

Morphology of the nucho-dorsal glands and related defensive displays in three species of Asian natricine snakes

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Keywords

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Abstract

Many animals are equipped with specialized defensive systems that function in a coordinated manner involving morphological structure, physiological processes and behaviour. Nucho-dorsal glands, unusual organs known in a few Asian natricine snakes, are believed to function in avoidance of predation, based on the defensive function of similar organs in a related Japanese species that sequesters prey toxins and stores them in the glands. We examined the arrangement of the nucho-dorsal glands of *Rhabdophis nuchalis*, *R. pentasupralabialis* and *Macropisthodon plumbicolor* and tested behavioural responses to tapping stimulation to investigate the spatial distribution of glands on the body and related defensive displays respectively. We confirmed the presence of glands that extend from the neck along the length of the body in all three species. The spatial arrangement of the glands was similar between the two *Rhabdophis* species, but it differed substantially in *M. plumbicolor*. In *M. plumbicolor*, there were two uninterrupted rows of glands throughout the full length of the body, whereas in the two *Rhabdophis* species, the position and size of the glands differed between the neck and trunk regions, with the two series separated by a spatial gap. In spite of these structural differences, *M. plumbicolor* and *R. pentasupralabialis* exhibited a similar defensive display, which we refer to as body lift, in response to a tapping stimulus on the body. Our study shows detailed morphological features of the nucho-dorsal glands and a novel display that are consistent with the presumed predator deterrent function of the glands, which have evolved as a unique defensive system in this lineage of snakes.

Introduction

Animals have evolved a variety of defensive mechanisms to evade predation. Some animals exhibit rapid escape, aided by morphological and physiological abilities adapted for agile movement (Caro, 2005), whereas others remain motionless to conceal their presence, relying on cryptic coloration (Stevens & Merilaita, 2011). Many animals have evolved novel defensive tactics that often involve specialized organs, such as the chemical spray of bombardier beetles (Eisner, 2003) and autotomy of a conspicuously coloured tail in some lizards (Ruxton, Sherratt & Speed, 2004). These specialized defences often involve a suite of correlated morphological, physiological and behavioural traits and would be used at specific stages of encounters with a predator. Investigation of such integrated defensive systems can provide clues to understand the evolutionary mechanisms underlying the diversification of those traits.

Several Asian snake species have a unique defensive system that relies on unusual organs, called nuchal glands (see Mori *et al.*, 2012 for review). The nuchal glands, which consist of a series of paired glands embedded under the skin of the neck region, were first described in a Japanese natricine snake, *Rhabdophis tigrinus* (Nakamura, 1935). He speculated that the nuchal glands have a defensive function because the fluid contained in the glands causes irritation to the mucous membrane of eyes. In the 1980s chemical compounds in the nuchal glands of *R. tigrinus* were identified as bufadienolides, a group of cardiotonic steroids typically found in the skin secretions of toads (Akizawa *et al.*, 1985; Azuma *et al.*, 1986). Recent studies have revealed that *R. tigrinus* sequesters bufadienolides from the skin toxins of toads consumed as prey (Hutchinson *et al.*, 2007, 2012). Fukada (1961) reported a peculiar body display of *R. tigrinus* that was considered to be associated with the presence of the nuchal glands. Extensive behavioural studies later demonstrated that *R. tigrinus* exhibits several unique

antipredator postures that enhance the effectiveness of the defensive glands (Mori, Layne & Burghardt, 1996; Mori & Burghardt, 2008). These lines of evidence indicate that the nuchal glands have evolved as a unique defensive system that consists of correlated morphological, physiological, ecological and behavioural characters (Mori *et al.*, 2012).

Smith (1938) reported similar organs from nine additional species of Asian natricine snakes. He notes that three species – *R. nigrocinctus*, *R. nuchalis* and *Macropisthodon plumbicolor* – possess a series of paired glands not only in the neck region but also along the entire length of the body. He collectively referred to these organs as nucho-dorsal glands. An anecdotal note, predating the discovery of the glands themselves, suggested the presence of toxic substances in the skin of *M. plumbicolor* (Fletcher, 1908). However, in contrast to the extensive studies on the nuchal glands of *R. tigrinus*, no attention has been paid to the nucho-dorsal glands of these species since the brief morphological description by Smith (1938).

Here, we describe the morphology of the nucho-dorsal glands and a defensive behaviour that may be related to the glands in three natricine species, *R. nuchalis*, *R. pentasupralabialis* and *M. plumbicolor*. *Rhabdophis pentasupralabialis* is a species that was originally described as a subspecies of *R. nuchalis* (Jiang & Zhao, 1983) and was subsequently elevated to a full species by Zhao (1995). Although none of these authors mentioned the presence of the nucho-dorsal glands in *R. pentasupralabialis*, we assumed their presence because the species was originally treated as a subspecies of *R. nuchalis*. We dissected specimens of all three species to determine the presence and arrangement of the glands, and we conducted behavioural tests to identify potential gland-related defensive behaviours, one of which has not been reported previously

from related species. The goal of our study was to clarify the morphological and behavioural features of snakes possessing nucho-dorsal glands and provide clues to understand the evolution of this peculiar defensive system in snakes.

Materials and methods

Morphological examination

For the morphological analysis, we examined ten *Rhabdophis nuchalis*, 10 *R. pentasupralabialis* and three *Macropisthodon plumbicolor*, collected from various localities in China and Sri Lanka (Table 1). Species of *Rhabdophis* were identified based on the numbers of infralabials and supralabials, following Zhao (1995). To ascertain the presence of fluid in the glands and to analyse its chemical components, dorsal regions of the neck and trunk that were presumed to have the glands were squeezed in several individuals with fingers or forceps and the fluid was collected. Results of chemical analyses will be presented elsewhere. Morphological characters were examined after euthanasia by injection of sodium pentobarbital. Snout-vent length (SVL) and tail length were measured to the nearest 1 mm. The number of ventral scales was counted following Dowling's (1951) method for use as landmarks for positions of glands on body. The numbers of subcaudal pairs and scale rows at midbody were also counted.

We carefully peeled the dorsal skin from each specimen from the posterior edge of the parietal scales to the anterior region of the tail. We recorded colour of the glands and counted the number of pairs of glands in the neck and trunk regions separately (see Mori *et al.* 2016 for a brief notation on the definition of neck of snakes). If a gap was present between

Table 1 Localities and sample sizes of snakes used in morphological examination (ME) and behavioural experiments 1 and 2 (BE 1 and BE 2). All individuals of *Rhabdophis* species were collected in China, and all *Macropisthodon plumbicolor* were collected in Sri Lanka. Numerals in parentheses in the column of ME are the numbers of snakes used for the detailed measurement of gland size. Numerals in parentheses in the columns of BE 1 and BE 2 are the numbers of snakes used for the later morphological examination

Species	Sex	Locality	Sample size		
			ME	BE 1	BE 2
<i>Rhabdophis nuchalis</i>	Male	Longnan, Gansu	1	1 (1)	0
		Baoji, Shaanxi	3 (1)	3 (2)	0
		Chengdu, Sichuan	1 (1)	1 (1)	0
		Jiguanshan, Sichuan	2 (1)	4 (2)	0
	Female	Panzhihua, Sichuan	1 (1)	0	0
		Baoji, Shaanxi	1 (1)	0	0
		Jiguanshan, Sichuan	0	3	0
<i>Rhabdophis pentasupralabialis</i>	Male	Panzhihua, Sichuan	1 (1)	3	0
		Jiulong, Sichuan	4 (2)	5 (4)	4 (3)
	Female	Panzhihua, Sichuan	2 (1)	2 (1)	0
		Jiulong, Sichuan	2 (2)	2 (2)	2 (2)
		Miyi, Sichuan	1	1 (1)	0
		Panzhihua, Sichuan	1 (1)	2 (1)	0
	Hatchling ^a	Jiulong, Sichuan	0	19	6
<i>Macropisthodon plumbicolor</i>	Male	Kandalama, Dambulla	1	2 (1)	1
	Female	Kandalama, Dambulla	2 (1)	3 (2)	1

^aNine males and 10 females were used for BE 1, and three each of males and females were used for BE 2.

the neck and trunk series, we measured the distance between the posterior edge of the posteriormost gland in the neck region and the anterior edge of the anteriormost gland in the trunk region (i.e. the diastema length between neck and trunk glands). The size of several glands was measured for six *R. nuchalis*, six *R. pentasupralabialis* and one *M. plumbicolor* (Table 1). In the *Rhabdophis* species, maximum length (measured along the major body axis) and maximum width (measured perpendicular to the major body axis) of a left side gland were measured to the nearest 0.05 mm using callipers for all glands in the neck region. These measurements were made for the first, last and every 25th pairs, beginning with the first pair in the trunk region. The relative anteroposterior position of these glands was determined by counting the number of ventral scales from the anteriormost scale. Because *M. plumbicolor* showed no morphological differences between the glands of the neck and trunk regions nor a diastema between them (see Results), measurements were made for every 10th pair of glands, beginning with the anteriormost pair. For all species the position of the glands in relation to body scale rows was recorded as follows: mid-dorsal scale row, along the vertebral line, was defined as zero, and the number of each scale row was counted in the ventrolateral direction until reaching the scale under which the glands were positioned.

Antipredator response test

To elicit antipredator responses by the snakes, we conducted two behavioural experiments. For experiment 1, we used 15 wild-caught *R. nuchalis*, 12 wild-caught and 19 laboratory-hatched *R. pentasupralabialis* and five wild-caught *M. plumbicolor*, of which 18 snakes were used for the above morphological examination afterward (Table 1). For experiment 2, we used six each of wild-caught and laboratory-hatched *R. pentasupralabialis* and two wild-caught *M. plumbicolor*. Each snake was used for only one trial except for six wild-caught *R. pentasupralabialis* and two *M. plumbicolor* that were used in both experiments. Snakes were collected in the field (Table 1), except for hatchlings that were originally collected as eggs (see below). Snakes were individually housed in various sizes of transparent plastic cages (c. 200 × 300 × 200 mm to 300 × 600 × 350 mm), with a paper substrate and water dish, at air temperatures of 21–30°C. Snakes were kept for 3–35 days until experiment 1. Mean SVL of wild-caught snakes was 449 mm in *R. nuchalis* (range, 312–523 mm), 323 mm in *R. pentasupralabialis* (range, 140–498 mm) and 444 mm in *M. plumbicolor* (range, 182–560 mm). To examine innate antipredator responses, we collected eggs of *R. pentasupralabialis* from Jiulong, Sichuan, China on 6 August 2014. These eggs were kept in a plastic cage in the laboratory at 25°C. They were put on moist soil substrate and were covered with damp cloth. As soon as we observed hatchling snakes, we moved them to individual, transparent plastic cages (c. 135 × 80 × 105 mm) with paper substrate and a water dish. Hatchlings were used for experiment 1 at the age of 10–16 days. Mean SVL was 156 mm (range: 140–165 mm). No

food was offered to hatchlings until all experiments were finished.

In experiment 1, we used a standard method developed for assessing levels and repertoires of antipredator reactions associated with the nuchal glands (Mori & Burghardt, 2000, 2008). One snake was gently removed from its cage and introduced into an arena (c. 190 × 290 × 255 mm, 300 × 500 × 330 mm or 430 × 600 × 320 mm, depending on the size of snakes) at air temperatures of 21–28°C. After leaving the snake undisturbed for 3 min, the antipredator test began. Each trial lasted 1 min, during which the anterior and posterior parts of the snake's body (excluding head and tail) were alternately pinned every 3 sec for a total of 20 stimuli. Snakes were gently pinned with a long metal snake hook (39–70 cm). This treatment simulates initial contact by a mammalian or avian predator that tries to subdue the snake with a foreleg or claw (see Tanaka & Mori, 2000 for natural predators of *R. tigrinus*). All trials were videotaped (30 frames/s) using a digital video camera (Sony, Handycam PJ760V).

To compare behavioural responses with those of the previous studies of natricine snakes (Mori *et al.*, 1996; Mori & Burghardt, 2000, 2008), we focused on the following four behavioural responses. Neck arch: the snake slightly raises the head and strongly bends the anterior part of the neck ventrally, so that the snout is pointing down and is in contact with the substrate. Neck butt: the snake exhibits erratic movements, with the head and the neck raised off the substrate; with each movement in response to the stimulus the snake swings the head backward so that the dorsal part of the neck is butted against the stimulus. Neck flattening: the snake flattens the anteriormost part of the body (approximately the same length as the head), just posterior to the head, dorsoventrally. Body flattening: the snake flattens the entire body, from behind the neck region to the cloaca, dorsoventrally. Analysis of the video recordings was conducted to quantify these responses. If a given type of behaviour was observed immediately after stimulus contact, we scored it as 1, otherwise as 0. Thus, maximum and minimum scores for each behaviour for each trial would be 20 and 0 respectively.

While conducting experiment 1, we noticed another putative antipredator response which we had not observed before. When we pinned a part of the body, the snake raised that part off the substrate and bent the body dorsally, making a small, shallow arch with the body. We refer to that behaviour as body lift. To systematically evaluate the occurrence of this novel display in relation to the presence of the nucho-dorsal glands, we designed a new experimental method. We introduced a snake into a wooden arena (635 × 520 × 90 mm for *R. pentasupralabialis* and 750 × 350 × 100 mm for *M. plumbicolor*) and left it undisturbed for three minutes. In each trial, we stimulated a snake by tapping its dorsal side with the tip of the right index finger of an experimenter. Each trial consisted of three sessions, each of which was comprised of five units. In each unit within a session, we tapped the dorsal side of either neck (N), anterior trunk (A), middle trunk (M), posterior trunk (P) or tail (T) continuously for 5 sec, with a frequency of

approximately four taps per sec. Accordingly, each snake had three units each for N, A, M, P and T (1 unit per session \times 3 sessions). The interval between units within a session was 1 sec, and that between sessions was 10 sec. The order of the tapping part is N-A-M-P-T for one session, T-P-M-A-N for another session and either M-P-T-N-A, M-A-N-T-P or T-N-A-M-P for the remaining one session for each snake. The occurrence of body lift in response to the tapping stimulus was easily observed and was felt through the tapping finger because the raising movement of the body was obvious and the tension of the muscles was quite strong. We also recorded all trials using a digital video camera (Sony, Handycam PJ760V) for later confirmation. We recorded the occurrence of body lift for each unit and compared the frequency of body lift among the five body parts. We considered that body lift in response to the stimulus on the neck is behaviourally equivalent to neck arch. Air temperature during the experiment was 21.8 and 27.3°C for *R. pentasupralabialis* and *M. plumbicolor* respectively. In adult *R. pentasupralabialis*, experiment 2 was conducted on the day following experiment 1. Hatchlings were tested at the age of 10–12 days. Because of the small sample size ($n = 2$), we conducted experiment 2 twice for each *M. plumbicolor* (six

sessions in total for each snake), with intervals of approximately 20 min and approximately 1 h after experiment 1.

Results

Morphological examination

When we pinched the glands in the neck region of *R. nuchalis* and *R. pentasupralabialis* with forceps, fluid squirted out (Fig. 1a, b), whereas when we pinched those in the trunk region, fluid only oozed out (Fig. 1c). Colours of these fluids were similar, showing milky yellow or yellowish orange. We did not attempt to squirt fluid in *M. plumbicolor* to collect as much fluid as possible from the limited number of individuals. The fluid was yellowish.

We confirmed the presence of two longitudinal rows of the glands that extended anteroposteriorly throughout the full length of the body in all three species. No glands were present on the tail in any species. Colour of the glands of *Rhabdophis* was milky yellow to yellow or yellowish brown, and that of *M. plumbicolor* was milky yellow. The spatial arrangement and morphological characteristics were different between the *Rhabdophis* species and *M. plumbicolor*.

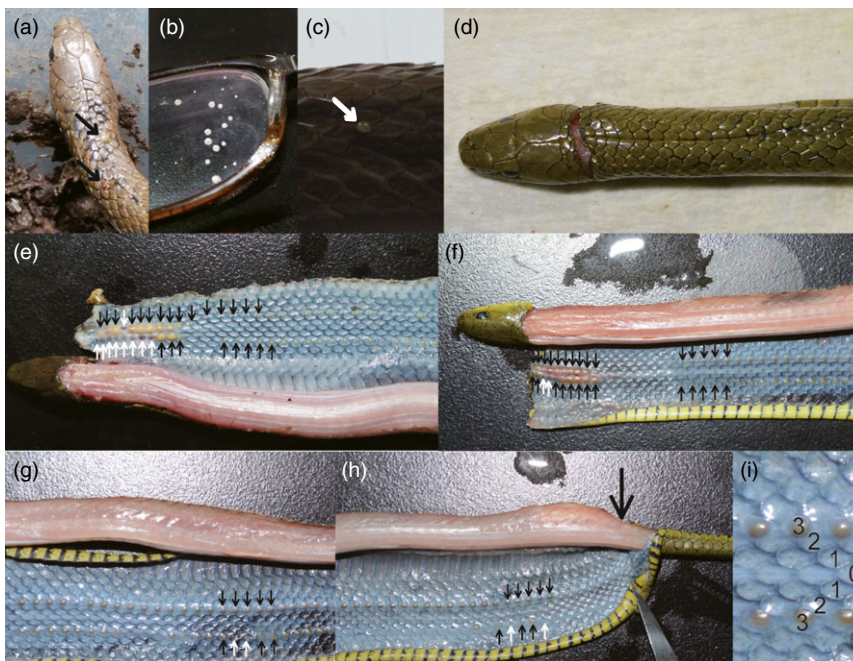


Figure 1 Nucho-dorsal glands of two species of *Rhabdophis*. (a) *R. pentasupralabialis* with fluid droplets (indicated by black arrows) released from its neck glands. (b) Droplets of neck gland fluid (white dots) squirted onto the lens of the eyeglasses worn by the person who pinched the gland with forceps. (c) Dorsal trunk of *R. pentasupralabialis* showing a fluid droplet (indicated by white arrow) released from the gland. (d) Neck and anterior trunk of *R. pentasupralabialis*, showing the absence of the mid-dorsal scale row on the neck due to the presence of a groove. (e and f) Dissection of neck and anterior trunk of *R. nuchalis* (e) and *R. pentasupralabialis* (f), showing two rows of nucho-dorsal glands. All neck glands and the first five trunk glands in each row are indicated by arrows. White arrows indicate glands that were broken to express the fluid. (g and h) Dissection of middle (g) and posterior (h) regions of the body of *R. pentasupralabialis*, showing two rows of trunk glands. Five pairs of glands are indicated by arrows as examples. White arrows indicate glands that were broken to express the fluid. The large black arrow in (h) indicates the cloaca. (i) Magnified view of trunk glands showing the position in association with the dorsal scale rows. Mid-dorsal scale row was defined as 0, and the number of each scale row was counted in the ventrolateral direction (1 to 3).

Rhabdophis

In both species, an obvious diastema between the neck and trunk glands was present (Fig. 1e, f), although the length of the diastema was highly variable among individuals (7.2–169 mm; Table 2). This variation was partially attributable to the difference in SVL, and population (rather than species) was also an obvious source of this variation, especially in *R. nuchalis* (Fig. 2a). The spatial arrangement of the glands was basically symmetrical, but a few individuals showed asymmetry in the length of the diastema, reflecting different numbers of trunk glands on the right and left sides (Fig. 1e). The neck and trunk glands can be distinguished easily because of the clear difference in size and arrangement of the two series of glands (Fig. 1). The number of neck glands varied from 7 to 11 (Table 2), but there were no clear differences either between sexes or between the species (species, Median test, $z = 0$, $P > 0.999$; no statistical test was made for sex because of small sample size). The number of trunk glands ranged from 97 to 150 (Table 2), which partially reflected the difference in the number of ventral scales, but also reflected the condition in individuals from Baoji, which deviated from this correlation (Fig. 2b). No clear differences in the number of trunk glands were observed between sexes, but a statistical test was precluded by the small sample size. *Rhabdophis pentasupralabialis* had a significantly larger number of trunk glands than *R. nuchalis* (Median test, $z = 2.61$, $P = 0.009$).

In the neck region, the two rows of glands started near the posterior edge of the parietal plates (which corresponded to the first or second ventral scale) and extended to the level of the 6th to 10th ventral scales in both species (Fig. 1e, f). Each row lay alongside the mid-dorsal scale row of the neck (Fig. 1d–f), and within each row the glands were in contact with the anteroposteriorly adjacent glands. Mean \pm SE of length and width of these glands were 1.38 ± 0.42 (range, 0.6–2.7) mm and 1.21 ± 0.35 (range, 0.65–2.1) mm respectively. Most of these glands were spherical in shape, but the first and last one or two glands were relatively small and more oval (Figs 3 and 4). One to two glands in each row were associated with each ventral scale.

The anteriormost trunk glands were situated at the level of the 10th to 58th ventral scales. The last glands were always located at the level of the last ventral scale or the anal plate. The two rows were located on the third body scale row throughout the body (i.e. there were five scale rows between the gland rows), but the ventrolateral position in relation to the third scale varied irregularly: some glands were positioned beneath the centre of the scale, whereas others were positioned below the lateral edge of the scale (Fig. 1g–i). Each gland in a row was separated from the anteroposteriorly adjacent glands (Fig. 1e–i). Mean \pm SE of length and width of the trunk glands were 1.24 ± 0.38 (range, 0.3–2.0) mm and 0.98 ± 0.30 (range, 0.4–1.6) mm respectively. The size of the glands varied along the body, and particularly in *R. pentasupralabialis* the glands in the midbody region were larger than those in the anterior and posterior regions (Figs 3 and 4). Approximately, one gland in each row was associated with each ventral scale.

Table 2 Morphometrics of *Rhabdophis nuchalis*, *R. pentasupralabialis* and *Macropisthodon plumbicolor*. Sample sizes (below sex) and ranges are shown in parentheses. Mean \pm SE are shown. Because there was no apparent distinction between the neck and trunk series of glands in *M. plumbicolor*, the total number of nucho-dorsal glands is shown as trunk glands

Species	Sex	SVL (mm)	TL (mm)	VEN	SC	DSR	NG	TG	DNT (mm)
<i>Rhabdophis nuchalis</i>	Male (8)	444.5 \pm 28.8 (282–523)	112.5 \pm 10.3 (49–134)	157.8 \pm 2.7 (144–169)	54.3 \pm 3.3 (35–61)	15 \pm 0 (15)	10.0 \pm 0.5 (7–11)	129.3 \pm 4.6 (108–150)	61.3 \pm 11.8 (11.2–107.4)
	Female (2)	332, 468	83, 119	143, 163	52, 57	15, 15	11, 7	131, 97	9, 169
<i>Rhabdophis pentasupralabialis</i>	Male (6)	351.0 \pm 37.8 (238–469)	94.0 \pm 8.6 (68–116)	153.5 \pm 1.2 (148–156)	58.0 \pm 2.7 (46–64)	15 \pm 0 (15)	10.7 \pm 0.2 (10–11)	135.3 \pm 2.8 (124–144)	20.4 \pm 5.3 (7.2–35)
	Female (4)	370.3 \pm 16.3 (336–405)	78.3 \pm 7.7 (60–97)	144.8 \pm 4.2 (137–155)	44.0 \pm 3.6 (34–51)	15 \pm 0 (15)	10.5 \pm 0.3 (10–11)	130.8 \pm 3.8 (120–138)	15.1 \pm 3.2 (7.3–22.4)
<i>Macropisthodon plumbicolor</i>	Male (1)	491	78	142	40	21	na	259	na
	Female (2)	182, 556	30, 83	147, 147	40, 39	21, 21	na	286, 279	na

SVL, snout-vent length; TL, tail length; VEN, number of ventrals; SC, number of subcaudals; DSR, dorsal scale rows at midbody; NG, number of neck glands; TG, number of trunk glands; DNT, distance between neck and trunk glands (i.e. diastema length).

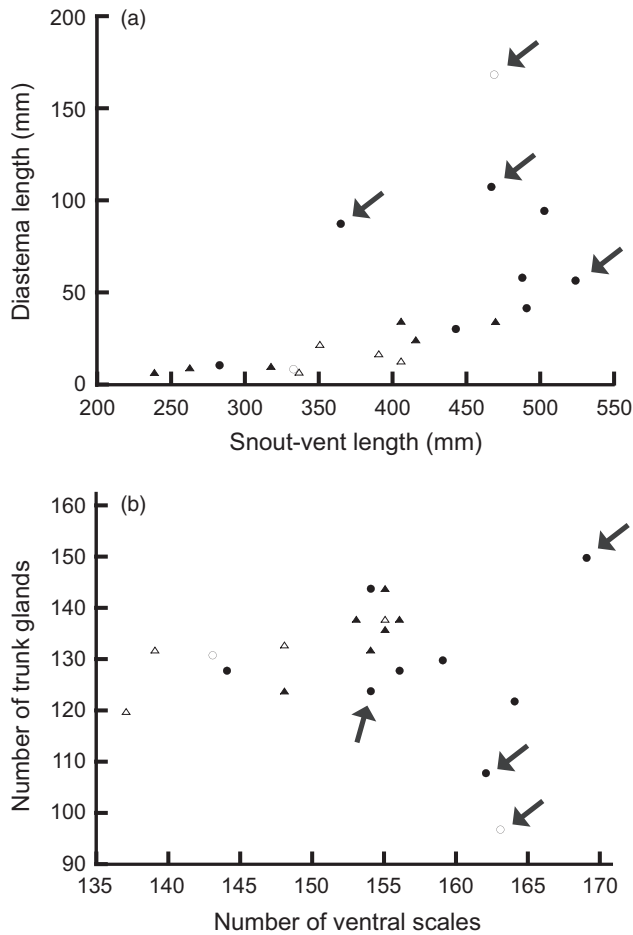


Figure 2 Relationships between snout-vent length and length of diastema between the neck and trunk series of glands (a) and between the number of ventral scales and the number of trunk glands (b) in two species of *Rhabdophis*. Circles and triangles indicate *R. nuchalis* and *R. pentasupralabialis* respectively. Closed and open symbols indicate males and females respectively. Arrows indicate snakes collected from Baoji.

Macropisthodon

Two rows of glands extended continuously from the posterior edge of the parietal plates, at the level of the first ventral scale, to the level of the anal plate in *M. plumbicolor*, without an obvious diastema between the neck and trunk regions (Fig. 5). The number of gland pairs was 259–286 (Table 2). Generally two glands in each row corresponded to each ventral scale. These glands were located on the second rows of body scales (i.e. three scale rows between the gland rows) for the first 40–50 glands and on the third rows more posteriorly.

In the anterior body region each gland in a row was well developed and closely apposed to anteriorposteriorly adjacent glands (Fig. 5b). In the middle and posterior body regions, each gland in a row was clearly separated from adjacent glands (Fig. 5c–f). Mean \pm SE of length and width of the glands were 1.81 ± 0.27 (range, 1.2–2.3) mm and 1.14 ± 0.29 (range,

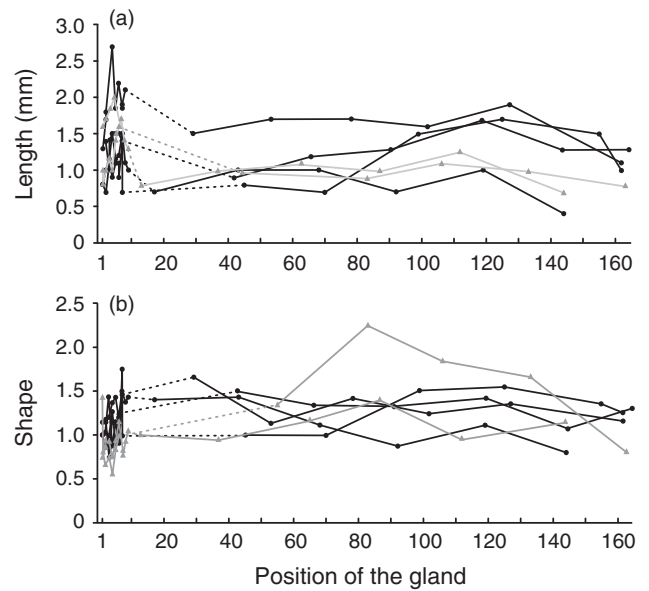


Figure 3 Changes in length (a) and shape (b: length/width) of glands in the left row along the body axis in *R. nuchalis*. Position of the gland corresponds to the number of ventral scales from the anteriormost one. Black and grey lines indicate males and females respectively. Broken lines indicate the diastema between the neck and trunk glands.

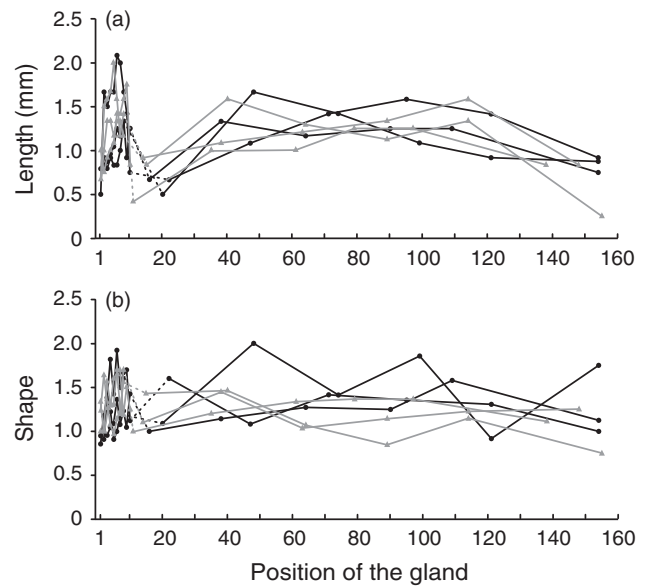


Figure 4 Changes in length (a) and shape (b: length/width) of glands in the left row along the body axis in *R. pentasupralabialis*. Position of the gland corresponds to the number of ventral scales from the anteriormost one. Black and grey lines indicate males and females respectively. Broken lines indicate the diastema between the neck and trunk glands.

0.7–2.0) mm respectively. The glands tended to be smaller in the posterior region, but those in the anteriormost region were not necessarily largest. Within a row, successive glands

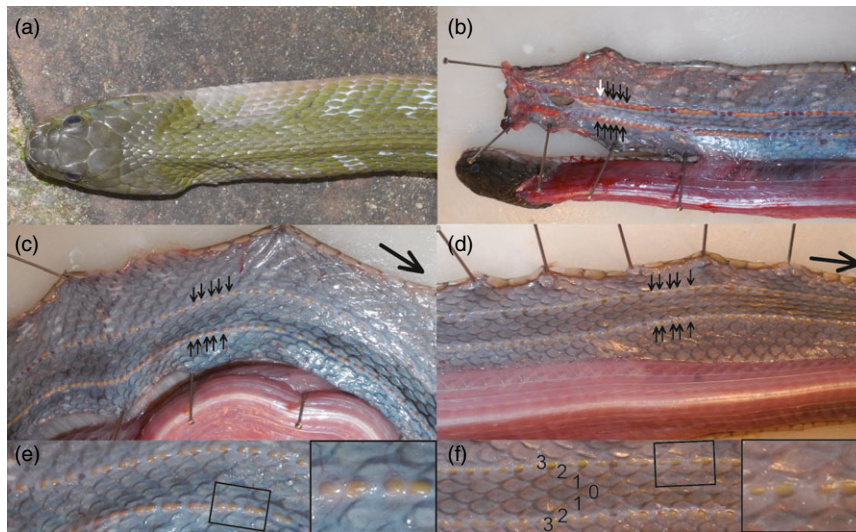


Figure 5 Nucho-dorsal glands of *Macropisthodon plumbicolor*. (a) Neck and anterior regions of the body flattened in a defensive display. (b–d) Dissection of neck and anterior (b), middle (c) and posterior regions of the body (d). Small black arrows indicate glands. White arrow in (b) indicates a gland that was broken to express the fluid. Large black arrows in (c) and (d) indicate the direction to the tail. (e and f) Magnified view of glands in the middle (e) and posterior regions of the body (f). Note the difference in size of alternate glands in the right panels. Numerals in (f) indicate the count of the scale rows.

alternated between larger and smaller sizes, particularly in the anterior and posterior body regions (Fig. 6).

Antipredator response test

In experiment 1, only *R. pentasupralabialis* (one wild-caught and four laboratory hatched individuals) showed neck arch. However, we observed neck arch display by at least one

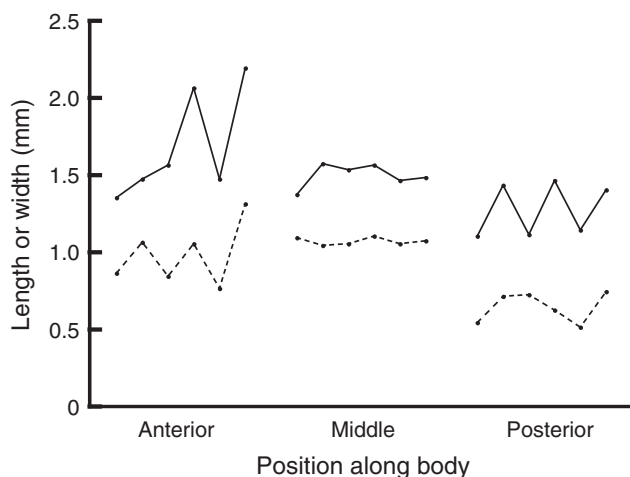


Figure 6 Size change of six consecutive nucho-dorsal glands in the left row in *Macropisthodon plumbicolor*, showing the change in length (solid lines) and width (broken lines) of successive glands. Anterior: measured 5 cm from the posterior edge of the head. Middle: measured at midbody. Posterior: measured 5 cm anterior to the cloaca.

R. nuchalis and one *M. plumbicolor* while we were handling them after the experiment. None of the three species showed neck butt display. Neck flattening was observed only in one *R. nuchalis* and one *M. plumbicolor*. Body flattening was observed in one wild-caught snake of each species. No hatching *R. pentasupralabialis* showed neck flattening or body flattening.

In experiment 2, we pooled the data of wild-caught and laboratory hatched *R. pentasupralabialis* because there were no apparent differences in the response pattern between them. When they were stimulated on their neck, all 12 snakes showed body lift (neck arch) in all the three sessions (Fig. 7). Body lift was never observed when we stimulated the tail. Among three trunk parts, body lift was most frequently elicited when we stimulated the anterior part, and was least frequently observed when we stimulated the posterior part.

In *M. plumbicolor*, both individuals showed body lift (neck arch) in response to the stimulus to the neck (in two and three sessions each). One individual showed body lift in four, six and four sessions when its anterior, middle and posterior trunk regions were stimulated respectively, whereas the other individual performed body lift only once when its mid-trunk was stimulated. No body lift was observed when the tail was stimulated.

Discussion

Since the original description by Smith (1938), which is based on one or two specimens of each species, our study is the first confirmation of the occurrence of nucho-dorsal glands in snakes, except for a recent report of similar glands in *R. adleri* (Mori et al., 2016). We also presented the first quantitative

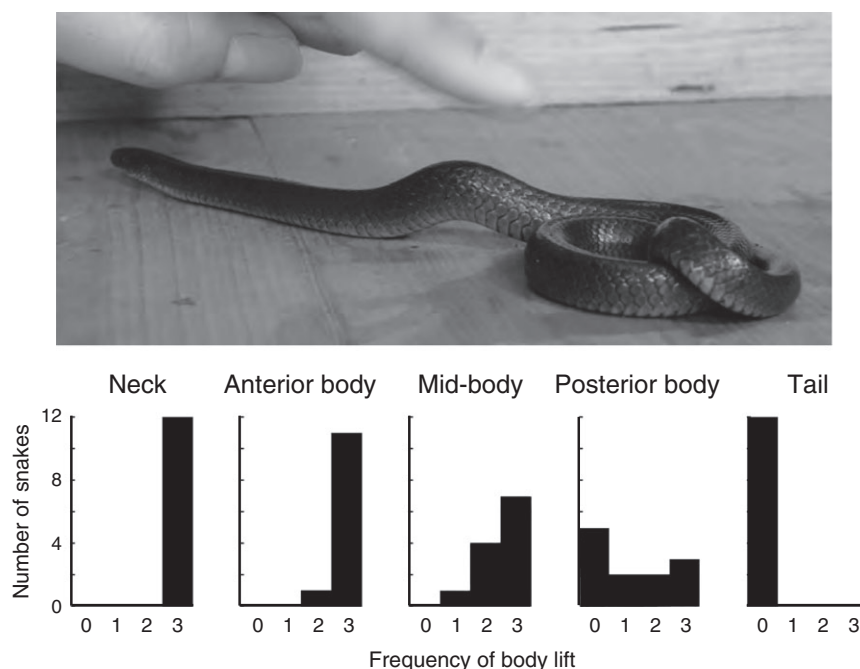


Figure 7 Body lift display of *R. pentasupralabialis*, elicited by tapping with a finger (upper photo), and the relationships between body part tapped and the frequency of body lift in experiment 2 (lower graphs).

description of the size and spatial arrangement of the nucho-dorsal glands. Furthermore, we showed the first confirmation of these glands in *R. pentasupralabialis*. Our observations essentially agree to those by Smith (1938), except for the following points. In the two species of *Rhabdophis*, we found an obvious diastema between the neck and trunk glands, whereas Smith (1938) did not mention the presence of such a gap (also see Fig. 1 in Smith, 1938). In *M. plumbicolor*, Smith (1938) mentioned that the smaller glands in the alternating pairs of each series eventually disappear in the posterior region of the body, whereas we did not observe that loss; the alternating small-large pairs of glands in each row continued to the posterior end of the trunk. It remains unknown whether these differences reflect individual or geographic variation or whether they are attributable to other factors, such as ontogenetic change. High levels of individual and geographic variation in diastema length observed in this study, especially in snakes from Baoji, suggest the former possibility, at least for that character.

The general spatial arrangement of the nucho-dorsal glands was similar between *R. nuchalis* and *R. pentasupralabialis*, whereas the arrangement in *M. plumbicolor* was quite different from that of the two *Rhabdophis* species. In *M. plumbicolor* there were no distinct differences in size or arrangement of glands between the neck and trunk regions, and each gland row extended uninterrupted from the neck to the cloaca. On the other hand, the glands in the neck and trunk regions in *Rhabdophis* were positioned beneath different body scale rows with a diastema between them, and the neck glands were generally larger than the trunk glands. Furthermore, in the former each ventral scale corresponded to two glands in a row, which alternated in size, whereas in the latter each ventral scale

corresponded to one gland in a row except for the neck region. These differences may suggest different developmental process and genetic mechanisms, implying independent evolutionary origin or, at least, extensive modification during the evolution.

The spatial arrangement of the neck glands is similar to that of the nuchal glands of *R. tigrinus* (Nakamura, 1935; Hutchinson *et al.*, 2007). It is noteworthy that the nuchal glands of *R. tigrinus* are ontogenetically of mesodermal origin (Fukada, 1958), unlike the primary secretory tissue of all other skin glands of terrestrial vertebrates, which is of ectodermal origin (Mori *et al.*, 2012). No organs homologous to the nuchal glands have been recognized in any other vertebrates. Observations of embryonic development of the nucho-dorsal glands will help clarify the proximate genetic and developmental mechanisms that govern the different spatial arrangements and may reveal the evolutionary origin of these unusual organs.

In spite of their morphological differences, both *M. plumbicolor* and *R. pentasupralabialis* showed a peculiar display, body lift, as well as neck arch. As far as we know, a defensive display similar to body lift has never been observed in any other species of snakes (Greene, 1988; Mori & Burghardt, 2008). Considering that body lift was never elicited when the snakes were stimulated on the tail, where no glands exist, and that this display is observed only in species that have the nucho-dorsal glands, we assume that body lift functions to enhance the effectiveness of predator deterrence by the trunk glands. This display may facilitate oozing of the fluid from the glands by applying pressure to the glands by the underlying trunk muscles. The absence of neck butt in these species may be related to the possession of glands along the entire body, under which condition it may not be necessary to attract the

target of attack by predators specifically to the neck, in contrast to snakes that have defensive glands only in the neck region. The similarity in defensive responses between *M. plumbicolor* and the two *Rhabdophis* species implies either the persistence of common ancestral behavioural traits or the parallel independent evolution of a similar behaviour. Phylogenetic analysis of species of *Rhabdophis* and *Macropisthodon*, as well as related genera, is necessary to elucidate how their morphological and behavioural traits may have evolved in concert. Comparative investigation of the embryonic development of these glands, determination of the chemical components of the glandular fluid, and investigation of the possible sequestration of prey toxins are highly desired to unravel the evolutionary steps of this unique defensive system.

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