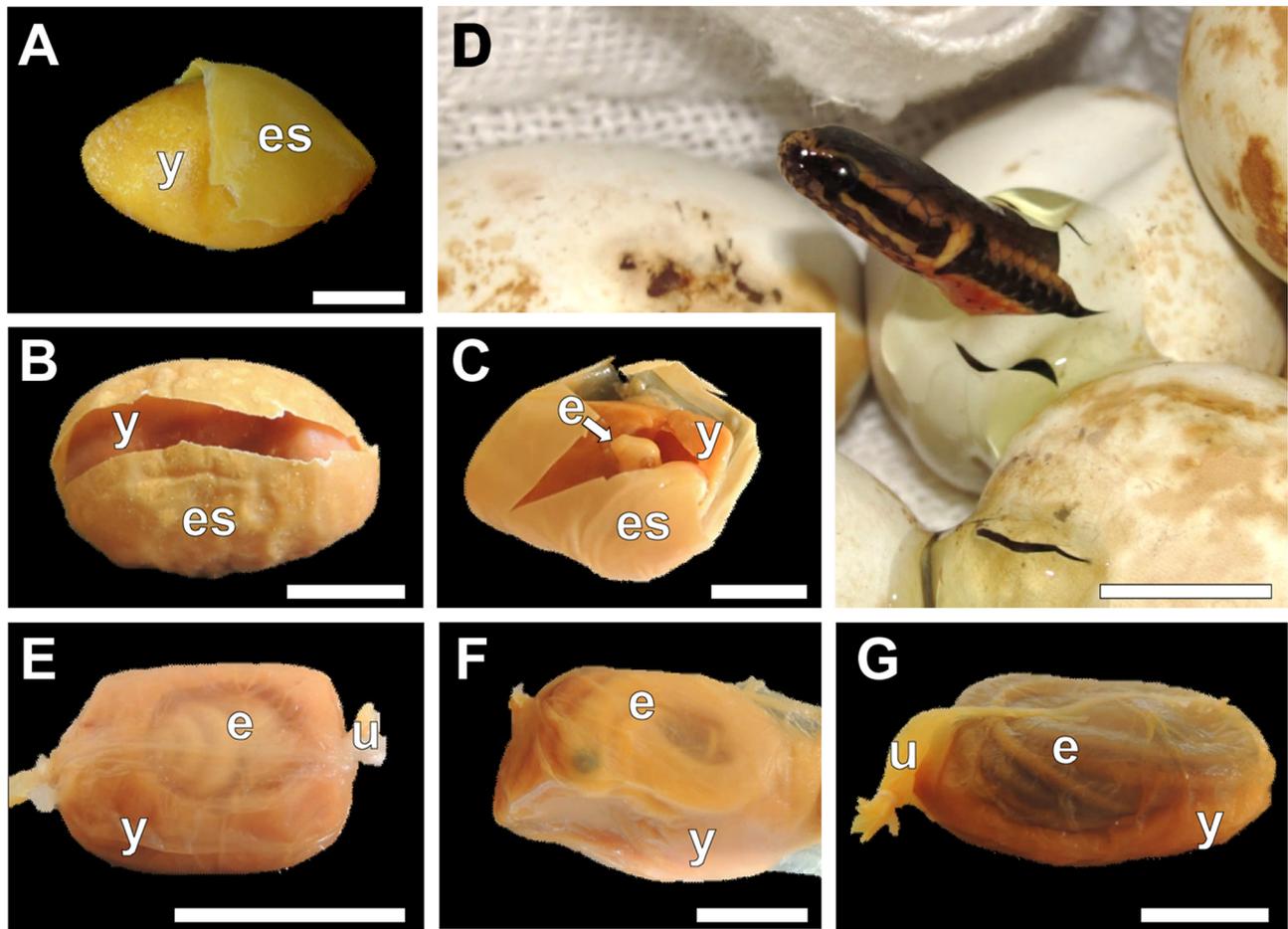


Fig. 1. Representative examples of macroscopic appearance of egg coverings in Hydropsini. (A–D) Thick, opaque, and leathery shells indicating oviparity. (A) *Hydrops martii*. (B) *Helicops gomesi*. (C) *Helicops hagdmani* (The embryo was reinserted into the yolk cavity after it has been removed following eggshell dissection, but notice how it is partially covered by the eggshell). (D) Hatchling of *Pseudoeryx plicatilis* emerging from thick-shelled egg found in nature (Rondônia, Brazil). (E–G) Thin and transparent membranes surrounding the yolk and developing embryos indicating viviparity (all embryos are enclosed in the uterus and their fetal membranes). (E) *Helicops modestus*. (F) *Helicops carinicaudus*. (G) *Helicops modestus*. Abbreviations: e, embryo; es, eggshell; u, uterus; y, yolk. Scale bars: 1 cm. Photo by F.F. Curcio (D).

developing embryo was readily visible through egg covering. After, we dissected egg covering and recorded its gross morphology as “thick” or “thin” and “opaque” or “transparent”. These distinctions are macroscopically easy to make (Figs. 1 and 3; see also Section 2.3). We then examined the eggs under a stereomicroscope to check for the presence of a developing embryo and its developmental stage. To draw general comparisons with other studies on lizards, we staged embryos using the staging system for the lizard *Zootoca vivipara* (Dufaure and Hubert, 1961; see also Blackburn, 1995; Shine, 1983 for similar uses in snakes). In the Dufaure and Hubert’s staging system (hereafter DH system), embryonic development is divided into a series of 40 stages, with stage 1 marking the beginning of cell division and stage 40 indicating a full-term embryo (Dufaure and Hubert, 1961). We also reported embryonic stages using the staging system for the snake *Thamnophis sirtalis* (Linnaeus, 1758), which divides embryonic development into a series of 37 stages (Zehr, 1962). A small fragment of eggshell of some specimens was collected, dehydrated through a series of increasing ethanol concentrations (70–100%), cleared in xylene, and embedded in paraffin for histological examination (Kiernan, 2008). Histological sections were cut at 7 μm using a rotary microtome, mounted on slides, and stained with haematoxylin and eosin (Kiernan, 2008). Sections of the eggshells were examined mainly to verify the presence of an inorganic layer, which is present only in oviparous species (Blackburn, 1998). Histological slides were photographed with an Olympus Q-Color 5 digital camera

(Olympus Corporation, Japan) mounted on an Olympus BX51TF light microscope (Olympus Corporation, Japan). The software Image-Pro Express, version 5 was used for image capture. Images were cropped and labelled with Microsoft PowerPoint 2013.

Data on the product at parturition were obtained from pregnant females ($n = 19$) collected at various sites in Brazil, and donated to the Instituto Butantan (São Paulo, Brazil). Animals were kept for a short time in the laboratory until parturition, and the reproductive product (eggs or young) was recorded. Most parturition observations ($n = 11$) were performed by one of us (RRS) between 2001 and 2005. Parturition events occurred before this period ($n = 8$) were recovered from the record books of the herpetological collection of the Instituto Butantan. In these cases, we examined all specimens to confirm their species identification and postpartum status (uteri with empty incubation chambers; Blackburn, 1998).

Additional information on the reproductive product at parturition and on oviductal contents was obtained from unpublished (though documented) data provided by other herpetologists.

2.2. Literature data

To clarify several ambiguous and confusing reports, we extensively reviewed the literature for information on reproductive modes or oviductal contents of Hydropsini. This review also enabled us to increase the number of observations per species and to obtain records for species in which we found no original data. Our

survey relied heavily on internet searches using Zoological Record and Google Scholar databases, and the species name were used as searching terms. Then, we surveyed the literature cited in the works as well as their citation records on Google Scholar to find additional records. We included in the literature review the specimens reported in two previous studies (Albuquerque and Camargo, 2004; Cunha and Nascimento, 1981) that we were not able to examine. We evaluated all published information that mentioned the reproductive mode of a Hydropsini species and/or showed information on the reproductive product at parturition or on the content of reproductive tract of dissected females.

2.3. Criteria for the classification of reproductive modes

To characterize the reproductive modes of each of the original and literature observation, we used a standard set of criteria established for squamate reptiles (Blackburn, 1993), which has been widely adopted in the literature (e.g., Cadle, 2009; Lobo and Espinoza, 2004; Smith and Shine, 1997). We classified as viviparity the cases in which we observed parturition of fully formed young surrounded (or not surrounded) by thin and transparent membranes followed quickly by emergence, and the presence of advanced or fully formed oviductal fetuses (Blackburn, 1993). We classified as oviparity the cases of oviposition of shelled eggs, hatching of eggs with hatchlings taxonomically identified, and egg-tending behaviour (Blackburn, 1993). The lack of a thick and opaque shell surrounding the egg and embryo is a definitive feature of viviparity (Blackburn, 1998). However, in eggs with very early development (without visible embryos), the lack of a thick eggshell is not necessarily indicative of viviparity, as there may not have been enough time for shell deposition (Blackburn, 1993). Thus, for eggs with thin and transparent membranes, we included (and thus classified as viviparous) only the specimens with relatively advanced embryos (\geq DH stage 28; Blackburn, 1993; Smith and Shine, 1997). At the other extreme, the presence of a thick and opaque eggshell even in early eggs can be indicative of oviparity. Despite the fact that substantial shell membranes are said to occur in a few viviparous squamates (particularly in early development eggs), which could mislead interpretations (Blackburn, 1993), shell membrane thickness is always substantially thinner in viviparous squamates (Blackburn, 1998; Heulin et al., 2005; Stewart et al., 2010). Moreover, shell membrane in all viviparous squamates typically lacks the external inorganic layer present in oviparous species (Blackburn, 1998). As a result, macroscopic distinctions between oviparous eggshells and viviparous shell membranes in oviductal eggs are easily made (e.g., Heulin, 1990; see also Figs. 1 and 3). Indeed, the simple observation of thick and opaque shells has been widely and reliably adopted as an indicator of oviparity (e.g., Ineich et al., 2006; Stafford, 2005; Webb et al., 2000). Thus, we also classified as oviparous those specimens that had thick and opaque shells surrounding oviductal eggs, with or without developing embryos (Ineich et al., 2006; Stafford, 2005; Webb et al., 2000), as well as thick, parchment-like or coriaceous shells mentioned in the literature. The existence of an external inorganic layer in those thick shells (as determined histologically) was also considered an indicator of oviparity.

We considered as inconclusive the literature reports based on insufficient evidence to draw a conclusion (e.g., females containing “eggs”, “oviductal eggs”, “ovarian eggs” or lack of shell membrane in early eggs) and the statements on reproductive modes made without explicit or documented evidence (Blackburn, 1993; Cadle, 2009).

2.4. Reconstruction of ancestral character states

The most comprehensive phylogenetic hypotheses available for the Hydropsini include seven species; one of *Hydrops*, one of *Pseudoeuryx*, and five of *Helicops* (Grazziotin et al., 2012; Pyron et al., 2013). Despite being incomplete, mapping of reproductive modes on these phylogenetic hypotheses allows some interpretation of the evolution of reproductive modes. Four different relationships among species were recovered in these works (Grazziotin et al., 2012; Pyron et al., 2013), but we reconstructed reproductive modes using three of them because one topology was not fully resolved (Grazziotin et al., 2012). The monotypic *Manolepis putnami* (Jan, 1863) was recovered as the sister group of Hydropsini in some of these analyses, but its phylogenetic placement is still uncertain (Grazziotin et al., 2012; Pyron et al., 2013), and no information on its reproductive mode is available. Therefore, we excluded this taxon from the trees. Reconstructions were performed using linear parsimony in Mesquite software, version 3.04 (Maddison and Maddison, 2015). Reproductive modes were considered as discrete binary characters (coded as [0] oviparity and [1] viviparity), and treated as unordered states with one step for each change with equal weights. For *H. angulatus*, which is reproductively bimodal (see Section 3.1.), we assumed oviparous and viviparous populations represent two closely related evolutionary lineages. Reproductive modes of outgroups were obtained from the literature (Marques et al., 2004).

3. Results

3.1. Reproductive modes

Out of 1424 dissected specimens, 116 (from 12 species) contained oviductal eggs/embryos with enough features to consistently classify their reproductive mode (Table 1; see also Appendix B for voucher numbers of dissected specimens). We re-examined 18 out of 31 *H. martii* (Wagler, 1824) females and 36 out of 51 *H. triangularis* (Wagler, 1824) described as gravid in a previous study (Albuquerque and Camargo, 2004), and found that no female of *H. martii* and only four females of *H. triangularis* had oviductal eggs (see below). All the remaining specimens reported by Albuquerque and Camargo (2004) contained only enlarged ovarian follicles. We also collected 21 original observations on the reproductive product at parturition of five species: *H. angulatus*, *H. carinicaudus* (Wied, 1825), *H. infrataeniatus* (Jan, 1865), *H. modestus* (Günther, 1861), and *P. plicatilis*. Our literature search found 54 publications mentioning the reproductive mode, reproductive product, or oviductal contents of Hydropsini (Appendix C). These works account for 77 reports for 17 species, including generalizations for the three genera. By applying the criteria to identify reproductive modes, we found that 39 of these literature reports showed insufficient evidence to classify reproductive mode and consequently they were not considered in this study (see Table A.1 in Appendix C). Thirty-eight published reports about 13 species contained enough evidence to identify reproductive modes (see Table A.2 in Appendix C). By combining our original data with all conclusive published records, it was possible to characterize reproductive modes of 15 species. We could not find any conclusive data (original or published) for six species: *P. relictuialis* (Schargel et al., 2007), *H. apiaka* (Kawashita-Ribeiro et al., 2013), *H. pastazae* (Shreve, 1934), *H. petersi* (Rossman, 1976), *H. tapajonicus* (Frota, 2005), and *H. yacu* (Rossman and Dixon, 1975).

Six species of Hydropsini are oviparous (*H. gomesi*, *H. hagmanni*, *P. plicatilis*, *H. caesurus*, *H. martii*, and *H. triangularis*; Table 2). Of these, we did not obtain original information only for *H. caesurus*. However, two literature records of hatchlings emerging from leathery shelled-eggs found in nature confirm oviparity in this

Table 1

Total of specimens examined, clutch/litter size, eggshell characteristics, embryonic stage, and reproductive mode of water snakes of the tribe Hydropsini.

Species	Total of specimens		Clutch/litter size range	Eggshell	Embryonic stage range			RM
	Dissected	Gravid/pregnant			DH stage	Zehr stage	n	
<i>Hydrops martii</i>	103	1	13	Thick, opaque, and leathery	28	20	1	O
<i>Hydrops triangularis</i>	141	4	9–20	Thick, opaque, and leathery	–	–	–	O
<i>Pseudoeryx plicatilis</i>	70	3	5–33	Thick, opaque, and leathery	32	26	1	O
<i>Helicops gomesi</i>	18	1	3	Thick, opaque, and leathery	30	25	1	O
<i>Helicops hagmanni</i>	112	3	16–19	Thick, opaque, and leathery	34	30	1	O
<i>Helicops angulatus*</i>	114	19	1–21	Thick, opaque, and leathery	28–30	21–24	5	O
		6	1–12	Thin and transparent	28–40	20.5–37	6	V
<i>Helicops carinicaudus</i>	129	4	8–17	Thin and transparent	29–36	21–31	4	V
<i>Helicops infrataeniatus</i>	148	23	5–36	Thin and transparent	30–40	24–37	23	V
<i>Helicops leopardinus</i>	188	7	3–21	Thin and transparent	32–40	26–37	7	V
<i>Helicops modestus</i>	264	35	4–23	Thin and transparent	28–40	20–37	35	V
<i>Helicops polylepis</i>	87	5	7–31	Thin and transparent	34–40	30–37	5	V
<i>Helicops trivittatus</i>	50	5	5–13	Thin and transparent	34–40	30–37	5	V

"DH stage" denotes embryonic stage following Dufaure and Hubert (1961), and "Zehr stage" following Zehr (1962). Sample size (n) of the embryonic stage range refers to the number of females found with developing embryos at least from DH stage 28. See Appendix B for a list of specimens examined. Asterisks indicate species with oviparous and viviparous populations.

Table 2

Summary of all available information on reproductive modes of Hydropsini.

Taxa	RM	Oviductal contents			Product at parturition			Total of records
		Literature	Present study	Total	Literature	Present study	Total	
<i>Hydrops caesurus</i>	O	0	0	0	2	0	2	2
<i>Hydrops martii</i>	O	0	1	1	0	0	0	1
<i>Hydrops triangularis</i>	O	0	4	4	1	0	1	5
<i>Pseudoeryx plicatilis</i>	O	0	3	3	2	1	3	6
<i>Helicops gomesi</i>	O	0	1	1	0	0	0	1
<i>Helicops hagmanni</i>	O	1	3	4	0	0	0	4
<i>Helicops angulatus^a</i>	O	2	19	21	5	1	6	27
	V	1	6	7	0	0	0	7
<i>Helicops carinicaudus</i>	V	3	4	7	≥2*	2	≥4*	≥11*
<i>Helicops danieli</i>	V	1	0	1	0	0	0	1
<i>Helicops infrataeniatus</i>	V	2	23	26	≥3*	6	≥9*	≥35*
<i>Helicops leopardinus</i>	V	16	7	23	9	0	9	32
<i>Helicops modestus</i>	V	0	35	35	3	11	14	49
<i>Helicops polylepis</i>	V	1	5	6	1	0	1	7
<i>Helicops scalaris</i>	V	0	0	0	2	0	2	2
<i>Helicops trivittatus</i>	V	4	5	9	1	0	1	10

^a Species with both oviparous and viviparous representatives. RM: Reproductive mode. O: Oviparous. V: Viviparous. Asterisks indicate the minimum number of records obtained from the literature, as it was not possible to determine the exact number of records from some works.

species (Table A.2 in Appendix C). All gravid females of *H. martii*, *H. triangularis*, *P. plicatilis*, *H. gomesi*, and *H. hagmanni* contained only oviductal eggs surrounded by thick, opaque, and leathery shells (Fig. 1A–C; Table 1). We could not evaluate histologically the eggshells of *H. triangularis*. The eggshells of *H. martii*, *H. gomesi*, and *H. hagmanni* (one per species) consisted of a thick fibrous layer overlain by a thinner inorganic layer (Fig. 2A–C). Except for *H. triangularis*, we found discernible developing embryos in the eggs of at least one female of each of these species (Table 1), and they were visible only after the eggshell was removed (see Fig. 1C for an example). One of the examined specimens was the *P. plicatilis* female (voucher number MPEG 297), which was described by Cunha and Nascimento (1981) as simultaneously oviparous and viviparous. Only two of the 33 eggs previously reported by the authors were still in the oviduct. The other eggs were in a plastic bag in the vial. All of these eggs had thick, opaque and leathery shells composed of a thick fibrous layer overlain by a thinner inorganic layer (Fig. 2D). We dissected two of these eggs, but no developing embryo was found. Original information on the reproductive product at parturition provided additional support of oviparity in *P. plicatilis*. A female was found coiled around a clutch of at least 31 shelled eggs on the banks of the Madeira River, in the surroundings of Porto Velho city (Rondônia, Brazil). Eggs were maintained until hatching, and hatchlings were identified as *P. plicatilis* (F. F. Curcio, pers. comm.; Fig. 1D). Indisputable published evidence also supports oviparity in

P. plicatilis, *H. triangularis*, and *H. hagmanni* (Table A.2 in Appendix C).

Viviparity occurs only in *Helicops*. Eight species of *Helicops* are viviparous: *H. carinicaudus*, *H. danieli* (Amaral, 1937), *H. infrataeniatus*, *H. leopardinus*, *H. scalaris* (Jan, 1865), *H. trivittatus* (Gray, 1849), *H. polylepis* (Günther, 1861), and *H. modestus* (Table 2). We did not obtain new data for *H. danieli* and *H. scalaris*. We suggest that *H. danieli* is viviparous based on a literature report of a preserved female showing apparently near-term embryos (Table A.2 in Appendix C). A literature report on two parturition events indicates that *H. scalaris* is viviparous (Table A.2 in Appendix C). Original information was obtained for the other six species above. In all preserved pregnant females of *H. carinicaudus*, *H. infrataeniatus*, *H. leopardinus*, *H. modestus*, *H. polylepis*, and *H. trivittatus*, we found thin and transparent membranes surrounding yolk masses and embryos (Table 1 and Fig. 1 E–G). In contrast to oviparous species, developing embryos in these viviparous specimens were always visible through extraembryonic membranes (Fig. 1C, E–G). Fully developed young were found in all these viviparous species, except for *H. carinicaudus* (Table 1). Additional support for viviparity was obtained from observations of freshly wild-caught females of three species, which gave birth in the laboratory (see Appendix B). Parturition of young was observed in two females of *H. carinicaudus* (litter size = 3 and 4), six females of *H. infrataeniatus* (mean litter size = 13.2 ± 7.3 ; range 3–26), and 11 females of

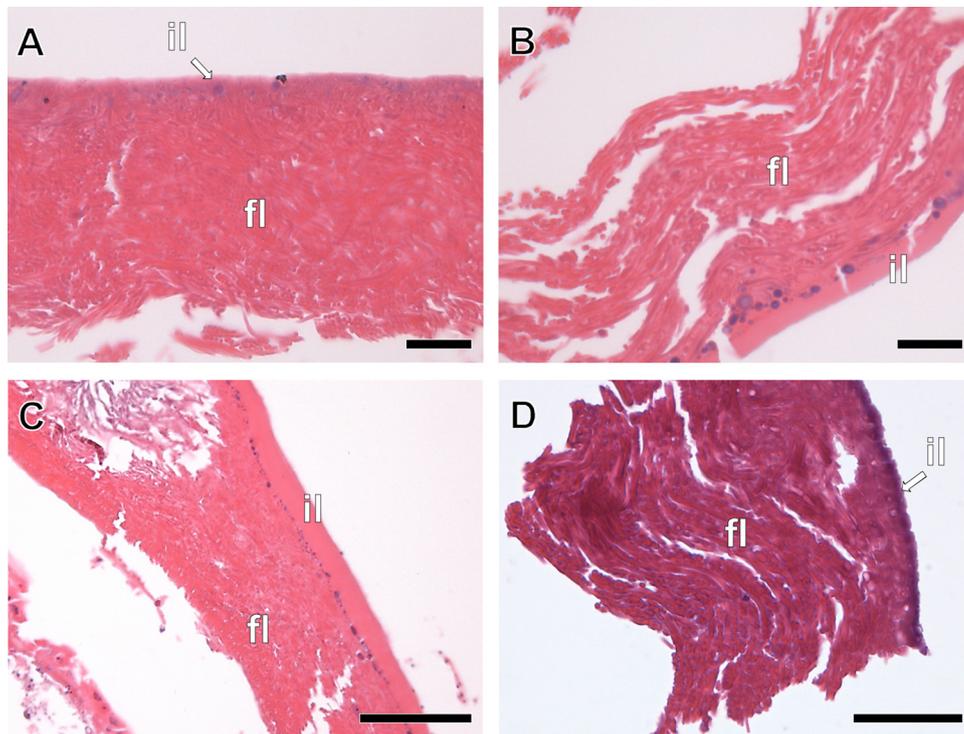


Fig. 2. Histology (Haematoxylin-eosin) of the eggshell of four oviparous Hydropsini. (A) *Hydrops martii*. (B) *Helicops hagmanni*. (C) *Helicops gomesi*. (D) *Pseudoeryx plicatilis*. Abbreviations: fl, fibrous layer; il, inorganic layer. Scale bars: A, B = 30 μ m; C = 100 μ m; D = 50 μ m.

H. modestus (mean litter size = 16.7 ± 6.8 ; range 10–28). Reliable literature reports also indicate that the six species above are viviparous (Table A.2 in Appendix C).

We found evidence of reproductive bimodality only for *H. angulatus*. Of the 114 dissected specimens, 27 females were gravid/pregnant (Table 1). Reproductive modes of 25 of these females could be unequivocally identified. Nineteen gravid females had oviductal eggs surrounded by thick, opaque, and leathery shells, indicating oviparity (Table 1; Fig. 3A–B). These eggshells consisted of a thick fibrous layer overlain by a thinner inorganic layer (Fig. 3C; Table 1). Developing embryos were found in eggs of five oviparous *H. angulatus* females (Table 1) and were visible only after eggshells was removed. An additional record of oviparity was obtained from an original observation of a clutch of seven eggs found on 1 February 2010 partially buried in the soil near a stream in Salvador (state of Bahia, Brazil). These eggs were collected and incubated, and hatchlings were positively identified as *H. angulatus* (E. Felix, pers. com.; Fig. 3D). Six indisputable records of oviparity in *H. angulatus* are available in the literature (Table A.2 in Appendix C).

Six females of *H. angulatus* were classified as viviparous. These females had only thin and transparent membranes surrounding yolk masses and/or embryos (Table 1 and Fig. 3 E–G), and developing embryos or fully developed young were always readily visible through extra-embryonic membranes (Fig. 3E–G). Embryos were partially developed (DH stages 28, 30.5, and 31) in three of these females and near term (DH stage 39.5) in a female from Colombia (M.C. Amorocho-Montaña, pers. comm.; Fig. 3F). Two other females contained fully developed young (DH stage 40). In one of these females, the young had almost no residual yolk (M.C. Santos-Costa, pers. comm.; Fig. 3G). Additionally, we found two likely records of viviparity in *H. angulatus*. Two pregnant females had thin and transparent membranes surrounding the yolk masses, but no discernible developing embryos. One reliable record of viviparity was obtained from a literature report of fully developed young found in the uterus of a preserved female (Table A.2 in Appendix C).

We mapped all original and published records of oviparity ($n=27$) and viviparity ($n=7$ confirmed and 2 likely) to generate a distribution map for each reproductive mode of *H. angulatus*. We used the location of collection of all other *H. angulatus* specimens examined in this study to estimate the species' geographic distribution range. The species is widely distributed in Tropical South America, and occurs to the east of the Andes, from the northern region of the continent, up to northern Bolivia, mid-western and north-eastern Brazil (Fig. 4). The mapping revealed that the geographic distribution of the reproductive modes of *H. angulatus* is allopatric (Fig. 4). We did not find records of co-occurrence of oviparity and viviparity within a single population (Fig. 4). Oviparous populations occur from northern to north-eastern and eastern South America, with records in Venezuela, Colombia, French Guyana, Trinidad, and in several states of northern, north-eastern and central Brazil (Fig. 4). Viviparous populations are found from north-western to mid-western South America, with records in Colombia, Peru, and in the Brazilian states of Acre, Rondônia, and Mato Grosso (Fig. 4).

3.2. Evolution of reproductive modes

Parsimony reconstructions on the three phylogenetic trees suggest that the common ancestor of Hydropsini was oviparous (Fig. 5A–C). In two of these topologies, oviparity was unambiguously reconstructed as the ancestral state for each deeper node, and viviparity evolved independently three times in *Helicops*, one in *H. carinicaudus*, one in *H. infrataeniatus*, and one in the viviparous *H. angulatus* (Fig. 5A–B). In one topology (Fig. 5C), reconstruction recovered two equally parsimonious results. The common ancestor of *Helicops* could be oviparous, and viviparity has evolved three times independently (Fig. 5C). Alternatively, viviparity would be the ancestral reproductive mode of *Helicops*, one reversion to oviparity occurred in the ancestor of the clade formed by *H. hagmanni*, *H. gomesi*, and *H. angulatus*, and viviparity re-evolved in viviparous

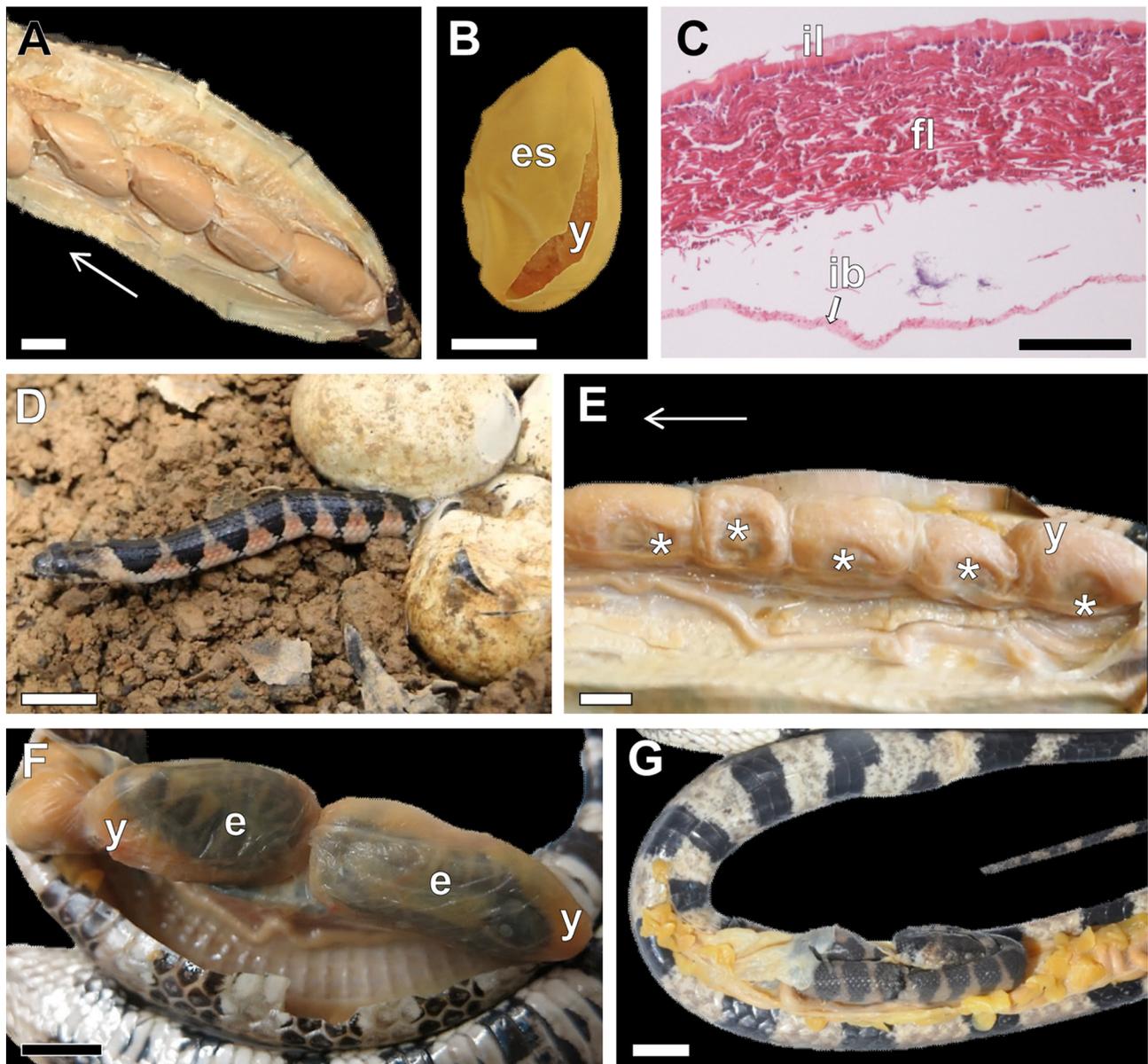


Fig. 3. Reproductive bimodality in *Helicops angulatus*. (A–D) Oviparity. (A) Gravid female of *Helicops angulatus* with oviductal eggs with thick, opaque, and leathery shells. (B) Thick, opaque, and leathery shells surrounding the yolk. (C) Histology (Haematoxylin-eosin) of the eggshell. (D) Hatchling *Helicops angulatus* emerging from thick and leathery egg collected in nature (Salvador, Bahia, Brazil). (E–G) Viviparity. (E) Pregnant female of *Helicops angulatus* with oviductal embryonated eggs surrounded by thin and transparent membranes (notice the residual yolk remaining). (F) Female of *Helicops angulatus* with a fully developed young with almost no residual yolk (embryo was removed from the uterus and shell membrane). Asterisks indicate developing embryos and arrows indicate head orientation. Abbreviations: e, embryo; es, eggshell; fl, fibrous layer; il, inorganic layer; ib, inner boundary; y, yolk. Scale bars: A–B, E–G = 1 cm; C = 100 μ m. Photos by E. Felix (D), M. C. Amorocho-Montaña (F), and M. C. Santos-Costa (G).

H. angulatus (Fig. 5C). Both possibilities required three evolutionary changes (Fig. 5C).

4. Discussion

Taken collectively, our original data and the literature review allowed us to identify the reproductive modes of 15 out of 21 species of Hydropsini. The genus *Hydrops* is oviparous, as well as *P. plicatilis*. Only in the genus *Helicops*, we found strong evidence for intrageneric variation in reproductive modes. In addition, our data confirm that one species, *H. angulatus*, has both reproductive modes, and indicate that such variation is geographic. Lastly, we found that viviparity evolved independently at least three times in

Helicops. These results confirm Hydropsini is an excellent model system for investigating the evolution of viviparity.

Because all specimens of *H. martii* and many of *H. triangularis* previously thought to be gravid (Albuquerque and Camargo, 2004) were in fact in secondary vitellogenesis, oviparity in these species was confirmed in this study (see also Boos, 2001 for a record of oviparity in *H. triangularis*). Of the two species of *Pseudoeryx*, we gathered data only for *P. plicatilis*, and we did not find support for the suggestion that this species is viviparous or reproductively bimodal (Abuys, 1986; Cunha and Nascimento, 1981). Available data indicate that *P. plicatilis* is oviparous. The previous conclusion that a specimen of *P. plicatilis* is simultaneously oviparous and viviparous (Cunha and Nascimento, 1981) was likely an evaluation mistake as we found this specimen had only thick-shelled

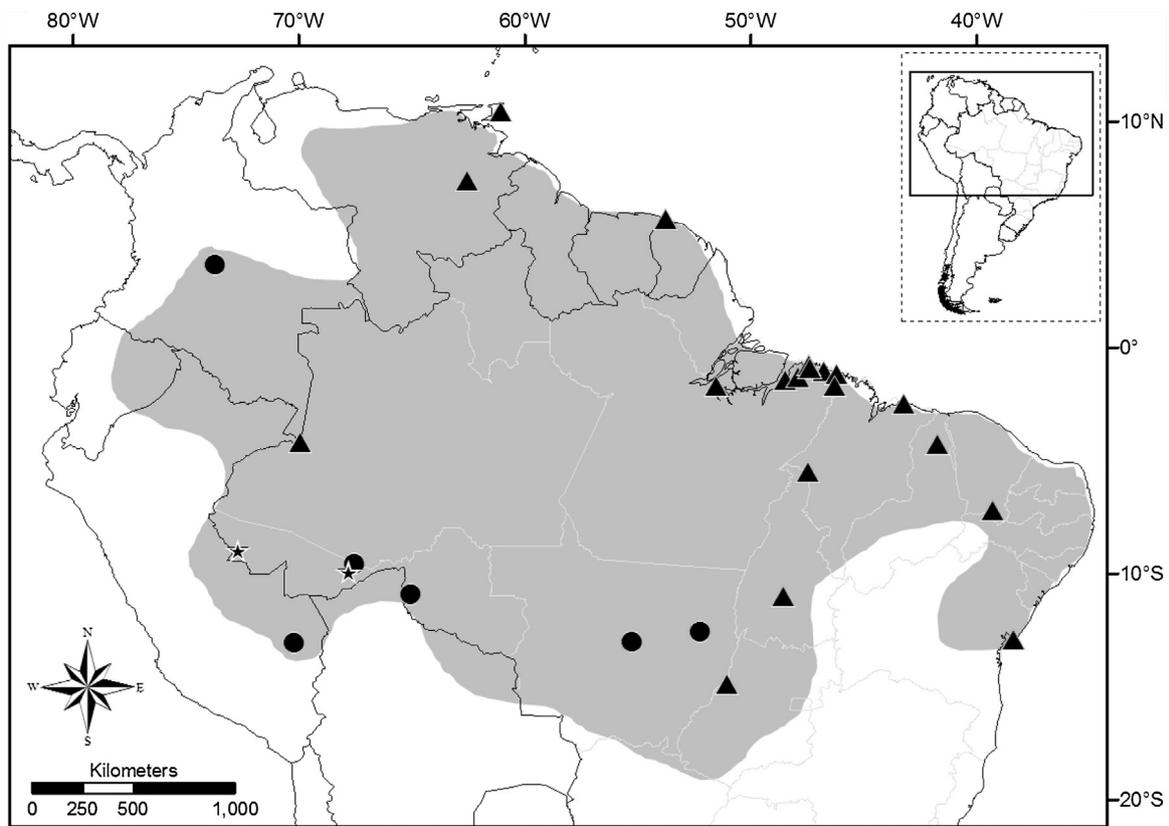


Fig. 4. Geographic distribution of the reproductive modes of *Helicops angulatus* across South America. Triangles indicate records of oviparity and circles indicate records of viviparity. Stars indicate probable records of viviparity. Grey shaded area indicates the estimated species occurrence based on the location of collection of specimens examined in this study.

eggs, indicating oviparity. Available evidence for other squamates strengthens the idea that their observation may have been misjudged. Despite at least 115 origins of viviparity in Squamata (Blackburn, 2015a), there is no record of a species exhibiting simultaneously shelled eggs and non-shelled eggs in the uterus (Shine, 1985). This is true even for the three lizard species in which reproductive bimodality is strongly documented (Heulin et al., 2005; Qualls et al., 1995; Smith and Shine, 1997; Stewart et al., 2010). In these reproductively bimodal lizards, oviparous and viviparous forms always occur allopatrically, as also observed for the bimodal *H. angulatus* (see below). An interesting trend that emerges from the data gathered for *P. plicatilis* is the apparent existence of parental care. Females of this species have been reported to remain with the eggs, guarding them during incubation (Hoge, 1980). Unlike other oviparous Hydropsini (*H. caesurus*, *H. triangularis*, and *H. angulatus*) in which all reported nests were always found unaccompanied of any female (Boos, 2001; Etchepare et al., 2012; Scrocchi et al., 2005; present study), eggs of *P. plicatilis* were found accompanied by mothers in the two available records (Frota and Yuki, 2005; present study). In one case, eggs were about to hatch (Frota and Yuki, 2005). Although we were not able to confirm whether females exhibit some sort of aggressive behaviour (Hoge, 1980), these data suggest that females of *P. plicatilis*, at least, remain with their eggs during incubation period.

We found evidence of both intrageneric and intraspecific variation in reproductive modes only within *Helicops*. Sixteen species of *Helicops* are recognized (Uetz and Hošek, 2015), and reproductive modes are now properly documented for 11 of them. The five species of *Helicops* (*H. apiaka*, *H. pastazae*, *H. petersi*, *H. tapajonicus*, and *H. yacu*) whose reproductive modes remain unknown have narrow geographic distribution and little is known about their

biology (Frota, 2005; Kawashita-Ribeiro et al., 2013; Rossman and Abe, 1979; Rossman, 1976; Schargel et al., 2007). Eight species (*H. carinicaudus*, *H. danieli*, *H. infrataeniatus*, *H. leopardinus*, *H. modestus*, *H. scalaris*, *H. polylepis*, and *H. trivittatus*) are viviparous and two species (*H. gomesi* and *H. hagmanni*) are oviparous. The reproductive modes of *H. danieli* and *H. gomesi* were confirmed for the first time in this study. Moreover, we present here the first documented evidence of parturition in *H. carinicaudus*.

Our results confirm that *H. angulatus* is reproductively bimodal and extend the number of records of viviparity in *H. angulatus* to seven and of oviparity to 27. We were not able to obtain parturitions in the laboratory and to find published data on parturition. Boos (2001) cites an unpublished manuscript reporting a live birth in the species, but provides no documentation on the phenomenon and its location. However, the existence of four females containing near-term and fully developed young, including one with almost no residual yolk and another with no residual yolk (Rossman, 1984; present study), is strong evidence of viviparity in the species. We identified all examined specimens of *H. angulatus* using taxonomic keys and a range of diagnostic characters, and all oviparous and viviparous individuals fit within the current diagnosis of the species (Amaral, 1921; Frota, 2005; Kawashita-Ribeiro et al., 2013; Rossman, 1973, 1970). Additionally, a literature survey on many diagnostic characters of *H. angulatus* across its distribution indicates no noticeable variation in colour pattern or pholidosis that might be correlated to the geographic variation in reproductive mode. Variation in ventral coloration occurs within populations and in different areas (e.g., Ford and Ford, 2002; Starace, 2013). Diagnostic pholidosis characters (e.g., number of dorsal scale rows in mid-body, ventral scales, and cephalic scales) seem to be similar between individuals from areas where oviparity occurs

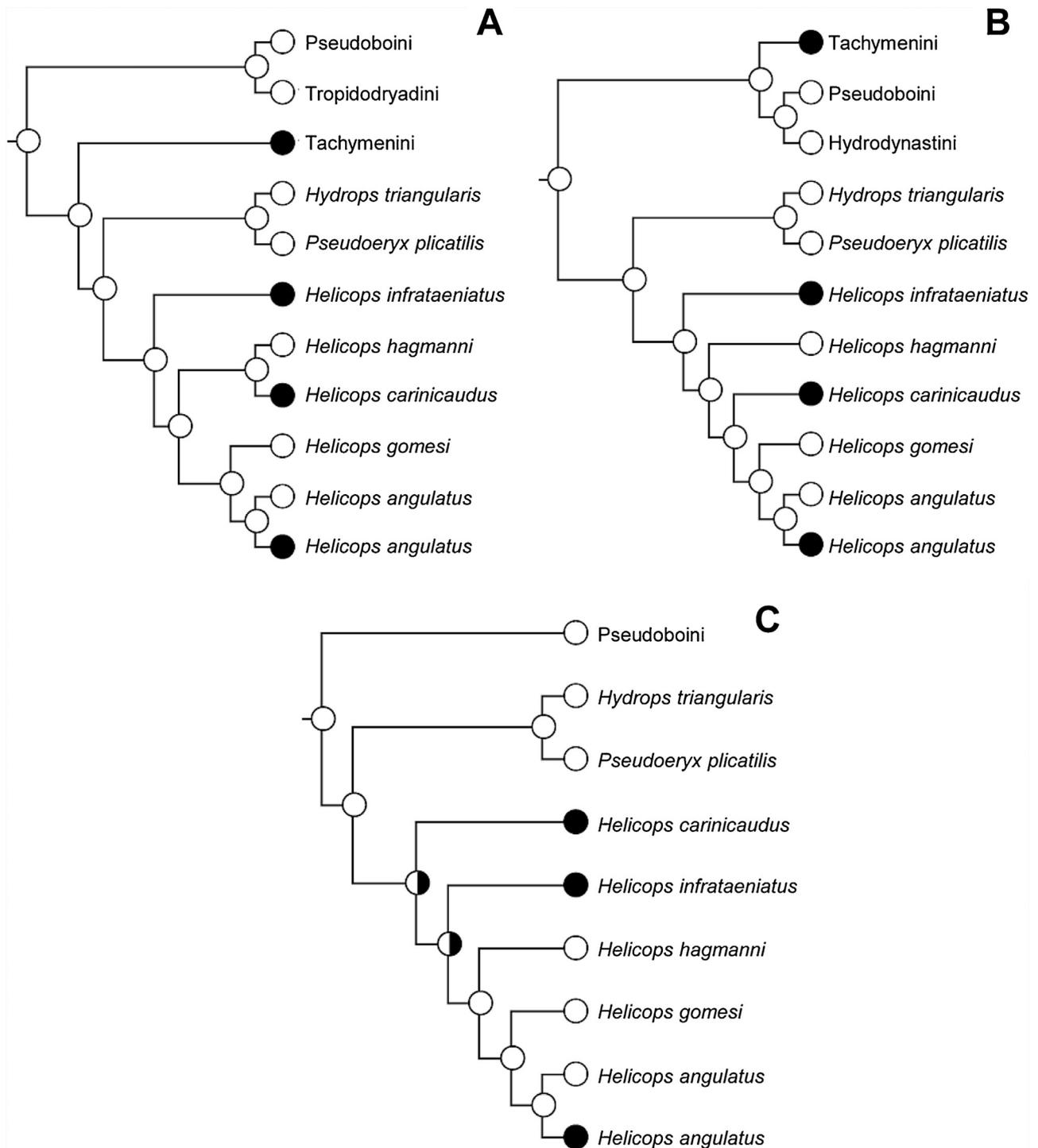


Fig. 5. Reconstruction of the evolution of reproductive modes in South American water snakes of the tribe Hydropsini. Reproductive modes were mapped onto phylogenetic trees by (A) Pyron et al. (2013) and (B–C) Grazziotin et al. (2012) using unordered parsimony. Open circles: oviparity. Solid circles: viviparity.

(e.g., Venezuela, French Guiana, north and north-eastern Brazil: Cunha and Nascimento, 1993; Gorzula and Señaris, 1998; Schmidt and Inger, 1951; Starace, 2013; H.B. Braz; pers. obs.) and individuals from areas where viviparity occurs (e.g., Peru, Colombia, central and eastern Brazil: Frota, 2005; Lehr, 2002; Pérez-Santos and Moreno, 1988; H.B. Braz, pers. obs.). Indeed, the pholidosis of *H. angulatus* has been generally reported to exhibit little variation within a population and among distant populations (Cunha and Nascimento, 1993), including areas in which reproductive mode is unknown (e.g., Suriname: Abuys, 1983; Ecuador: Pérez-Santos and Moreno,

1991). Further studies could evaluate the degree of genetic differentiation as well as of reproductive isolation between oviparous and viviparous populations of *H. angulatus*.

A number of squamate species are suggested to present both oviparity and viviparity (Blackburn, 2015a; Shine, 1985; Tinkle and Gibbons, 1977), but evidence is anecdotal for many of them. Well-documented cases are available only for three lizard species: the European lacertid *Z. vivipara* (Heulin, 1990; Heulin et al., 2005), and the Australian scincids *Lerista bougainvillii* (Qualls et al., 1995) and *Saiphos equalis* (Smith and Shine, 1997). Thus, *H. angula-*

tus is only the fourth confirmed case of reproductive bimodality in squamates, but the first for the New World and the first in snakes. Some authors have suggested that a possible reproductive mode difference in *H. angulatus* would not vary geographically, but that the species could be facultatively viviparous if, for example, suitable nest sites are unavailable (Duellman, 2005; Rossman, 1984, 1973). If so, we could expect to find oviparous and viviparous *H. angulatus* randomly distributed across species' occurrence area. Our results did not support this assumption. Similarly to the three reproductively bimodal lizards (Qualls et al., 1995; Smith and Shine, 1997; Surget-Groba et al., 2006), we found that the geographic distribution of reproductive modes in *H. angulatus* is allopatric. This pattern of variation does not support the hypothesis of facultative viviparity in an individual (Cunha and Nascimento, 1981; Duellman, 2005; Rossman, 1973).

In addition to the geographic variation in reproductive modes, we suggest that the variation in the time eggs are retained in the uterus (and then in embryonic stage at oviposition) also occurs in *H. angulatus*. This suggestion derives from observations on the duration of egg incubation in the species. Although the incubation period is strongly affected by temperature (reviewed in Birchard, 2004), it may provide some clues about the stage at oviposition. Long incubation periods suggest that eggs are laid with embryos at early stages, whereas short incubation periods suggest eggs are laid with embryos at later stages. Incubation duration in oviparous *H. angulatus* was 109 days in Venezuela (Gorzula and Señaris, 1998), 39–45 days in Trinidad (Ford and Ford, 2002), and 17–18 days in Colombia (Rossman, 1973). In the latter, well-developed embryos were readily visible through the shells at oviposition (Rossman, 1973). This suggests that this species may also have populations that are intermediate between typically oviparous and viviparous populations, as has been observed in two scincid lizards (Qualls et al., 1995; Smith and Shine, 1997). Interestingly, two other Hydropsini species seem to exhibit prolonged egg retention in uterus. Embryonic stages observed in oviductal eggs of *P. plicatilis* (DH stage 32) and *H. hagmanni* (DH stage 34) represent at least a minimum embryonic stage attainable in uterus, and both are past the usual stage at oviposition (i.e., DH stages 26–32) observed for the majority of oviparous squamates (Andrews and Mathies, 2000; Blackburn, 1995; Shine, 1983). This suggests that eggs of these two species are also likely to be laid at more advanced stages than typical oviparous squamates. Data on embryonic stages at oviposition are needed to test the hypothesis of prolonged egg retention in oviparous *Helicops* and *Pseudoeryx*.

Oviparity is the plesiomorphic condition of Hydropsini, and our results agree with early suggestions that viviparity evolved multiple times in *Helicops* (Blackburn, 1985; Shine, 1985). However, our analyses indicate three separate evolutionary transitions to viviparity in *Helicops* instead of two origins previously suggested (Blackburn, 1985; Shine, 1985). This difference is explained by the higher number of taxa studied here. The evolution of viviparity from oviparity is traditionally viewed as an irreversible phenomenon (Blackburn, 2015b; Shine, 1985). Although this traditional view has been challenged by phylogenetic and model-based analysis (e.g., de Fraipont et al., 1996; Pyron and Burbrink, 2014), biological evidence derived from several studies strongly supports the idea that the evolution of viviparity is not only more frequent but also less difficult than the re-evolution of oviparity (Blackburn, 2015b; Griffith et al., 2015). Therefore, we interpret the multiple (three) origins of viviparity in *Helicops* as a much more plausible scenario than the alternative hypothesis (though equally parsimonious) of one origin followed by a reversion back to oviparity, and re-evolution of viviparity, as recovered in one of our ancestral reconstructions. Inferences on the number of origins of viviparity made here obviously depend on the accuracy of the available phylogeny (Griffith et al., 2015).

It should be noted that the phylogenetic hypotheses for the tribe are still incomplete, and include one-third of the currently known Hydropsini species (Grazziotin et al., 2012; Pyron et al., 2013). Additionally, whereas the two known oviparous species of *Helicops* (*H. gomesi* and *H. hagmanni*) are represented in the phylogenies used in our analyses, only two (*H. carinicaudus* and *H. infrataeniatus*) of the eight exclusively viviparous species are included (Grazziotin et al., 2012; Pyron et al., 2013). However, two of the viviparous *Helicops* (*H. leopardinus* and *H. modestus*) not included in these phylogenies seem to be more closely related to the viviparous *H. infrataeniatus* (Camolez and Zaher, 2010; Nunes et al., 2005) and therefore viviparity in *H. leopardinus* and *H. modestus* would not be explained by additional origins. More comprehensive phylogenies will allow more refined estimates about the origins of viviparity in the tribe. Nevertheless, even if we adopt a conservative standpoint of all exclusively viviparous species of *Helicops* come to be recovered as a monophyletic group, viviparity is likely to have evolved multiples times (at least twice) in the genus, since we found that reproductive mode varies in *H. angulatus*, and its sister relationship with an oviparous species, *H. gomesi*, is strongly supported by different molecular phylogenies (Grazziotin et al., 2012; Pyron et al., 2013; Zaher et al., 2009).

This study has investigated the reproductive modes of Hydropsini and clarified several ambiguities present in the literature. The lack of a clear overview on the reproductive modes of Hydropsini has had several important implications. For example, the lack of clear knowledge has led some researchers to mistakenly consider (or infer) viviparity as a synapomorphy of the tribe or even of the genus *Helicops*. This includes studies on the phylogenetic systematics (Zaher et al., 2009) and analytical studies on the evolution of viviparity (Feldman et al., 2015; Pyron and Burbrink, 2014). By clarifying the reproductive modes of Hydropsini, we not only contribute to the biological knowledge about the group but also confirm that they are an excellent model system for studying the evolution of viviparity in squamates. Hydropsini and, more importantly, the genus *Helicops* and the bimodal *H. angulatus* are an excellent model for several reasons. First, they form a well-supported monophyletic group (Grazziotin et al., 2012; Pyron et al., 2013) in which intrageneric and intraspecific variation in reproductive mode is now well established. Such variation and the multiples origins of viviparity in *Helicops* provide ideal material for comparison of closely related oviparous and viviparous taxa. Second, representatives of the tribe and the bimodal genus *Helicops* (and *H. angulatus*) are widely distributed throughout tropical South America (Uetz and Hošek, 2015), thus providing an appropriate setting for testing hypotheses about the selective pressures favouring origins of viviparity. Third, oviparous and viviparous water snakes are relatively ecologically similar, at least in terms of habitat use (aquatic) and dietary habits, preying on fishes and anurans (Aguilar and Di-Bernardo, 2004; Albuquerque and Camargo, 2004; Ávila et al., 2006; Ford and Ford, 2002; Marques and Sazima, 2004), which minimizes environmental influences on reproductive traits. Lastly, some species are widespread and very abundant in some areas (e.g., França et al., 2012; Strüssmann and Sazima, 1993), which facilitates collection of a reasonable number of specimens for experimental studies.

Although snakes account for nearly one-third of the origins of viviparity in squamates (Blackburn, 1985; Shine, 1985), studies on the evolution of squamate viviparity have focused mostly on lizards (Blackburn and Stewart, 2011). However, in view of the multiple origins of viviparity in the group, we can expect a considerable diversity of ways on how species have reached viviparity (Blackburn, 2006, 2000). For example, reduction in eggshell thickness observed in viviparous populations of reproductively bimodal lizards has been associated with reduction in size of uterine shell glands in *Z. vivipara* (Heulin et al., 2005), but not in *S. equalis*

(Stewart et al., 2010). This suggests that the decrease in the number of shell glands may also play a role in the reduction of eggshell thickness (Guillette, 1993; Stewart et al., 2010). As another example, despite the strong correlation between viviparity and cold climates (Feldman et al., 2015; Shine, 1985; Watson et al., 2014), several origins of viviparity in squamates are associated with tropical taxa (Shine, 1985), and thus cannot be explained by low temperatures. Therefore, the South American water snakes are of great value to advance our understanding on the ways viviparity has evolved in squamates.

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Appendices A–C. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jcz.2016.04.003>.

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