CHEMOECOLOGY

Nuchal glands: a novel defensive system in snakes

Akira Mori · Gordon M. Burghardt ·
Alan H. Savitzky · Kathleen A. Roberts ·
Deborah A. Hutchinson · Richard C. Goris

Abstract Of the various chemical defensive adaptations of vertebrates, nuchal glands are among the most unusual. First described in a Japanese natricine snake, *Rhabdophis tigrinus*, in 1935, these organs are embedded under the skin of the neck region as a series of paired glands that have neither lumina nor ducts. The major chemical components of the glandular fluid are bufadienolides, which are cardiotonic steroids also found in the skin secretion of toads. Here we review early studies of nuchal glands and briefly introduce our recent findings on the sequestration of bufadienolides from consumed toads and the maternal provisioning of those sequestered compounds. We summarize behavioral studies associated with the antipredator function of the nuchal glands, which have been conducted during our more than decade-long collaboration. Results of preliminary analyses on the possible costs of toad-eating and on the ultrastructure of the nuchal glands are also presented. Finally, we discuss the evolutionary origin of the nuchal glands and suggest future directions designed to understand the biological importance of these novel vertebrate organs, which have evolved in a limited number of snake species.

A. Mori (✉)
Department of Zoology, Graduate School of Science,
Kyoto University, Sakyo, Kyoto 606-8502, Japan
e-mail: gappa@ethol.zool.kyoto-u.ac.jp

G. M. Burghardt
Departments of Psychology and Ecology and Evolutionary
Biology, University of Tennessee, Knoxville,
TN 37996-0900, USA

A. H. Savitzky · K. A. Roberts · D. A. Hutchinson
Department of Biological Sciences,
Old Dominion University, Norfolk, VA 23529-0266, USA

R. C. Goris
Hatsuyama 1-7-13, Miyamae, Kawasaki,
Kanagawa 216-0026, Japan

Present Address:
K. A. Roberts
Department of Natural Resources and Environmental Sciences,
Alabama A & M University, Normal, AL 35762, USA

Present Address:
D. A. Hutchinson
Department of Biology, Coastal Carolina University,
Conway, SC 29528, USA

Introduction

Chemical defense is a widespread strategy for organisms to avoid predation. Selection pressure has shaped defensive organs, coupled with morphologically and behaviorally coordinated features, to enhance their efficacy in chemical defense. Thus, we see a variety of defensive organs that have a unique suite of chemically, morphologically, and behaviorally integrated mechanisms. Nuchal glands are unusual organs of chemical defense that occur only in several species of specialized vertebrates, specifically snakes. In this review, we first summarize early studies of the nuchal glands. We then introduce a hypothesis that we have proposed and tested concerning the derivation of the chemical compounds of the glands. We also describe functional and evolutionary aspects of the nuchal glands, along with their behavioral and ecological correlates. Finally, we discuss future directions designed to increase

our understanding of the function and evolution of these unique vertebrate organs.

Discovery and early studies

The nuchal glands were first described in 1935 as integumentary glands in a Japanese colubrid snake, *Rhabdophis tigrinus tigrinus* (Nakamura 1935). The organs are embedded under the skin of the neck region as a series of paired glands that have neither lumina nor ducts. A yellowish, opaque fluid with a disagreeable odor spews out when pressure breaks the cell membranes and ruptures the glands' connective tissue capsules (Nakamura 1935). The discharged fluid is almost neutral in reaction and contains abundant small granules, isolated nuclei of the gland cells, blood, lymphatic cells, and pigment cells (Nakamura 1935). Three years later, Smith (1938) reported similar organs from nine additional species of natricine snakes in

three genera (*Rhabdophis*, *Macropisthodon*, and *Balanophis*), among 44 species of Colubridae and Elapidae examined. He divided the species containing nuchal glands into two groups, those having glands of sacculated form (seven species) and those with the non-sacculated form (three species: Table 1). In addition, he described glands extending the entire length of the body in two of the nine species (*R. nuchalis* and *M. plumbicolor*) and collectively called the glands "nucho-dorsal glands". He also reported the absence of the glands in four congeneric species (Table 1).

Nakamura (1935) surmised a protective function of the nuchal glands on the basis of his own aversive experience. When he decapitated an individual of *R. tigrinus*, yellow droplets that sprayed from the nuchal region on which the blades of the scissors were pressed splashed into his eyes, causing acute pain (Fig. 1). The first clinical note of ocular distress caused by unknown "skin secretions" of *R. tigrinus* predates his finding (Shibuya 1923), and a series of

Table 1 Occurrence of nuchal (nucho-dorsal) glands among species in the three natricine genera in which the glands have been reported

Species	Glands	Gland type	Gland site	Source
Rhabdophis callichromus	P	S	N	Smith (1938)
R. himalayanus	P	S	N	Smith (1938)
R. leonardi	P	–	WB[b]	Jiang and Zhao (1983)
R. nigrocinctus	P	S	N	Smith (1938)
R. nuchalis	P	S	WB	Smith (1938); Jiang and Zhao (1983)
R. pentasupralabialis	P	–	WB[b]	Zhao (1995)
R. subminiatus	P	S	N	Smith (1938)
R. tigrinus	P	S	N	Nakamura (1935); Smith (1938)
R. murudensis	A/P[a]	–	N	Smith (1938); Stuebing and Lian (2002)
R. adleri	A	–	–	Zhao (1997)
R. chrysargos	A	–	–	Smith (1938)
R. spilogaster	A	–	–	Smith (1938)
R. swinhonis	A	–	–	Mao and Chang (1999)
R. angelii	U	–	–	
R. auriculatus	U	–	–	
R. barbouri	U	–	–	
R. chrysargoides	U	–	–	
R. conspicillatus	U	–	–	
R. lineatus	U	–	–	
Macropisthodon flaviceps	P	US	N	Smith (1938)
M. plumbicolor	P	S	WB	Smith (1938)
M. rhodomelas	P	US	N	Smith (1938)
M. rudis	A	–	–	Smith (1938)
Balanophis ceylonensis	P	US	N	Smith (1938)

Taxonomy of snakes follows David and Ineich (1999)

A absent, *N* neck region, *P* present, *S* sacculated form, *U* unknown, *US* unsacculated form, *WB* whole body

[a] Smith (1938) did not find the glands, whereas Stuebing and Lian (2002) confirmed the presence of the glands

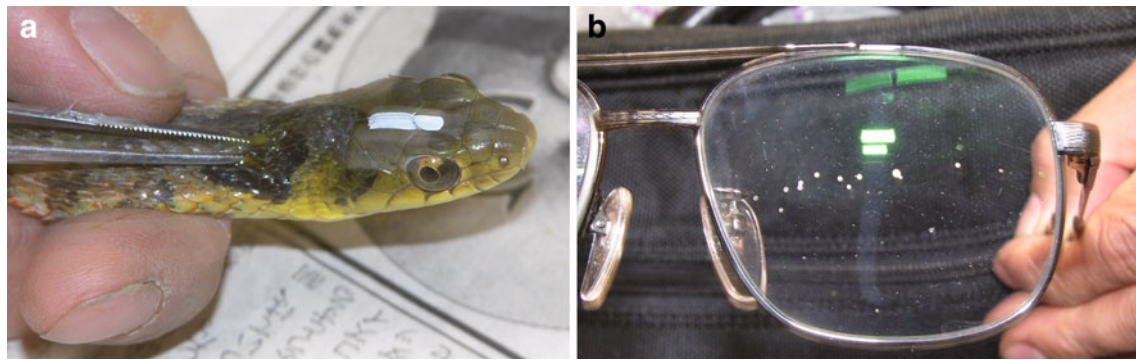[b] Based on the description of *R. nuchalis* that was formerly considered the same species

Fig. 1 a Pinching a nuchal gland with forceps to release contents. **b** Droplets of the nuchal gland fluid (*white dots*) squirted onto the lens of the eyeglasses worn by the person who pinched the gland. The *white line* on the head of the snake is a paint mark used for individual identification

similar clinical cases have been reported (Dobashi 1935; Tamura 1940a, b; Hikita 1947; Saitoh 1949; Ariga 1953; Kawashima 1957, 1959; Suzuki 1960; Watanabe et al. 1988; Kawamoto and Kumada 1989; Ogawa et al. 1992). Medical studies of the physiological actions of the chemicals in the glands began in the 1950s. Kitazume (1953, 1958) observed increased blood pressure caused by intravenous injection of the secretion in rabbits. Nakai (1953) reported that mice injected with the secretion intraperitoneally showed acute toxic reactions such as labored breathing with strongly paralyzed legs and delayed symptoms of mammary tumors. A recent toxicological study showed that the LD_{50} of the secretion in mice, through intraperitoneal injection, is 97.99 mg/kg (He et al. 2007). Ophthalmopathic studies have revealed corneal epithelial detachment and strong miosis in pupil movements when the secretion is instilled into the eyes of laboratory animals (Asahi et al. 1985).

Half a century passed before any knowledge of the chemical components of the secretion was obtained. Akizawa and his team purified and isolated bufadienolides, cardiotonic steroids also found in the skin secretion of toads, which are the major chemical components of the nuchal glands (Akizawa 1986; Akizawa et al. 1985a, b; Azuma et al. 1986). They determined the structures of ten compounds: seven of them were novel compounds found in *R. tigrinus*, and one was identified as gamabufotalin, which is commonly found in skin secretions of toads (*Bufo* spp.).

In spite of these intriguing medical and chemical reports, only a few researchers paid attention to ecological and behavioral aspects of the nuchal glands. Fukada (1961) described a peculiar behavior, neck arch, which appears unique to *R. tigrinus*. This is a behavior in which the snake arches its neck, conspicuously exposing the dorsal region where the nuchal glands are located (Fig. 2a). He considered "neck arch" an antipredator display that has a functional association with the nuchal glands. Mutoh (1983) reported death-feigning behavior of *R. tigrinus* and suggested a functional relationship between this behavior and the nuchal glands.

Derivation of the chemical compounds

Rhabdophis tigrinus is distributed in Japan, Korea, Southern Primorsky in Russia, southern continental China,

Fig. 2 Nuchal gland-related behaviors of *Rhabdophis tigrinus* (collected in Korea). **a** A snake exhibiting neck arch. **b–c** A snake performing neck butt against a human finger in response to a tapping stimulus by the finger. Note the two longitudinal rows of nuchal glands on the dorsal part of the neck region

and Taiwan, but most quantitative ecological and behavioral information is based on populations in Japan (e.g., Fukada 1959; Moriguchi and Naito 1982, 1983; Moriguchi 1985; Moriguchi and Toriba 1985; Mori 1997, 2006). This species mainly feeds on amphibians, especially frogs, and it often exploits toads (Mori and Moriguchi 1988; Kadowaki 1992; Hirai 2004; Mori and Vincent 2008). Toads are usually avoided by predatory animals because of their highly toxic skin secretions, including bufadienolides and bufotoxins (e.g., Licht and Low 1968; Shimada et al. 1977).

The aforementioned two features of *R. tigrinus*, that is, bufophagy (eating toads) and the presence of a chemical substance identical to a toad toxin (gamabufotalin) in the nuchal glands, led us to hypothesize that the chemical compounds of the nuchal glands are derived from the skin secretions of toads ingested by the snakes. Indeed, sequestration of toxins from the diet is a well-known phenomenon in animals (see Savitzky et al. in prep., Saporito et al. in prep. for reviews). To test this hypothesis we organized a Japan–US collaboration in the mid-1990s. Our primary objectives were not only to test the hypothesis experimentally, but also to clarify ultrastructural, functional, and physiological aspects of the nuchal glands from an evolutionary viewpoint. After a decade of alternative approaches and circumstantial evidence, we finally demonstrated evidence of the dietary sequestration of bufadienolides in nuchal glands (Hutchinson et al. 2007). In addition, we obtained an unexpected finding, that provisioning of these defensive compounds can occur between mothers and their unborn offspring (Hutchinson et al. 2008). More details on chemical aspects of the nuchal glands are presented in Hutchinson et al. (2011).

Nuchal gland-related behaviors

A major initial focus of our research project was to examine the antipredator behavior of *R. tigrinus*, to gain insights into how the nuchal glands may be incorporated into protective displays. We began by describing and categorizing 18 antipredator responses of *R. tigrinus* (Mori et al. 1996). In addition to the previously noted "neck arch", we found that the snakes exhibit a series of peculiar displays such as "neck butt" and "dorsal facing posture". In neck butt, a snake raises its head and neck off the substrate and swings the head backward with erratic movements so the dorsal part of the neck is forcibly pressed against the stimulus (Fig. 2b, c). In dorsal facing posture, a snake slightly or highly elevates its head and neck above the substrate and keeps the dorsal neck region directed toward the stimulus, typically as a result of three-dimensional movements of the anterior part of the body.

Coupled with the results of a subsequent comparative study among natricines from around the world (Mori and Burghardt 2008), we concluded that neck arch, neck butt, and dorsal facing posture are antipredator behaviors unique to snakes having nuchal glands and that these behaviors enhance the deterrent effects of chemicals in the glands. The behavioral suite appears to be innate, because newly hatched snakes perform these behaviors shortly after hatching. We also showed that dependency of these "nuchal gland-related behaviors" varies according to internal and external factors, such as body temperature of snakes, body part stimulated, and type of stimulus (Mori et al. 1996; Mori and Burghardt 2001).

A most relevant behavioral finding was our discovery of geographic differences in the dependency on nuchal gland-related behaviors. Hatchling snakes from Kinkasan Island, where no toads occur, exhibit neck arch and neck butt less frequently and perform simple escape responses more frequently than hatchling *R. tigrinus* from populations sympatric with toads (Mori and Burghardt 2000). This result is congruent with a prediction deduced from the aforementioned hypothesis: If *R. tigrinus* sequesters toad toxins in the nuchal glands, it would be disadvantageous for snakes on Kinkasan Island to expose their neck region to predators because they lack defensive compounds in their nuchal glands due to the unavailability of toads. Therefore, nuchal gland-related behaviors should experience negative selection on Kinkasan Island. In fact, subsequent chemical analysis of nuchal gland fluid from *R. tigrinus* on this toad-free island confirmed the lack of bufadienolides (Hutchinson et al. 2007). *Rhabdophis tigrinus* on Kinkasan Island exemplifies a clear case of correlated microevolution among ecological, physiological, and behavioral traits.

Possible costs of toad-eating

Because *R. tigrinus* not only tolerates the toxic effects of toad skin secretions, but also utilizes them for its own defense, this species obviously has overcome a primary defense of toads, essentially winning an evolutionary arms race between predators and prey. However, it is possible that bufophagy incurs some physiological costs in the snakes. For example, garter snakes that have evolved physiological tolerance against the tetrodotoxin of certain newts (see Williams et al. in prep. for review) show a temporary reduction of locomotor performance and obvious behavioral symptoms of poisoning after ingesting toxic newts, making the snakes more vulnerable to their predators for some hours (Brodie and Brodie 1999; Williams et al. 2003). Similarly, *R. tigrinus* may incur some costs from eating toads that are not immediately evident.

To explore this possibility, we conducted preliminary tests to identify short-term effects of bufophagy on locomotor performance. We also compared the survivorship of juvenile snakes reared on different diets to examine the long-term effects of bufophagy.

Possible short-term effects of bufophagy were examined by comparing crawling and swimming performances. Crawling speeds of ten wild-caught snakes (body mass 3.1–125 g, five males and five females) were measured immediately before, and 1, 2, and 3 days after the snakes ate a prey item (4.5–35.1% of snake body mass). Each snake was fed approximately the same mass of either toads (*Anaxyrus terrestris* or *A. woodhousei*, formerly members of the genus *Bufo*), frogs (*Acris crepitans*, *Hyla squirella*, *Lithobates palustris*, or *L. sphenocephala*), or fish (*Pimephales promelas* or *Poecilia reticulata*) every 4 days in counterbalanced order (repeated measures design). In each trial, a snake was gently moved from its home cage and introduced onto a track (2 m length × 0.3 m width) with artificial floor covering (Astroturf) at an air temperature of $24 \pm 1°C$. The snake was then encouraged to traverse the track by gently tapping the tail tip or the substrate just behind the tail. The duration of the crawl (1.5 m for adults and 1 m for juveniles) was measured with a stopwatch, and the crawling speed was calculated. Trials for each snake were repeated three times with 3-min intervals, and the average speed of each snake was compared among food types. Size-adjusted speed (crawling speed/snout-vent length of snake) did not show any clear differences among food type on any trial day (Friedman test: baseline, chi square = 0.84, $p = 0.66$; day 1, chi square = 0.25, $p = 0.88$; day 2, chi square = 1.75, $p = 0.42$; day 3, chi square = 0.45, $p = 0.80$; Fig. 3).

Swimming performance tests were conducted using wild-caught snakes and laboratory-born hatchlings. Snakes
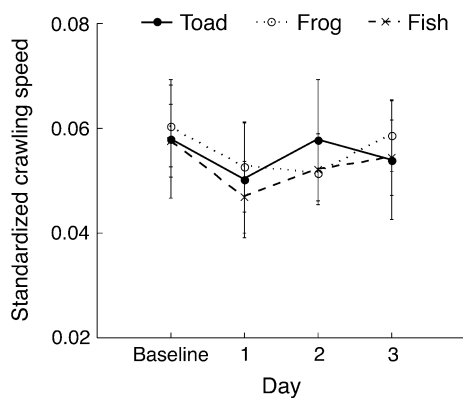
were introduced into a circular pool (1.3 m in diameter and 0.35 m in water depth at 19.0–22.5°C water temperature), and were encouraged to swim for ten circular laps (wild-caught snakes) or five laps (hatchlings). The time taken to complete each lap or half-lap was measured. Three people stood outside the pool to encourage the snakes to swim by tapping the water just behind the snakes' tails. Eight wild-caught adults and juveniles (body mass 3.4–115 g, three males and five females) were tested three times: immediately before eating, and 1 and 2 days after eating either toads (*A. terrestris*) or frogs (*L. sphenocephala*, *L. clamitans*, or *H. squirella*). Each snake was tested once with toads and once with frogs of similar body mass (17.1–34.0% of snake body mass) with a four-day interval, in a counterbalanced order. Of the hatchlings (snout-vent length, 178–248 mm), three were offered toads (*A. quercicus* or *A. terrestris*), three were offered frogs (*Scaphiopus holbrookii*), and eleven were offered fish (*Pimephales promelas*) of similar body mass; swimming tests were conducted 1, 2, and 3 days after feeding. In wild-caught snakes, only six individuals accepted toads and four individuals accepted frogs under the experimental schedule; thus, no statistical analyses were applied, but the results showed no clear differences in swimming speeds between toad- and frog-fed snakes (Fig. 4a). Hatchlings also did not show obvious effects of toad-feeding on swimming speed, although again no statistical tests were conducted due to the small sample size (Fig. 4b).

Before examining the effects of toad-feeding on the survivorship of juveniles, we compared the initial (innate) prey preferences of hatchling *R. tigrinus* for toads between a toad-free population (six clutches from Kinkasan Island) and toad-present populations (two clutches each from Shiga and Chiba Prefectures and six clutches from Ishima Island). We divided the hatchlings of each population into three feeding groups and introduced either a dead toadlet (previously frozen *Bufo japonicus*), dead froglet (previously frozen *Hyla japonica*), or small live fish (*Oryzias latipes*) into their individual cages when the hatchlings were approximately 3 weeks old. Twenty-four hours later we recorded whether or not they ate the food. As shown in Table 2, most snakes from both toad-present and toad-free populations accepted toads as well as frogs. These hatchlings were then raised on either toad-and-fish, frog-and-fish, or fish-only diets for 6 months to examine their survivorship. Food was offered to each group every 2–3 days, keeping the body mass of all prey items as equivalent as possible on each feeding day. If snakes left the food until the subsequent day, we force-fed them with the same amount of food. At the age of 3 months, snakes from both the toad-present and toad-free populations showed the highest survivorship in the toad-and-fish group (Table 3). At the age of 6 months, similar results were

Fig. 3 Comparisons of crawling speeds of adult and juvenile *Rhabdophis tigrinus* after one-time ingestion of toads, frogs, or fish. Baseline speeds were measured immediately before feeding. Standardized crawling speeds were calculated by dividing crawling speed by snout-vent length of the snake. *Bars* indicate standard errors

Fig. 4 Comparisons of swimming speeds of *Rhabdophis tigrinus* after one-time ingestion of toads, frogs, or fish (**a** adults and juveniles, **b** hatchlings). *Bars* indicate standard errors
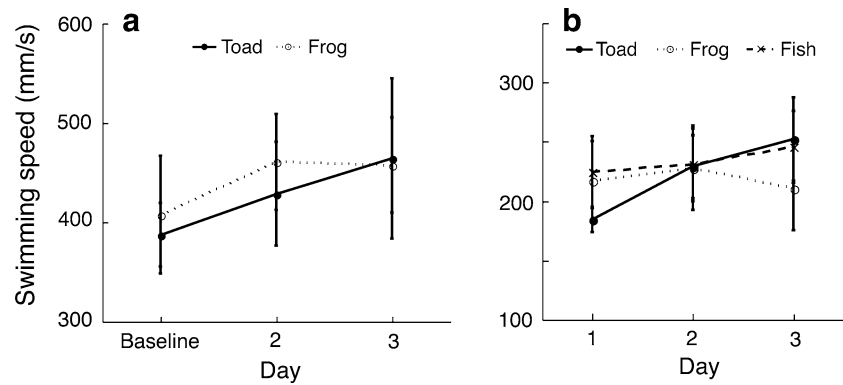

Table 2 Percentage of acceptance of the initial food offered to hatching *Rhabdophis tigrinus* from toad-present and toad-free populations

Population	Food type		
	Toad	Frog	Fish
Toad-present	95.1% (41)	90.0% (40)	16.7% (24)
Toad-free	100% (24)	100% (24)	45.8% (24)

Numbers in parentheses are sample sizes

Toad, *Bufo japonicus*; Frog, *Hyla japonica*; Fish, *Oryzias latipes*

Table 3 Survivorship of juvenile *Rhabdophis tigrinus* reared with different diet regimens

Population	Age (month)	Food type		
		Toad and fish	Frog and fish	Fish only
Toad-present	3	92.7% (38/41)	50.0% (20/40)	62.5% (15/24)
	6	61.0% (25/41)	50.0% (20/40)	62.5% (15/24)
Toad-free	3	87.5% (21/24)	41.7% (10/24)	50.0% (12/24)
	6	70.8% (17/24)	33.3% (8/24)	45.8% (11/24)

Numbers in parentheses are sample sizes (surviving individuals/total sample)

Toad, *Bufo japonicus*; Frog, *Hyla japonica*; Fish, *Oryzias latipes*

obtained for the toad-free population, although the fish-only group showed slightly, but not statistically significantly (Fisher exact probability test: $p > 0.9$), higher survivorship than the toad-and-fish group in the toad-present populations (Table 3). Importantly, snakes from the toad-free island exhibited survivorship patterns similar to those of toad-present populations.

These experiments failed to identify any clear physiological costs of toad-eating in *R. tigrinus*. Even snakes from the toad-free island showed a survival rate similar to that of snakes from toad-present populations. However, this does not necessarily demonstrate that *R. tigrinus* incurs no physiological effects of toad toxins. For example, we have often witnessed a phenomenon we refer to as "sudden

death" in our attempts to maintain captive *R. tigrinus*, and this phenomenon may reflect side effects or associated costs of physiological mechanisms to accommodate to toad-feeding. *Rhabdophis tigrinus* is generally voracious in captivity, but even well-fed snakes exhibit this syndrome, making this species difficult to keep in captivity for a long period, in contrast to many other species of snakes. Death often follows a sudden refusal of food and occurs a few weeks later without preceding signs of emaciation. At other times a hatchling or adult that has eaten avidly the day before will be found dead the next day. This sudden death can occur in adults and hatchlings, and in wild-caught and laboratory-born snakes, irrespective of food type (live or dead, frogs or fish, and even frozen chicken hearts). Although we have no evidence that links toad-feeding habit directly to this enigmatic sudden death, detailed pathological and physiological investigations should be pursued to determine whether it reflects an evolutionary trade-off between the innovation of the nuchal glands (benefit) and some form of physiological stress (cost). Preliminary studies of the cardiac responses of *R. tigrinus* and nontoad-eating snakes to the consumption of toads suggest that *R. tigrinus* exhibits a distinctive response that includes an elevated but regular heart rate (Savitzky, Hutchinson, and Mori unpublished data). Such a response may constitute another physiological cost of bufophagy in this species.

Ontogeny, ultrastructure, and evolution

Sequestration and redeployment of dietary toxin is not an uncommon phenomenon among animals. Most animals that rely on sequestered toxins for defense store the chemicals in evolutionarily pre-existing organs, such as skin, muscles, and viscera. In this respect, the nuchal glands are unique because these organs appear to have evolved de novo as a protective device for storing sequestered defensive chemicals. Ontogenetically, the glands are of mesodermal origin and are fully developed in hatchlings (Fukada 1958). To the best of our knowledge,

the primary secretory tissue of all other skin glands of terrestrial vertebrates is ectodermal in origin. Thus, the nuchal glands are quite distinctive from an ontogenetic and evolutionary viewpoint.

Our examinations of microstructure and cellular morphology by light microscopy confirmed the characteristics of the glands noted by Nakamura (1935), including the lack of lumina and ducts and the holocrine nature of the glands (Roberts 2000; Hutchinson et al. 2007). Especially notable for a vertebrate integumentary gland is the absence of a distinct secretory epithelium (Fig. 5a), an attribute
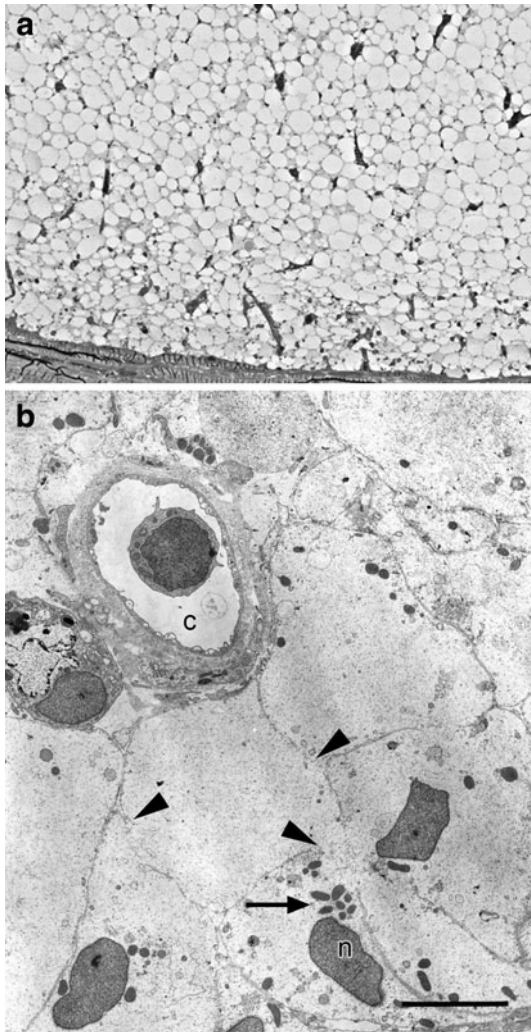

Fig. 5 Histology and ultrastructure of the nuchal glands of *Rhabdophis tigrinus*. **a** Low-magnification micrograph of a resin-embedded thick section, stained with Richardson's stain. The *dark band* in the lower left is the collagenous capsule of the gland. Note the nearly uniform cells of the gland and the absence of a secretory epithelium adjacent to the gland capsule. The *dark streaks* within the gland are collagen. **b** Transmission electron micrograph of nuchal gland tissue, showing cells filled primarily with indistinct cytoplasm. Note the absence of secretory organelles. *c* capillary, *n* nucleus, *arrow* points to a cluster of mitochondria; *arrowheads* indicate regions where cell membranes are incomplete. *Scale* 5 μm

consistent with the reported non-epithelial origin of the glands (Fukada 1958). In addition, transmission electron microscopy revealed that the nuchal gland cells frequently exhibit deteriorating cell membranes and are filled primarily with indistinct cytoplasm, relatively small nuclei, a few mitochondria, and some unidentified structures (Fig. 5b). However, the cells conspicuously lack many organelles, such as the Golgi apparatus and rough endoplasmic reticulum, that are associated with protein synthesis and steroid production or modification (Roberts 2000). The vascular morphology associated with the nuchal glands, revealed by casts obtained by perfusion with Microfil, a fine latex, includes circumferential blood vessels and dense central capillary beds (Hutchinson et al. 2007), suggesting that defensive compounds are delivered to the glands via the circulatory system. These ultrastructural and vascular characteristics are consistent with the sequestration of chemical compounds delivered via the circulatory system and the inability of the nuchal glands to biosynthesize bufadienolides. Furthermore, they contrast sharply with the attributes of other vertebrate skin glands that contain bufadienolides, such as the parotoid glands of toads (Hutchinson and Savitzky 2004).

Currently, 13 species of three Asian natricine genera (*Rhabdophis*, *Macropisthodon*, and *Balanophis*) are known to possess nuchal (nucho-dorsal) glands, and the absence of the glands in congeners has been reported in *M. rudis* and at least four species of *Rhabdophis* (Table 1). This phylogenetically scattered occurrence of the glands may suggest multiple independent origins and/or the evolutionary loss of the glands in some species, but a lack of authoritative phylogenetic information about species in these genera precludes a definitive conclusion at this time. In addition, even basic biological information on all species of the three genera, except for *R. tigrinus*, is quite limited. Since Smith (1938), almost no work has been conducted on the morphology and physiology of the glands except for those of *R. tigrinus*. The only available comparative chemical information is a brief statement by Akizawa (1986), who mentioned the occurrence of bufadienolides in the glands of *R. subminiatus*, and recent brief reports on *R. tigrinus* from China (formerly recognized as *R. t. lateralis*: He et al. 2007; He and Yu 2008). Quantitative ecological and behavioral information on the three genera are also quite meager. Bufophagy has been described in all four species of *Macropisthodon*, irrespective of the presence or absence of the glands (Pope 1935; Smith 1943; Daniel 1983; David and Vogel 1996; Zhao et al. 1998; Stuebing and Inger 1999; Whitaker and Captain 2004), but has been reported only in a few species of *Rhabdophis*, including *R. tigrinus* and *R. subminiatus* (Pope 1935; Smith 1943; Campden-Main 1970; Sura 1981; Mori and Moriguchi 1988; David and Vogel 1996; Cox et al. 1998; Jiang 2002; Schleich and

Kästle 2002; Whitaker and Captain 2004). Nuchal gland-related behaviors have been confirmed only for *R. tigrinus* and *R. subminiatus*. Neck flattening behavior, a display that often accompanies the nuchal gland-related behaviors (Mori and Burghardt 2008), is known in all four species of *Macropisthodon* (Pope 1935; Smith 1943; Taylor 1965; Daniel 1983; Tweedie 1983; Lue et al. 1999; Stuebing and Inger 1999; Whitaker and Captain 2004; Baker and Lim 2008) and in at least four species of *Rhabdophis*, including species that either possess or lack the glands (*R. subminiatus*: Schleich and Kästle 2002; Whitaker and Captain 2004; Mori and Burghardt 2008, *R. tigrinus*: Mori et al. 1996; Mori and Burghardt 2008, *R. murudensis*: Stuebing and Lian 2002, *R. swinhonis*: Lue et al. 1999). To formulate a working hypothesis regarding the origin and evolution of the nuchal glands, comparative studies on ecology, behavior, physiology, chemistry, and phylogeny are required.

Whatever the evolutionary origin of the nuchal glands, it is almost certain that exploitation of toads predated the evolution of the glands. Comprehensive phylogenetic information on Asian natricine snakes based on molecular analyses is lacking, but a putative phylogenetic tree based on available information suggests the widespread occurrence of bufophagy among natricines (Fig. 6). In addition, relatively high tolerance of toad toxins in a natricine species that is currently allopatric with toads (Phillips et al. 2003) suggests that toad-feeding, or at least toad tolerance, is an ancestral trait that evolved early in natricine snakes. Similarly, neck flattening behavior must have predated the evolution of the nuchal glands because neck flattening as a threatening display is widespread not only in natricines but also in other groups of snakes (Greene 1988; Mori and Burghardt 2008). Accumulation of sequestered toad toxins in the neck region, to which predators' attention is especially attracted, may have had a selective advantage in an ancestral species, which may have led to the evolution of the nuchal glands.

Further questions and perspectives

As shown above, considerable knowledge of the nuchal gland system has accumulated during the past decade. However, many questions and topics remain to be explored and clarified. In addition, our findings have raised new questions that are worth pursuing. Virtually nothing is known of the physiological mechanisms of uptake, modification, storage, and tolerance of bufadienolides. A preliminary examination has shown that *R. tigrinus* has a relatively large heart and large adrenal glands compared with related species, suggesting a possible physiological association between these organs and bufophagy.
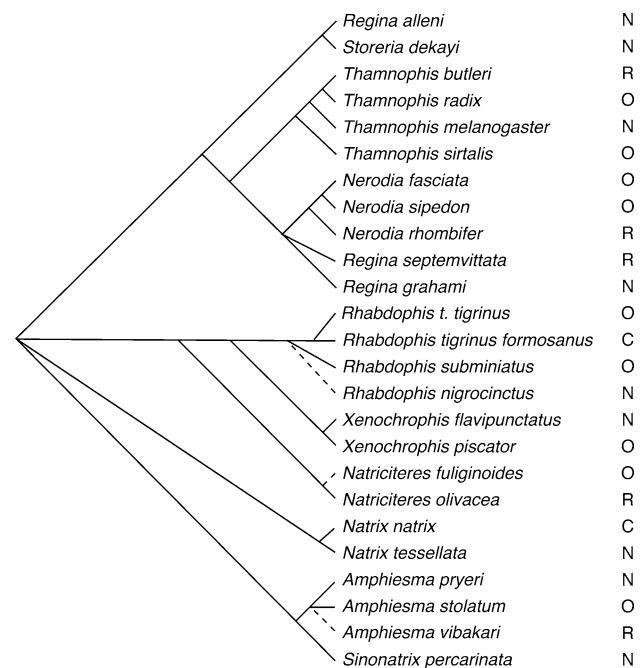

Species	
Regina alleni	N
Storeria dekayi	N
Thamnophis butleri	R
Thamnophis radix	O
Thamnophis melanogaster	N
Thamnophis sirtalis	O
Nerodia fasciata	O
Nerodia sipedon	O
Nerodia rhombifer	R
Regina septemvittata	R
Regina grahami	N
Rhabdophis t. tigrinus	O
Rhabdophis tigrinus formosanus	C
Rhabdophis subminiatus	O
Rhabdophis nigrocinctus	N
Xenochrophis flavipunctatus	N
Xenochrophis piscator	O
Natriciteres fuliginoides	O
Natriciteres olivacea	R
Natrix natrix	C
Natrix tessellata	N
Amphiesma pryeri	N
Amphiesma stolatum	O
Amphiesma vibakari	R
Sinonatrix percarinata	N

Fig. 6 Phylogenetic hypothesis of the relationships among snake taxa used in Mori and Burghardt (2008) and occurrences of bufophagy. *Atretium schistosum* was excluded from the tree due to absence of phylogenetic information. The branching pattern is based on published molecular phylogenies (Lawson 1986; Kraus and Brown 1998; Alfaro and Arnold 2001; de Queiroz et al. 2002; Vidal and Hedges 2002) and an unpublished phylogeny for several Old World species (Chang, unpubl. data). Frequency of toads in diets was evaluated as either common (C), occasional (O), rare (R), or none (N). References for feeding habits are: Akani and Luiselli 1999/2000 (*Natriciteres fuliginoides*); Bowers 1966 (*Nerodia rhombifer*); Broadley 1983 (*Natriciteres olivacea*); Campden-Main 1970 (*Rhabdophis subminiatus*, *Sinonatrix percarinata*, and *Xenochrophis piscator*); Cox et al. 1998 (*R. nigrocinctus* and *R. subminiatus*); David and Vogel 1996 (*R. subminiatus* and *X. piscator*); De Silva 1990 (*Atretium schistosum*); Disi et al. 2001 (*Natrix tessellata*); Ernst and Barbour 1989 (*Nerodia fasciata*, *N. rhombifer*, *N. sipedon*, *Regina alleni*, *R. grahami*, *R. septemvittata*, and *Storeria dekayi*); Gruber 1989 (*Natrix natrix* and *N. tessellata*); Jiang 2002 (*Rhabdophis t. tigrinus*); Karsen et al. 1986 (*Sinonatrix percarinata*); Lee and Lue 1996 (*Amphiesma stolatum*, *R. t. formosanus*, *S. percarinata*, and *X. piscator*); Loveridge 1958 (*Natriciteres olivacea*); Luiselli 2003 (*N. fuliginoides*); Manthey and Grossmann 1997 (*X. flavipunctatus*); Mori and Moriguchi 1988 (*A. pryeri*, *A. vibakari*, and *R. t. tigrinus*); Nikol'skii 1964 (*Natrix tessellata*); Pope 1935 (*A. stolatum*, *R. subminiatus*, *R. t. tigrinus*, *S. percarinata*, and *X. piscator*); Rossman et al. 1996 (*Thamnophis butleri*, *T. melanogaster*, *T. radix*, and *T. sirtalis*); Schleich and Kästle 2002 (*A. stolatum*, *Atretium schistosum*, and *X. flavipunctatus*); Smith 1943 (*Amphiesma stolatum*, *R. nigrocinctus*, *R. subminiatus*, *S. percarinata*, and *X. piscator*); Spawls et al. 2002 (*Natriciteres olivacea*); Steward 1971 (*Natrix natrix* and *N. tessellata*); Sura 1981 (*R. t. tigrinus*); Taylor 1965 (*R. nigrocinctus*)

Interestingly, North American hognose snakes (*Heterodon*), which are well-known bufophagous snakes, have been reported to possess enlarged adrenal glands (Smith and White 1955; Spaur and Smith 1971).

The vasculature of the nuchal glands should be examined with more specimens because our observations to date are limited. If the highly developed blood vessels reflect delivery of bufadienolides to the glands via the circulatory system, it is expected that blood plasma will contain similar chemical compounds to those that are stored in the nuchal glands; we have obtained a preliminary result supporting this mechanism (Hutchinson et al. unpublished data). Furthermore, clarification of the function of the unknown organelles found in the cells of the nuchal glands is necessary. Because discharge of the glandular fluid involves destruction of the cells in the glands, those cells must be regenerated if the snake is to use the organs again. Alternatively, each nuchal gland may be an "expendable" organ that can be used only once in the snake's lifetime.

Although the functional role of the nuchal glands as an antipredator device is beyond doubt, the effectiveness of the discharged fluid and the characteristic displays, such as neck arch, to deter predators, as well as the role that the discharged fluid plays in the process of deterrence, have not been demonstrated. Direct field observations on the interaction between snakes and their predators are virtually lacking (Mori et al. 2009). We suggest two scenarios, not mutually exclusive, concerning the process of predator deterrence. One possibility is that predators, such as raptors (Tanaka and Mori 2000), that bite the neck region of the snake would release the snake because of the unpalatability of the fluid discharged by the biting force. The other possibility is that predators that restrain the snake by holding it with their claws or feet, or by gripping it with their beaks, exert pressure on the nuchal glands, which causes the glandular fluid to squirt into the predator's eyes. Presumably, the predator would then release the snake as the result of the irritation caused by the nuchal gland fluid. Staged encounter tests with predators are necessary to clarify the actual interactions.

The provisioning of sequestered chemical compounds by mothers to their offspring (Hutchinson et al. 2008, 2011) implies the occurrence of indirect maternal care by female snakes. Dams with large quantities of bufadienolides in their nuchal gland fluid produce offspring with large quantities of these compounds, but dams that lack bufadienolides, or possess small quantities of them, produce hatchlings that lack these defensive compounds (Hutchinson et al. 2008). Newly hatched snakes, which appear in the wild around the end of August, may not be able to consume juvenile toads due to gape limitation. That is, the juvenile toads that metamorphosed the previous spring may already have grown too large for hatchling snakes to eat. If this is the case, juvenile snakes would not be able to obtain bufadienolides from ingested toads until the subsequent spring, when newly metamorphosed toadlets become available. Thus, maternal provisioning would

be an essential route to arm the hatchlings with toxic compounds until they are able to consume toads themselves. Therefore, it is predicted that females (or gravid females) are more likely to feed on toads than males (or non-gravid females) because of the need for a sufficient amount of sequestered compounds for provisioning (Hutchinson et al. 2008). However, we do not know the quantity of bufadienolides required for deterrence of predators by hatchlings or the delivery route of sequestered chemical compounds from dams to offspring. Although several routes appear to be involved in the transfer of toxins to embryos (Hutchinson et al. 2008), it is not known whether the provisioned toxins must be freshly ingested or whether they can be mobilized from stores in the nuchal glands or elsewhere in the female's tissues. Behavioral studies both in the field and laboratory, along with a physiological investigation of the provisioning system in *R. tigrinus*, may uncover a type of maternal care unique among terrestrial vertebrates.

The possibility that individual snakes exhibit behavioral adjustments according to the amount of toxins stored in the nuchal glands is worthy of exploration. If snakes are able to recognize how toxic they are based on the amount of bufadienolides accumulated in their glands, they may modify their antipredator responses accordingly. They may rely more heavily on nuchal gland-related behaviors when they possess a sufficient amount of toxins to deter predation than when they do not have enough. A preliminary experiment addressing this question revealed that juvenile snakes that have been fed toads exhibit neck arch more frequently than those that have been reared on other types of foods (Mori and Burghardt in prep.). This may imply self-recognition of toxicity, whatever proximate mechanism is involved, and, if so, would provide an interesting case of a plastic behavioral response coordinated with toxicity level.

An investigation of the antipredator behavior of additional species is necessary to understand the evolution of the unique displays associated with nuchal glands. It would be worth examining the antipredator responses of species other than *R. tigrinus* that possess nuchal glands, including those species that have glands extending the entire length of the body (nucho-dorsal glands). Additionally, the antipredator displays of congeneric species and closely related outgroups that lack nuchal glands should be studied to elucidate the correlated evolution of morphology and behavior. To implement these approaches, broad phylogenetic analyses of Old World natricine snakes, including the three genera containing species with nuchal glands (*Rhabdophis*, *Macropisthodon*, and *Balanophis*), are required. The phylogenetically independent occurrence of death feigning behavior in *R. tigrinus*, *Heterodon nasicus*, *H. platirhinos*, *Natrix natrix*, and *Hemachatus haemachatus* (Greene 1988),

which are all toad-eaters (Steward 1971; Broadley 1983; Ernst and Barbour 1989), suggests the possible, but uncertain, functional association between bufophagy and death feigning. A comparative study of these snakes may reveal unexpected physiological and neuroethological links.

We have observed considerable individual variation in the size of the nuchal glands, composition of bufadienolides, and the propensity to perform nuchal gland-related behaviors that are not readily explained by differences in sex or body size of the snakes. Clarification of the physiological, genetic, developmental, and functional factors that underlie such individual variation would shed light on the adaptive nature of the nuchal glands.

Overall, we believe that research on nuchal glands and their related features is a fruitful avenue not only for understanding these unique squamate glands, but also for comprehending broad mechanisms of physiology, biochemistry, and evolution of phenotypic traits that may be applicable to many other vertebrates. We hope that this review will stimulate researchers in various fields of biology and chemistry to address the many outstanding questions and will foster integrated collaborative approaches.

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