VENOM COMPOSITION AND DIET OF THE CANTIL *AGKISTRODON BILINEATUS HOWARDGLOYDI* (SERPENTES: VIPERIDAE)

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ABSTRACT—The southernmost subspecies of the cantil, *Agkistrodon bilineatus howardgloydi,* occurs in almost all terrestrial habitats of the Sector Santa Rosa, in Guanacaste Conservation Area, Costa Rica. We obtained 21 specimens during several visits to the park from 1993 to 1996. Neonates were observed only during late May through July, despite searching in other months, suggesting that birth in this subspecies occurs at the beginning of the rainy season. Fecal analyses revealed differences in diet composition between juveniles and adults. Juveniles prey primarily on lizards and various species of frogs, whereas adults eat rodents. This ontogenetic change in diet does not seem to trigger any corresponding change in venom composition with snake age. Venom of this subspecies is highly toxic (LD_{50} = 1.25 μg/g), and has elevated hemorrhagic, hemolytic, and myotoxic activities. Extreme deforestation of dry forest habitats in the Pacific lowlands of Central America has made *A. bilineatus* a rare species over most of its range. Sector Santa Rosa in Costa Rica is the only area within its distributional range where the species still can be regularly observed.

RESUMEN—La subespecie más austral del cantil, *Agkistrodon bilineatus howardgloydi,* ocupa casi todos los habitats terrestres del Sector Santa Rosa, Area de Conservación Guanacaste, Costa Rica. Se obtuvieron 21 especímenes durante varias visitas al área desde 1993 a 1996. Neonatos fueron observados sólo desde fines de mayo hasta julio, aunque se buscaron en otros meses. Esto sugiere que los nacimientos de esta subespecie coinciden con el inicio de la temporada de lluvias. Un análisis de restos fecales reveló diferencias en la composición de dieta entre juveniles y adultos. Los juveniles depredan principalmente lagartijas y varias especies de ranas, mientras que los adultos comen roedores. Esta variación en dieta no parece estar relacionada con cambios en la composición de los venenos con la edad de la serpiente. En esta subespecie, el veneno es altamente tóxico (DL_{50} = 1.25 μg/g) y tiene elevada actividad hemorrágica, hemolítica y miotóxica. La destrucción acelerada de los bosques xéricos en las tierras bajas del Pacífico Centroamericano ha convertido *A. bilineatus* en una especie rara en la mayoría de su rango de distribución. El Sector Santa Rosa, en Costa Rica, es la única área dentro de su rango de distribución donde la especie aún puede ser observada con regularidad.

The cantil, *Agkistrodon bilineatus,* has received considerable attention in terms of distribution (Campbell and Lamar, 1989), diagnosis (Burger and Robertson, 1951; Gloyd, 1969), morphological variation (Gloyd, 1969), and phylogenetic relationships (Knight et al., 1992; Parkinson et al., 1997). Most of our knowledge on this species was summarized by Gloyd and Conant (1990) in their monumental review of the *Agkistrodon* complex. They provided information from more than four decades of research on this and related genera (Gloyd and Conant, 1990). Despite these efforts, most aspects of the natural history of the southernmost subspecies of the cantil, *A. b. howardgloydi,* are poorly known.

Agkistrodon bilineatus howardgloydi is the only member of the genus occurring south of Honduras. It inhabits dry forest and its various successional stages, including tropical deciduous

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forest, thorn forest, and savannas. *Agkistrodon b. howardgloydi* is distributed from the lowlands of Golfo de Fonseca (Departments of Valle and Choluteca) in Honduras, along the Pacific coast of Nicaragua, including the Departments of Granada and Jinotega (according to J. Villa, cited in Gloyd and Conant, 1990), and then south to Golfo de Papagayo (Guanacaste Province) in northwestern Costa Rica. The species was first reported in Costa Rica by Bolaños and Montero (1970) from a specimen collected 5 km E of Playa Naranjo (Golfo de Papagayo), in what is today Sector Santa Rosa of the Guanacaste Conservation Area (GCA). Subsequently, Conant (1984) examined a specimen collected from the same place ("near Mirador Cañón del Tigre in Santa Rosa National Park, Guanacaste, Costa Rica"), and recognized it as a new subspecies, geographically isolated from other populations. Because it is a snake that is rarely encountered in most of its range, information pertaining to natural history, toxicology of the venom, and snake bite risk is sparse. We suspect that bites from this species probably are capable of causing human fatalities; however, envenomation by *A. bilineatus howardgloydi* has not been reported in detail. Here, we summarize field observations on the natural history of this subspecies in the region of its type locality in Sector Santa Rosa of the Guanacaste Conservation Area and provide a characterization of the composition and effects of its venom.

MATERIALS AND METHODS—*Specimens and Study Site*—Twenty-one specimens of *Agkistrodon bilineatus howardgloydi* were collected in Sector Santa Rosa of the GCA between June 1993 and January 1996. Sector Santa Rosa of the GCA, formerly known as Santa Rosa National Park, lies between the Interamerican Highway and Playa Naranjo, 30–35 km north of Liberia, Guanacaste Province, Costa Rica. Two life zones are included in this area: humid Premontane Tropical Forest and Dry Tropical Forest (Tosi, 1969). These life zones are similar climatically, receiving an annual precipitation of 1,500 to 2,000 mm that is strongly seasonal (Hartshorn, 1983). Eight vegetational associations are recognized from this area: riparian vegetation, mangroves, *Prosopis* swamps, semi-deciduous alluvial forest, deciduous forest, evergreen forest, *Quercus* forest, and jaragua grasslands (*Hyparrhenia rufa*). The GCA is 120,000 ha of conserved dry forest and associated wetter and marine habitats. In Sector Santa Rosa, *A. b. howardgloydi* is not encountered frequently (Sasa and Solórzano, 1996).

Two of us (A. S. and M. S.), periodically visited the GCA to record activity patterns, habitat selection and behavior. Specimens were weighed, sexed, and their lengths measured to the nearest 0.5 cm. Captured individuals were maintained in individual aquaria for fecal analysis, venom extraction, and further studies of reproductive biology. Representative preserved specimens were cataloged in the Collection of Vertebrates, University of Texas at Arlington (UTA R-42837-39) or the Museo de Zoología, Universidad de Costa Rica (UCR 8062, 13071, 13496-99).

Venom and Antivenom—Venom was extracted from all specimens at Instituto Clodomiro Picado, Universidad de Costa Rica. Sufficient venom was collected from juvenile snakes for electrophoretic analysis, but only adult venom was used for the other analyses. Venoms were lyophilized and stored at −20°C. The polyvalent (Crotalinae) antivenom (batch 2571294 LQ) produced at Instituto Clodomiro Picado, according to Bolaños and Cerdas (1980), was used in neutralization experiments.

Ontogenetic variation in venom was analyzed by conducting electrophoresis (SDS-PAGE) of individual venom samples, performed under reducing conditions on 12% polyacrylamide gels. Proteins were stained with Coomasie Blue G-250.

Median lethal dose (LD_{50}) was estimated by the intraperitoneal route in 16 to 18 g Swiss Webster mice. Groups of six mice were injected with various amounts of venom dissolved in 0.5 ml of phosphate-buffered saline solution, pH 7.2 (PBS). Deaths were recorded after 48 h and the LD_{50} was estimated by the Spearman-Kaber method (WHO, 1981). Neutralization experiments were performed by preparing mixtures of various ratios of venom and antivenom. Mixtures were incubated at 37°C for 30 min and 0.5-ml aliquots (containing 4 LD_{50}s of venom) were injected intraperitoneally into groups of six mice (16 to 18 g). A group of control mice received venom and no antivenom. Deaths were recorded after 48 h and the effective dose 50% (ED_{50}) was estimated. ED_{50} is defined as ml volume of antivenom required to neutralize 1 mg of venom.

Hemorrhagic activity was studied using a skin test (Kondo et al., 1969), as modified by Gutiérrez et al. (1985). Groups of four mice (18 to 20 g) were injected intradermally with various amounts of venom dissolved in 100 μl of PBS. Two hours after injection, mice were sacrificed, their skin removed and the area of the hemorrhagic spot on the inner side of the skin measured. The minimum hemorrhagic dose (MHD) was the amount of venom that induced a hemorrhagic area of 10 mm diameter. For neutralization experiments, mixtures containing various ratios of venom and antivenom were prepared and incubated at 37°C for 30 min. Then, hemorrhagic ac-

TABLE 1—Prey items recorded for 13 *A. b. howardgloydi* from Sector Santa Rosa, Guanacaste Conservation Area, Costa Rica. Numbers of prey items are presented by snake age category. Each snake contained just one prey item.

	Age category		
Prey	Newborn (<380 mm)	Juveniles (380 to 500 mm)	Adults (>500 mm)
Mabuya unimarginata	2	—	—
Ameiva undulata	1	—	—
Ctenosaura similis	—	1	2
Hypopachus variolosus	1	—	—
Leptodactylus poecilochilus	—	1	—
Liomys salvinii	—	1	3
Sigmodon hispidus	—	—	1

tivity was estimated as described. A control group of mice received venom alone. Neutralization of venom activity for hemorrhagic activity was expressed as effective dose 50% (ED_{50}), defined as the ratio of ml antivenom/mg venom in which the activity of venom was reduced 50%.

The agarose-erythrocyte-egg yolk assay described by Gutiérrez et al. (1988) was used to detect indirect hemolytic activity. Various amounts of venom, dissolved in PBS, were applied to wells on agarose gels containing sheep erythrocytes and egg yolk. After a 20 h incubation period at 37°C, diameters of the hemolytic halos were measured. The MHD was the amount of venom that induced a hemolytic halo of 20 mm diameter. For neutralization experiments, mixtures with various ratios of venom and antivenom were prepared and incubated at 37°C for 30 min. Aliquots of each mixture were then assayed for indirect hemolysis as described above. A control containing venom and no antivenom was included, as well as a control of PBS alone. After 20 h of incubation at 37°C, hemolysis was quantified as described. Neutralizing ability of antivenom was expressed as ED_{50}, defined as the ratio of ml antivenom/mg venom in which the diameter of the hemolytic halo was reduced by 50% when compared to the halo induced by venom alone.

Myotoxic activity of the venom of *A. b. howardgloydi* was evaluated histologically. Venom (50 μg dissolved in 50 μl PBS) was injected intramuscularly in the right gastrocnemius of mice (18 to 20 g). A group of control mice received PBS alone. Three and 24 h after injection, groups of mice were sacrificed and a sample of injected muscle was obtained, fixed in Karnovsky's fixative and processed routinely for histological evaluation (Gutiérrez et al., 1991).

RESULTS—*Habitat, Behavior, and Diet*—Almost all snakes were found at night, except for two found at noon and late afternoon. *Agkistrodon b. howardgloydi* were found in various macrohabitats in Sector Santa Rosa. From our data and the confirmed observations of this species by resident biologists at the ACG, it seems that this snake occurs in all sorts of habitats. No association with temporary or permanent water was recorded and snakes were found from rocky ridges to the banks of dry temporary streams.

Eleven specimens maintained in captivity showed similar striking behaviour. In all cases, they bit, injected venom, and released the offered prey (mostly white mice). No gravid females were encountered during our visits to the park. However, neonates a few months old (aged by body size and the presence of an umbilical scar) were observed from late May to July, which are the first 3 months of the rainy season.

Fecal samples collected early in the rainy season in 1994 indicated age-related variation in diet of these snakes (Table 1). Neonates and juveniles preyed on small lizards (*Mabuya unimarginata, Ameiva undulata*) that are common in the leaf litter, and on several frog species that are particularly abundant during the rainy season (*Hypopachus variolosus, Leptodactylus* sp.). Specimens with lengths greater than 500 mm (adults) contained rodents (*Liomys salvinii, Sigmodon hispidus*) and large lizards (*Ctenosaura similis*).

Activities of the Venom and Neutralization by Antivenom—A similar, but not identical, electrophoretic pattern was found in venom proteins of adults and juveniles (Fig. 1). In both cases, nine bands were observed, with molecular

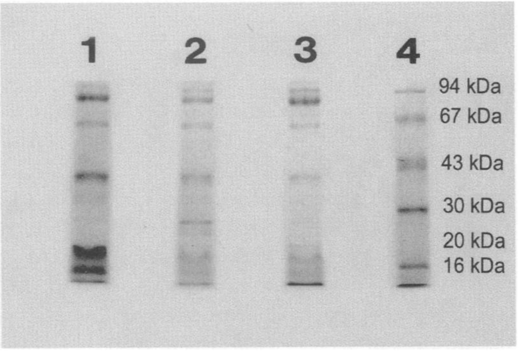

FIG. 1—Gel electrophoresis of the venom of *Agkistrodon bilineatus*. Electrophoresis was run in 12% polyacrylamide gels under reducing conditions. 1) Adult *A. b. bilineatus* from Guatemala; 2) Adult *A. b. howardgloydi* from Costa Rica; 3) Juvenile *A. b. howardgloydi* from Costa Rica; 4) Molecular weight markers.

weights of 95, 90, 82, 67, 42, 28, 23, 20, and 16 kDa. Along with these common bands, venom of adults had an additional band (26 kDa) that was not present in juveniles. The 28 kDa band was brighter in venom of adults than in juveniles. The electrophoretic pattern of venom of adults of *A. b. howardgloydi* from Costa Rica was identical to the pattern in *A. b. bilineatus* from Guatemala (Fig. 1).

Lethal dose (LD_{50} by the intraperitoneal route) of venom of adult *A. b. howardgloydi* was 1.25 μg/g mouse. Antivenom neutralized the lethal effect of venom with an ED_{50} of 0.98 ml antivenom/mg venom. Venom had a strong hemorrhagic activity with a MHD of 0.12 μg. Antivenom also neutralized this activity with an ED_{50} of 1.06 ml antivenom/mg venom. Indirect hemolytic activity was observed, and the MHD was estimated at 0.20 μg. Antivenom neutralized this activity, with an ED_{50} of 2.17 ml antivenom/mg venom. Histological analysis of mouse skeletal muscle injected with *A. b. howardgloydi* venom showed evidence of conspicuous myonecrosis with muscle fibers having hypercontracted masses of myofibrils in the cellular space. This pattern was qualitatively similar to that induced by the venom of *Bothrops asper* (Gutiérrez et al., 1984). In addition, hemorrhage was evidenced by abundant erythrocytes in the interstitial space. In samples collected 24 h after injection, a prominent inflammatory infiltrate was observed with abundant polymorphonuclear leukocytes and macrophages.

DISCUSSION—*Agkistrodon b. howardgloydi* occurs in many types of dry forest, especially along the ecotonal edge with tropical scrub habitat. Contrary to the general belief [as stated in Villa (1983)] that this snake is semiaquatic, we found it in a variety of terrestrial habitats, never associated with water. The northern subspecies, *A. b. taylori*, also seemed to prefer the ecotonal area between tropical scrub and semi-deciduous forest, and not the riparian habitats (Burchfield, 1982).

In Santa Rosa, births in *A. b. howardgloydi* apparently occur between late May and August, coincident with the first half of the rainy season. A similar reproductive cycle was described for *Crotalus durissus* in that region (Solórzano and Cerdas, 1988), with copulation taking place early in the dry season (December to February) and births occurring early in the rainy season. Burchfield (1982) observed copulations of captive *A. b. taylori* from Tamaulipas in late January and February. In this subspecies, litters of four to nine neonates were born from late June to October, at the beginning of the cold rainy season in that region.

Fecal analyses indicated that the cantil preys on a diverse group of taxa (Table 1). This observation is further supported by previous observations summarized by Gloyd and Conant (1990) that diets of cantils include snakes (*Imantodes gracillimus* [=*gemmistratus*], *Thamnophis* sp.), rodents (*Liomys pictus, L. irroratus, Mus musculus, Peromyscus leucopus, Oryzomys palustris*), frogs (*Hyla squirella*), and grasshoppers. Moreover, our data indicate that a change in diet appears to occur as the snakes mature. Ontogenetic shifts in diet from frogs and lizards to mammals also have been reported for other pitvipers (i.e., *Bothrops asper, B. moojeni, C. viridis*, see also Greene [1992]), and usually are related to changes in venom composition within different age groups (Mackessy, 1988). In *Cerrophidion godmani* (Sasa, 1997), no age-related changes in diet or differences in the venom composition of adult and juveniles are evident. Similarly, dietary shift in *Agkistrodon bilineatus* may not be related to a change in toxin composition, as suggested by the similarities of venom electrophoretic patterns of juveniles and adults. However, the low amount of venom collected from juvenile specimens precluded other analyses and, therefore, it is not clear if biological activities of their venom differ from those of adults as reported here. Whether ontogenetic changes in venom and diet composition reflect a phylogenetic trend or are influenced by selective forces acting in an ecological context is still unclear.

Lethal doses in mice were slightly lower than those reported by Bolaños (1972) for two specimens of *A. b. howardgloydi* collected in northern Costa Rica (1.70 μg/g I.P.), and by Burchfield (1982) for *A. b. taylori* (2.30 μg/g). Venom of *Agkistrodon b. howardgloydi* has highly lethal, hemorrhagic, indirect hemolytic and myotoxic activities. Almost identical lethal and hemorrhagic effects were described for the venom of *A. b. bilineatus* from Guatemala (Rojas et al., 1987). It is likely that this venom would induce prominent local tissue damage in humans. A hemorrhagic metalloproteinase, named bilitoxin, was isolated from this venom (Ownby et al., 1990), and a myotoxic phospholipase A_2 also was purified (Mebs and Samejima, 1986). On the other hand, indirect hemolytic effect is due to the action of phospholipases A_2 on egg yolk phospholipids with the consequent release of lysolecithin, which in turn induces lysis of erythrocytes by detergent action (Tu, 1977).

No well-documented clinical reports of envenomation by this species in Costa Rica are available, although it is likely that they would be characterized by local and systemic alterations similar to that reported for other North American species of this genus (Gómez and Dart, 1995). The polyvalent antivenom produced at Instituto Clodomiro Picado was effective in the neutralization of lethal, hemorrhagic and indirect hemolytic activities in our experiments, despite the fact that *A. bilineatus* is not included in the immunizing mixture used for production of this antivenom (Bolaños and Cerdas, 1980). Lomonte et al. (1987) observed that antibodies to the lancehead, *Bothrops asper*, myotoxins cross react against proteins in the venom of *A. bilineatus*. Thus, in agreement with an earlier study (Rojas et al., 1987), our results strongly suggest that this polyvalent antivenom is likely to be effective in the treatment of envenomation by *A. bilineatus* in Central America.

Despite the extensive distribution of the cantil, it is difficult to find in most parts of its range. Recent efforts made by one of us (M. S.) to find specimens in the places where it is known to occur, including the dry valleys of Río Lagarteros (Department of Huehuetenango) and Río Chixoy (Department of Alta Verapaz) in Guatemala, and in several localities in Golfo de Fonseca (Honduras) and Granada (Nicaragua), yielded no specimens. Furthermore, it was noted that most locals in these regions were not aware of the presence of this species there, thus confirming that low population densities (or local extinction) may be the trend in most of the range of the species. Unfortunately, large portions of habitat for *A. bilineatus* have been destroyed for agricultural purposes within the last few centuries. Habitat disturbance is the main reason that "the cantil, except in still remote areas or where it is protected, as in the Parque Nacional Santa Rosa, Costa Rica, is now almost certainly a rare snake," (Conant, 1992:29).

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