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Identification of a Neurotoxic Venom Component in the Tiger Rattlesnake, *Crotalus tigris*

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ABSTRACT.—Previous toxicological and immunology assays have strongly suggested that a neurotoxic component is present in the venom of the Tiger Rattlesnake, *Crotalus tigris*. However, there has been no direct identification of this neurotoxin. We obtained 18 blood samples from Arizona *C. tigris* and analyzed them by PCR and DNA sequencing using primers specific for the acidic and basic subunits of Mojave toxin. All 18 samples demonstrated the presence of both subunits. Venom collected from five of the 18 snakes that provided blood samples were additionally tested for the presence of Mojave toxin with monoclonal antibodies. These anti-Mojave toxin antibodies recognized all five venoms. We conclude that, at least for the Arizona snakes sampled, the neurotoxin in *C. tigris* is Mojave toxin. Additional sampling and testing is necessary to determine the complete geographic distribution of Mojave toxin and Mojave toxin subunits in *C. tigris* populations.

Various authors have investigated venom from the Tiger Rattlesnake, *Crotalus tigris*, using toxicological and immunological assays. Minton and Weinstein (1984) reported venom of *C. tigris* as the most toxic of rattlesnake venoms based on LD₅₀ analysis. Klauber (1972) and Minton and Weinstein (1984) noted that venom of *C. tigris* was comparable in toxicity to the Mohave Rattlesnake, *Crotalus scutulatus* and the tropical rattlesnake, *Crotalus durissus*, both of which have extremely toxic venom. Neurotoxic properties of *C. tigris* venom have been suggested based on the effects of envenomated animals (Githens and Wolff, 1939; Minton and Weinstein, 1984). Glenn and Straight (1985) screened for antigen-antibody immunoprecipitation using Ouchterlony immunodiffusion and detected positive reactions for the basic subunit for Mojave toxin in *C. tigris*. Weinstein et al. (1985) used enzyme-linked immunosorbent assays (ELISA), Ouchterlony immunodiffusion, and immunoelectrophoretic (Western) transfer to show that venoms from *C. tigris* elicited positive responses for Mojave toxin. Henderson and Bieber (1986) using both (ELISA) and Ouchterlony immunodiffusion provoked positive responses to both the acidic and basic subunits for Mojave toxin in *C. tigris*. Polyclonal antibodies prepared against Mojave toxin from *C. scutulatus* indicated antigenic identity (Weinstein and Smith, 1990).

Mojave toxin is a neurotoxic venom component found in some *C. scutulatus* (Glenn and Straight, 1978; Glenn et al., 1983; Rael et al., 1984; Wilkinson et al., 1991). The expression of Mojave toxin in *C. scutulatus* in the United States has been shown to vary geographically. For example, various authors have identified populations of *C. scutulatus* in central Arizona that do not express Mojave toxin (Glenn and Straight, 1978; Glenn et al., 1983; Wilkinson et al., 1991). Mojave toxin

is a presynaptically acting neurotoxin (Gopalakrishnakone et al., 1980) that is composed of two peptides (Aird et al., 1985; Zepeda et al., 1985), a nontoxic acidic subunit (subunit A) and a phospholipase A₂ (PLA₂) basic subunit (subunit B), which is mildly toxic (Cate and Bieber, 1978). Both subunits must be present to produce Mojave toxin, which can increase the venom toxicity 10 to 30 times (Tu et al., 1982).

The Tiger Rattlesnake, *C. tigris* (Kennicott, 1859), is a medium-sized (45–90 cm) crotalid with a conspicuously small head and large rattle in proportion to its body size. *Crotalus tigris* inhabits desert canyons and foothills from sea level to 1400 m and occurs from south central Arizona southward to the southern area of Sonora, Mexico (Klauber, 1972; Stebbins, 1985). A relatively inoffensive snake, individual *C. tigris* usually do not rattle, strike, or bite unless significantly agitated (Klauber, 1972; Lowe et al., 1986). However, Weinstein and Smith (1990) reported it to be highly irritable and quick to strike.

Toxicological and immunological analysis suggests that a neurotoxic component is present in the venom of at least some *C. tigris*. Immunological data also suggest this neurotoxic component is closely related to Mojave toxin, the neurotoxin found in *C. scutulatus*. However, thus far there has been no direct identification of the neurotoxin in this species. The goal of this study was the identification of the neurotoxic venom component in *C. tigris* using DNA analysis of blood and toxin specific immunological analysis of venom.

MATERIALS AND METHODS

Blood ($N = 18$) and venom ($N = 5$) were obtained from *C. tigris* from the Arizona counties of Maricopa, Pima, and Santa Cruz (Fig. 1). During collection of blood and venom samples, snakes were immobilized in appropriately sized clear acrylic tubes that allowed for safe handling and prevented unnecessary injury to the snake (Murphy, 1971). Blood collection was via caudal

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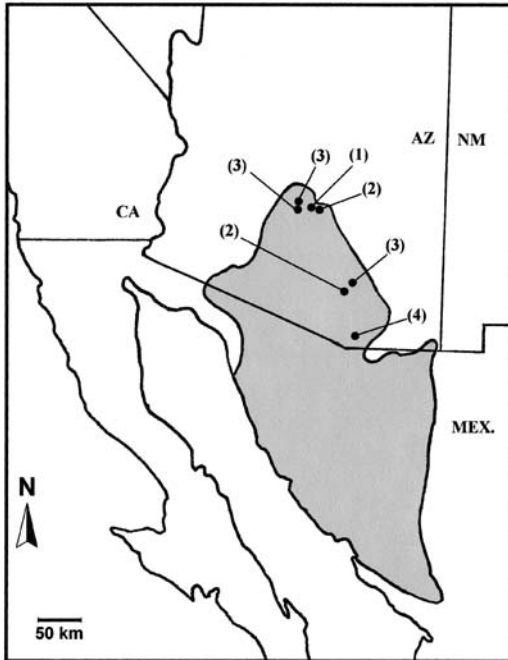


FIG. 1. Range map of *Crotalus tigris* with sample sites. The number of individuals sampled from each location appears in parentheses. Subunits A and B were present in all individuals sampled.

vein puncture as described by Esra (1975) and Bush and Smeller (1978). For venom collection, snakes were allowed to bite sterile 135-mL plastic collection containers that were covered with parafilm. No pressure was applied to the venom glands during the extraction procedure. Collection of venom from *C. tigris* proved to be difficult, as many individuals were reluctant to bite or failed to discharge venom. In addition, venom yields from *C. tigris* are low as compared to other rattlesnakes of equivalent length and body size (Klauber, 1972; Minton and Weinstein, 1984). Blood was stored in lysis buffer (Longmire et al., 1997) at ambient room temperature, out of direct light. Extracted venom was frozen at -80°C and lyophilized.

DNA was extracted using Masterpure MPC Protein Precipitation Reagent (Epicentre Technologies, Madison, WI) and phenol/chloroform/isoamyl alcohol (25:24:1). Primers for the amplification of gene sequences were used based on genomic DNA sequences for Mojave toxin acidic and basic subunits (John et al., 1994) and as described by Wooldridge et al. (2001). Both Mojave toxin acidic subunit primers and Mojave toxin basic subunit primers anneal to the second (sense) and fourth (antisense) exons. The specificity for these sites was verified using BLAST (National Center for Biotechnology Information). PuReTaq Ready-To-Go beads (Amersham Biosciences Corp., Piscataway, NJ) were used for DNA amplification with PCR parameters following Powell (2003); Subunit A, (32 cycles) 94.0°C for 30 sec, 48.0°C for 45 sec, and 72.0°C for 1 min; Subunit B, (32 cycles) 94.0°C for 30 sec, 53.0°C for 45 sec and 72.0°C for 1 min. Amplified product was electrophoresed in agarose and visualized with ethidium bro-

mid. Successful amplifications were purified using a Gene Clean kit (Bio 101 Systems, Carlsbad, CA). Purified DNA was visualized and quantified using Mass-Ruler DNA Ladder (MBI Fermentas Inc., Hanover, MD).

The DNA product was amplified by PCR using a SequiTherm Excel II DNA Sequencing Kit-LC (Epicenter Technologies, Madison, WI) with appropriate sequences using dye-labeled primers (LI-COR Biosciences, Lincoln, NE). PCR products were electrophoresed in a 3.7% polyacrylamide gel in 0.8% TBE buffer utilizing a LI-COR automated LongReadIR 4200 DNA sequencer (LI-COR Biosciences, Lincoln, NE) at 3000 V, 25.0 mA, 45.0 W at 45°C , for 10 h. Nucleotide sequences were analyzed and aligned using BLAST (National Center for Biotechnology Information) and the Vector NTI Suite program (InforMax, Inc., North Bethesda, MD). Sequences were compared to the published Mojave toxin acidic and basic subunits with specific sequence identities for the regions of the acidic subunit, intron 2, exon 3, and intron 3 (base sequence 1746-2795) and the basic subunit, intron 2, exon 3, and intron 3 (base sequence 947-1968) as previously identified by John et al. (1994).

Venom was analyzed for the presence of Mojave toxin using Western Blots as described by Rael et al. (1993). The primary antibody for the reaction was CSS12, a mouse monoclonal antibody that is specific to Mojave toxin (Rael et al., 1986; Martinez et al., 1989).

RESULTS

PCR products generated from successful amplifications resulted in similar fragments of two sizes. The Mojave toxin subunit A primer pair amplified a 1250-base sequence and the Mojave toxin subunit B primer pair amplified a 1150-base sequence which is as expected according to published sequences for both subunits in *C. scutulatus* (John et al., 1994). None of the individuals tested had amplified fragments that were of different size (smaller or larger) than the 1250 (A)/1150 (B) bases. Additionally, none of the individuals tested produced multiple fragments during amplification. All *C. tigris* tissue samples tested ($N = 18$) successfully amplified both the acidic and the basic subunits (Fig. 1).

The products we generated aligned correctly to the nucleotide sequences in the BLAST database search with the published Mojave toxin A and B subunits. Sequence identities for the 18 *C. tigris* that were amplified by the acidic and basic subunit primers produced an average overall similarity of 92.6% when compared to the published Mojave toxin A subunit for the intron 2, exon 3, and intron 3 (base 1746-2795) region. Sequence identities amplified by the basic subunit primers produced an average overall similarity of 95.7% when compared to the published Mojave toxin B subunit for the intron 2, exon 3, and intron 3 (base 947-1968) region.

Testing venom for Mojave toxin using the anti-Mojave toxin antibody (CSS12) produced results in accord with the PCR analysis. Venom from five of 18 (26%) specimens was tested. The anti-Mojave toxin antibodies recognized all of the venoms, indicating that Mojave toxin was detected by this assay also.

DISCUSSION

These data indicate that the neurotoxic constituent in venom of *C. tigris* is Mojave toxin, and that *C. tigris*

produce Mojave toxin over a range of sites in Arizona (Fig. 1). However, no specimens were tested from the western area of their range within the United States or from Mexico. Considering previous work showing that distributions of Mojave toxin and Mojave toxin subunits are not geographically uniform in other crotalids (Glenn et al., 1983; Wilkinson et al., 1991; Wooldridge et al., 2001; French, 2002; Powell, 2003), it is possible that Mojave toxin in *C. tigris* may not be universal across their entire range and there may be regions in Mexico or the United States where *C. tigris* may lack one or both of the subunits to produce the toxin. Additional sampling and testing is necessary to determine the complete distribution of Mojave toxin and Mojave toxin subunits in *C. tigris*.

Prior to Wooldridge et al. (2001), testing for the presence of Mojave toxin in snake venoms was based solely on antibody detection, both monoclonal and polyclonal. The anti-Mojave toxin antibody (CSS12) developed by Rael et al. (1986) used a monoclonal cell line that is, unfortunately, no longer extant. The anti-Mojave toxin monoclonal antibody (CSS12) is thus available in a limited and diminishing supply. Additionally, the monoclonal antibodies detect the product of two presumably independent genes. Only when both Mojave toxin subunits A and B are present and expressed is there antibody detection. Clearly, the ability to test for the presence or absence of each subunit independently is important for understanding the evolutionary and ecological genetics of the subunits and provides valuable information for interpreting gene flow and distribution. This information may also be useful for predicting where populations of neurotoxin-producing snakes could be expected to occur. Additionally, the anti-Mojave toxin antibody (CSS12) and PCR with sequencing have shown congruency in previous studies where both methods of detection of Mojave toxin have been employed (Wooldridge et al., 2001; French, 2002; Powell, 2003). Whereas testing venom with the anti-Mojave toxin antibody (CSS12) has previously been the standard for positive identification of the presence of Mojave toxin, verification of the presence of Mojave toxin with PCR analysis and subsequent sequencing of the amplified product is a viable procedural substitute and may supplant antibody testing (Powell, 2003).

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A Quantitative Analysis of the Ancestral Area of Rattlesnakes

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ABSTRACT.—Establishing the ancestral area of a taxon is a central theme of historical biogeography. We used the cladistic method of ancestral area analysis devised in 1992 by K. Bremer to determine the ancestral area of rattlesnakes (genera *Crotalus* and *Sistrurus*). We then extended this approach to determine the vegetation type of the ancestral area. The most probable ancestral area of rattlesnakes is the Madrean Occidental. The most probable vegetation of the ancestral area was pine-oak forest. Knowledge of the ancestral area and its vegetation was applied to macroevolutionary hypotheses regarding the evolution of the rattlesnake rattle.

Establishing the ancestral area of a group of organisms is a central theme of historical biogeography (Brown and Lomolino, 1998). The purpose of this paper is threefold. First, we used Bremer's (1992) cladistic approach to historical biogeography to provide the first quantitative assessment of the ancestral area of rattlesnakes. Second, we applied Bremer's approach to determine the vegetation type of rattlesnakes' ancestral area. Third, we applied the results of our historical analysis to macroevolutionary hypotheses.

Rattlesnakes are a monophyletic (Parkinson, 1999; Murphy et al., 2002), New World group of pitvipers comprising two genera, *Crotalus* and *Sistrurus* (Viperidae: Crotalinae). Approximately 32 species of rattle-

snakes are currently recognized (McDiarmid et al., 1999; Douglas et al., 2002). Rattlesnakes are distributed from Canada to Argentina and occur in most major habitat types including arid deserts, temperate marshes, and tropical forests (Klauber, 1972).

There are a few qualitative studies concerning the historical biogeography of rattlesnakes. Brattstrom (1964) implied in his figures 39 and 41 that rattlesnakes originated on the central plains of North America. Gloyd (1940) stated that the north-central portion of the Mexican Plateau was the center of origin of rattlesnakes and cited as evidence that the "... *triseriatus* group ... is almost entirely confined to the Mexican Plateau" and "... in most groups the probable 'stem' forms occupy at least a portion of the Mexican Plateau." Klauber (1972) and Greene (1997) each suggested that rattlesnakes originated in Mexico, primarily because several morphologically primitive species of rattlesnakes occur in

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Mexico. Klauber did not indicate a specific region within Mexico. Greene specified the Mexican Plateau as the ancestral area of rattlesnakes.

In addition to the lack of quantitative data on the historical biogeography of rattlesnakes, we could not find any literature on the habitat associations of ancestral rattlesnakes. Data on ancestral habitats is important for development of hypotheses concerning paleoecology of rattlesnakes. Vegetation patterns change dramatically over time. Thus, increased confidence in inferring the ancestral habitat type could emerge only if it can be shown that habitats in the ancestral area have changed little since the initial divergence. Palynological data indicate that pollen typical of the present pine-oak habitats of the region were already present in the middle to late Tertiary (Graham, 1987, 1989). Armstrong and Murphy (1979) give vegetational associations of extant rattlesnakes in Mexico.

Bremer's (1992) cladistic method makes two assumptions. The first assumption is that vicariance events cannot explain the current distribution of the taxon under investigation. The estimated date of divergence of rattlesnakes from their common ancestor with *Akistrodon* is recent enough (20–30 mya; Knight et al., 1993) to preclude vicariance via mountain forming in Central America (40–60 mya; Graham, 1989). Our application of Bremer's method meets this assumption.

The second assumption assumes the taxon being investigated dispersed from its ancestral area. The method is applied to taxa that have a worldwide distribution, but occur in smaller distinct locales. Our application of Bremer's method does not meet this assumption. However, although rattlesnakes are not cosmopolitan, we feel their distribution is sufficiently large to warrant application. The extension of this method to narrowly distributed taxa has been used by Rodriguez-Robles and Jesus-Escobar (1999) and Pitra et al. (2002) in their studies of lampropeltine snakes and otidid bustards, respectively.

MATERIALS AND METHODS

We limited our analysis to Mexico for two reasons. First, all but six of the approximately 32 recognized species of rattlesnakes occur there (Campbell and Lamar, 1989). Second, there is general consensus that rattlesnakes originated in Mexico (Armstrong and Murphy, 1979; Klauber, 1972; Greene, 1997).

Separate analyses were run to determine the ancestral area and vegetation type of the ancestral habitat of rattlesnakes. To determine the ancestral area, the physiographic regions of Mexico were used as characters. These regions were defined based on the data of Campbell and Lamar (1989; see their table 4) who reported the distribution of rattlesnakes by physiographic region. The habitat used by ancestral rattlesnakes was determined using the primary vegetation types in Mexico as described and given in Campbell and Lamar (1989; see their table 5) as characters. We treated habitat in a manner similar to area because we assumed species could disperse into other neighboring habitat types the same way they disperse into neighboring land areas. Palynological evidence suggests the current plant communities have changed little since the middle to late Tertiary (Graham, 1987, 1989).

Ancestral area was determined using Bremer's (1992) method. Physiographic regions were optimized

onto the preferred rattlesnake phylogeny of Murphy et al. (2002; see their fig. 6) using forward and reverse Camin-Sokal parsimony with MacClade 4.0 (Maddison and Maddison 2000). All rattlesnake taxa not inhabiting Mexico were trimmed from the phylogeny (i.e., *Crotalus adamanteus*, *Crotalus horridus*, *Crotalus vegrandis*, *Crotalus unicolor*, and *Sistrurus miliarius*) prior to optimization, leaving 25 taxa in the analysis. Comparing the gains (i.e., the presence of a taxon in an area) and losses (i.e., absence of a taxon in an area) of the two optimizations allowed us to infer the ancestral area. We rescaled the gain-to-loss ratios (referred to as AA, for ancestral area) for each character by dividing each ratio by the largest ratio among all the regions. The region with the largest AA has the highest probability of being the ancestral area (Bremer, 1992). The same procedure was followed using the vegetation type in place of the physiographic regions. Thus, the vegetation type with the highest AA is the most probable ancestral vegetation type.

Previous applications of Bremer's (1992) method have not used statistical tests to determine the significance of differences between the resulting gain-to-loss ratios or AA ratios. We assumed the gain-to-loss ratios in our analyses were drawn from normally distributed populations. One-sample Kolmogorov-Smirnov tests indicated the physiographic regions data did not differ from a normal distribution ($P = 0.200$). The vegetation data was not normally distributed ($P = 0.018$) but was normal following a \log_{10} transformation ($P = 0.200$). The ratios (or their transforms) were converted to Z-scores (Sheskin, 1997). Z-scores were then used to determine the probability of occurrence of a given ratio. The right tail probability of the standard normal curve indicates the probability of occurrence of a ratio more extreme than the one tested.

Previous studies have suggested that ratios may have undesirable statistical properties (Atchley et al., 1976). For example, as the coefficient of variation of the denominator of a ratio increases, the distribution of a ratio becomes right skewed and leptokurtic (Achely et al., 1976). The coefficients of variation of the losses (the denominator of our ratios) in both the physiography and the vegetation data are substantially smaller than the gains. Hence, the skewness and kurtosis of the distribution of our ratios changes little relative to the distributions of the original two variables (Atchely et al., 1976).

RESULTS

Table 1 shows that the Sierra Madre Occidental is the most probable ancestral area ($Z = 1.55$, $P = 0.060$) followed by the Mexican Plateau ($Z = 1.01$, $P = 0.157$). The Maderean Occidental is adjacent to the Mexican plateau and extends from the extreme southern regions of New Mexico and Arizona, United States, in the north to the Rio Grande de Santiago in Jalisco, Mexico, in the south (Campbell and Lamar, 1989). The next most probable ancestral areas in order of decreasing probability are the Sierra Madre Oriental ($Z = 0.94$, $P = 0.175$), Peninsula of California ($Z = 0.72$, $P = 0.236$), Transverse Volcanic Cordillera ($Z = 0.61$, $P = 0.271$), Pacific Coastal Lowlands ($Z = 0.46$, $P = 0.321$), Sierra Madre del Sur ($Z = -0.11$, $P = 0.546$), Rio de las Balsas Basin ($Z = -0.66$, $P = 0.745$), and Gulf Coastal Lowlands ($Z = -0.91$, $P = 0.819$). The Southern

TABLE 1. Estimated ancestral area of the rattlesnakes (*Crotalus* and *Sistrurus*) following the method of Bremer (1992) using the physiographic regions given in table 4 of Campbell and Lamar (1989) to define the area characters used in the analysis.

Character	Gains	Losses	G/L	AA	Z-score	P-value
Sierra Madre Occidental	9	10	0.90	1.00	1.55	0.060
Mexican Plateau	6	8	0.75	0.83	1.01	0.157
Sierra Madre Oriental	8	11	0.73	0.81	0.94	0.175
Peninsula of California	6	9	0.67	0.74	0.72	0.236
Transverse Volcanic Cordillera	7	11	0.64	0.71	0.61	0.271
Pacific Coastal Lowlands	6	10	0.60	0.67	0.46	0.321
Sierra Madre del Sur	4	9	0.44	0.49	-0.11	0.546
Rio de las Balsas Basin	2	7	0.29	0.32	-0.66	0.745
Gulf Coastal Lowlands	2	9	0.22	0.25	-0.91	0.819
Southern Mexico Highlands	1	7	0.14	0.16	-1.20	0.885
Isthmus of Tehuantepec	1	7	0.14	0.16	-1.20	0.885
Yucatan Peninsula	1	7	0.14	0.16	-1.20	0.885

Mexican Highlands, Isthmus of Tehuantepec, and the Yucatan Peninsula shared the lowest probability ($Z = -1.20$, $P = 0.885$) of being the ancestral area.

Table 2 shows that the most probable vegetation type of the ancestral rattlesnakes' habitat was pine-oak ($Z = 1.74$, $P = 0.041$) and is characteristic of much of the Mexican Plateau and its bordering mountain ranges—the Madrean Occidental and Oriental ranges (Campbell and Lamar, 1989). The remaining habitats in order of decreasing probability of being the ancestral vegetation type are desert ($Z = 1.24$, $P = 0.108$), mesquite-grassland ($Z = 0.88$, $P = 0.190$), and tropical deciduous forest ($Z = 0.29$, $P = 0.385$). Boreal forest, chaparral, and thorn forest shared the next lowest probability of being the ancestral vegetation type ($Z = 0.12$, $P = 0.454$). Arid tropical scrub was the next most probable vegetation type ($Z = -0.18$, $P = 0.571$). Cloud forest and savanna shared the next lowest probability ($Z = -0.62$, $P = 0.733$). Rain forest and tropical evergreen forest were the least probable ancestral vegetation type ($Z = -1.54$, $P = 0.938$).

DISCUSSION

Our results (1) provide the first quantitative assessment of the ancestral area of rattlesnakes; (2) provide the first estimates of the ancestral habitats of rattlesnakes; (3) support the hypothesis that the ancestral area of rattlesnakes is in North-Central Mexico; (4) demonstrate that the method of Bremer can be used to determine ancestral habitats of rattlesnakes and other taxa.

The results presented here suggest that the most probable ancestral area of the rattlesnakes is the Madrean Occidental. This is contrary to Brattstrom's (1964) suggestion of a central North American origin. Although Brattstrom did not make a quantitative analysis of the ancestral area of the rattlesnakes as we did, he indicated in his figures 39 and 41 that rattlesnakes diverged from ancestral pitvipers in central North America. Additionally, he suggests that the *Lachesis-Crotalus-Sistrurus* "stock" diverged soon after the arrival of an *Agkistrodon contortrix*-like ancestor in the New World via the Bering Land Bridge.

TABLE 2. Estimated ancestral habitat association of the rattlesnakes (*Crotalus* and *Sistrurus*) following a modification of the method of Bremer (1992) using the vegetation types given in table 5 of Campbell and Lamar (1989) to define the characters used in the analysis.

Character	Gains	Losses	G/L	AA	Z-score ¹	P-value
Pine-Oak forest	12	7	1.70	1.00	1.74	0.041
Desert	7	6	1.20	0.68	1.24	0.108
Mesquite-grassland	8	9	0.89	0.52	0.88	0.190
Tropical deciduous forest	4	7	0.57	0.33	0.29	0.385
Boreal forest	5	10	0.50	0.29	0.12	0.454
Chaparral	5	10	0.50	0.29	0.12	0.454
Thorn forest	4	8	0.50	0.29	0.12	0.454
Arid tropical scrub	4	10	0.40	0.23	-0.18	0.571
Cloud forest	2	7	0.29	0.16	-0.62	0.733
Savanna	2	7	0.29	0.16	-0.62	0.733
Rainforest	1	7	0.14	0.08	-1.54	0.938
Tropical evergreen forest	1	7	0.14	0.08	-1.54	0.938

¹ Z-score of the log₁₀ of G/L.

Recent molecular phylogenetic studies of pit vipers indicate monophyly in a New World temperate group (*Agkistrodon*, *Crotalus*, and *Sistrurus*) and in a tropical group (bothropoid genera and *Lachesis*; Parkinson et al., 2002). This is inconsistent with Brattstrom's hypothesis, because he proposed that *A. contortrix* was an Old World migrant and that *Crotalus* and *Sistrurus* were closely related to *Lachesis*. Clearly *Agkistrodon* is of New World origin, and *Lachesis* is more closely related to a bothropoid genus than to rattlesnakes (Parkinson et al., 2002). Our results support the notion that, following their divergence from the common ancestor with *Agkistrodon*, the rattlesnakes as a group dispersed northward into North America and southward into South America (Greene, 1997; Parkinson, 1999; Parkinson et al., 2002).

Our identification of an ancestral area for rattlesnakes based on a quantitative assessment allows us to begin to address macroevolutionary questions (Brown and Lomolino, 1998). How does, for example, the ancestral area and pattern of dispersal of rattlesnakes compare with that of other New World pitvipers? Moreover, are there specific characteristics of the Madrean Occidental or its associated ranges that facilitate speciation among pit vipers and other taxa?

Also of interest from a macroevolutionary perspective is the distribution of potential ancestral predators. It is widely accepted that predator pressure led to the evolution of the rattlesnake rattle (Klauber, 1972; Greene, 1988, 1997). Six families in the mammalian order Carnivora have been recovered from Miocene formations in North America (Savage and Russell, 1983). Most notable among these fossils is the species richness of mustelids and procyonids. Members of these families are typically active foragers, often probing into cavities in search of vertebrate and invertebrate prey. Klauber (1972, and references therein) identified as known predators of rattlesnakes: badgers (*Taxidea taxus*), raccoons (*Procyon lotor*), skunks (*Spilogale gracilis*, *Conepatus* sp.), and ring-tailed cats (*Bassariscus astutus*). All of these rattlesnake predators inhabit brushy or wooded habitats (Burt and Grossenheider, 1980).

Inferences about the ancestral predators and the ancestral habitat could be used to assess macroevolutionary hypotheses regarding the evolution of the rattlesnake rattle. Our data suggest that pine-oak forest was probably the habitat occupied by the first rattlesnakes. The Madrean-talus-predator hypothesis (Greene, 1997) predicts a talus-strewn habitat for early rattlesnakes, whereas the trampling-ungulate hypothesis (Hay, 1887; Barbour, 1922) predicts a grassy, prairie ancestral habitat. This second hypothesis can be discarded, whereas the former may, at most, be tentatively supported.

Campbell and Lamar's (1989) interpretation of pine-oak forest includes dense stands of pine forest, pine-oak woodlands, piñon-juniper woodlands, and oak scrub. As we accumulate information on habitat use in the basal lineages of rattlesnakes, we will be able to refine the description of the various pine-oak habitats and more sharply define the ancestral habitat.

The results presented here should be regarded as preliminary because only Bremer's method was used to determine ancestral area. Morrone and Crisci (1995) advocate using multiple methods (e.g., area clado-

grams, parsimony analysis of endemics) to address the same problem. Corroboration between several distinct techniques would make these conclusions more robust. The estimation of the ancestral areas of other New World pit viper genera will allow us to formulate and test hypotheses about modes of speciation and cladogenesis. Determining the historical biogeography of potential past predators will allow us to formulate hypothesis about ecological factors and their impact on species origins and radiation.

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Is Coloration of Juvenile Male Collared Lizards (*Crotaphytus collaris*) Female Mimicry?: An Experimental Test

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ABSTRACT.—Juvenile male Collared Lizards (*Crotaphytus collaris*) have orange, dorsolateral color patterns that closely resemble those of gravid, adult females, and it has long been hypothesized that they serve as a form of female mimicry, reducing aggression from adult males. We experimentally tested this hypothesis by painting juvenile males to remove or maintain orange coloration and measuring the agonistic response of adult males but found no significant differences between treatments. These results do not support the hypothesis that orange coloration of juvenile male Collared Lizards is used as a form of female mimicry to reduce aggression from adult males.

Ontogenetic color changes among lizards have received little attention (Cooper and Greenberg, 1992), and few studies detailing the adaptive significance of juvenile coloration exist (e.g., Clark and Hall, 1970; Huey and Pianka, 1977; Cooper and Vitt, 1985). Eastern Collared Lizards (*Crotaphytus collaris*) represent a good model to examine ontogenetic changes in coloration. Eastern Collared Lizards are sexually dichromatic with adult males being bright bluish-green dorsally with

a pale to near-fluorescent yellow head, whereas adult females are wholly brown to dull olive (McGuire, 1996; McCoy et al., 1997). During the reproductive cycle, females develop orange, dorsolateral bars that presumably cycle in intensity with ovarian steroid hormones (Cooper and Ferguson, 1972; Ferguson, 1976).

Many authors have suggested that in *C. collaris* the orange bars of females inhibit male courtship and/or aggression (e.g., Carpenter, 1967; Cooper and Ferguson, 1972; Cooper and Greenberg, 1992). However, there have been numerous observations of males courting females with orange bars (Yedlin and Ferguson, 1973;

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Cooper, 1984; Baird et al., 2001), and a recent experimental test by Baird (in press) revealed that the orange bars actually stimulate male courtship. Juvenile male Collared Lizards display orange markings on their dorsum and sides that are similar to those of gravid, adult females. This coloration is rarely observed in juvenile females. Juvenile males also lack the adult-typical green and yellow coloration until they begin to become sexually mature (Rand, 1986). Although the orange coloration of juvenile males is similar to that of adult females, the hormones that produce them differ in adult females and juvenile males (Rand, 1986).

It has been suggested that in *C. collaris* the orange coloration of juvenile males is a form of female mimicry that functions to inhibit aggression from larger adult males (Fitch, 1967; Cooper and Ferguson, 1972; Bontrager, 1980; Rand, 1986; Cooper and Greenberg, 1992; Carpenter, 1995; McGuire, 1996). This hypothesis was originally proposed primarily because the role of female orange coloration was ambiguous (Cooper and Greenberg, 1992). Similar female mimicry hypotheses have been proposed for juvenile males in other species (e.g., *Gambelia wislizenii*, Montanucci, 1978; *Tropidurus delanonis*, Werner, 1978; *Urosaurus ornatus*, Carpenter, 1995; *Sceloporus gadoviae*, Lemos-Espinal et al., 1996), but only Werner (1978) performed experimental manipulations and was able to provide evidence suggesting a form of female mimicry. Although the hypothesis of female mimicry has been repeatedly cited for *C. collaris*, it has not been empirically tested.

We tested the hypothesis that the dorsolateral orange coloration of juvenile male Eastern Collared Lizards reduces aggression from adult males, that is, adult males should respond more aggressively to juvenile males without orange coloration than to those with it. To test this hypothesis, we experimentally modified juvenile male color patterns using acrylic paint. We also predicted that adult males would respond more aggressively toward large intruders than small ones since a larger intruder presumably represents more of a threat to the resident.

MATERIALS AND METHODS

We conducted two experiments and a descriptive study of the ontogenetic development of orange coloration in juvenile males, each in different Oklahoma populations. The first experiment was conducted during the breeding season in June 1994, at the Wichita Mountains National Wildlife Refuge (WM) in Comanche County, Oklahoma. The second experiment was conducted during the breeding season from late May to early June 2000, at Sooner Lake dam (SL) in Pawnee County, Oklahoma, which is a rip-rap boulder dam substrate. Both populations were the subjects of ongoing studies of social behavior (Husak and Fox, 2003a; McCoy et al., 1997, 2003), allowing us to select subjects of known age. The experiments were not conducted soon after hatchlings emerge when orange coloration may be the brightest (August and September), because adult males are no longer active at this time of the year, and there should be no social pressure from adult males then. The few adult males that may still be active during this time of the year no longer exhibit heightened rates of territorial behavior characteristic of the breeding season (Baird et al., 2001). Juvenile males (less than one year old) were captured from areas away from the

study site to ensure that subject lizards were not familiar with intruders, eliminating any possible confound caused by parentage or dear enemy effects (Fox and Baird, 1992; Husak and Fox, 2003a).

The six intruders at WM were grouped into three size-matched pairs: small (snout-vent length [SVL] 75.0–77.0 mm), medium (SVL 80.0–84.0 mm), and large (SVL 90.0–90.5 mm). One intruder from each size class was selected at random to serve as the intruder with orange coloration and the other as the intruder with no orange coloration. We used acrylic paints to modify the color patterns of both types of intruders to achieve the greatest possible uniformity in color within their respective groups. All lizards had orange coloration when captured, which was subsequently painted over with brown or orange. Orange coloration was added to the lateral surfaces of half of the intruders so that the coloration of these individuals closely resembled the color pattern displayed by gravid female lizards. To the other half of the intruders, orange coloration was covered with paint that matched the background color of that portion of the lizard. Ultraviolet signals were likely not a problem because spectral reflectance data suggest minimal ultraviolet reflectance from the dorsum of male or female collared lizards (Macedonia et al., 2002; J. Macedonia, unpubl. data). Therefore, we feel that the acrylic paints used in our experiments successfully manipulated the potential visual signal as they have done in numerous other behavioral studies of lizards (e.g., Cooper and Vitt, 1985; Quinn and Hews, 2000).

We selected 10 territorial adult male lizards at WM (at least 1.5-years old; SVL > 95.0 mm) as subjects and presented intruders to them, recording the response of each territorial adult male. Each intruder was presented separately to each of the territorial adult males in random order when substrate temperatures were between 30° and 40°C, the optimal temperature range for *C. collaris* in central Oklahoma (Uzee, 1990). Intrusions were staged by tethering the intruder from the bottom of the adjustable central stem of a photographic tripod with approximately 5 cm of monofilament fishing line, allowing movement by the intruder. Intruders were placed 2–3 m from the point at which the subject lizard was observed. For each intrusion, we recorded all aggressive acts by the adult male subject for 10 min (or less in seven cases; see below) after the resident adult male established visual contact with the intruder.

We calculated several different measures of aggression. Total aggressive acts per minute was calculated by dividing the total number of aggressive acts displayed by the resident toward the intruder by the total number of minutes of the intrusion. We also calculated a graded agonism score, where certain acts that are more costly were given a greater weight (following table 1 in Husak and Fox 2003b). We recorded the time elapsed between establishment of visual contact and initiation of aggression (latency). The escalation of aggression (maximum aggression) was also scored as follows: fight then display (highest level of aggression), 5; display then fight, 4; display but not fight, 3; display then retreat, 2; no response, 1. During seven intrusions, the level of aggression by the adult male was so intense that the interaction was stopped before 10 min had elapsed to prevent serious injury to the intruder.

To avoid potential problems of intercorrelations among our measures of aggression, we analyzed the

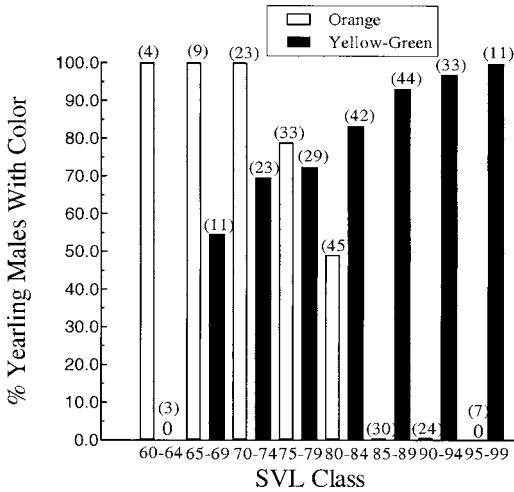


FIG. 1. Percentage of yearling Eastern Collared Lizard males with orange lateral markings (white bars) and/or yellow and green (black bars) on the dewlap and forelimbs (respectively) as a function of body size. Parenthetical numbers are the number of individuals in each size class. Minimum size at sexual maturity in this population as determined by swabs of cloacal fluids is 72 mm SVL (Baird and Timanus, 1998).

relationship between them using a correlation matrix, and discarded those variables that were highly correlated to retained variables (see Results). The results for each of the retained measures of aggression were analyzed using repeated-measures analysis of variance (ANOVA), in which each intrusion was treated as a repeated measure of aggression for one subject (one adult lizard). Size and color treatment of intruders were analyzed as factors within the repeated measures design, allowing us to test for differences in response based on the body size of the intruder, presence/absence of orange coloration on the intruder, and for interactions between these two factors.

The negative data from the WM experiment (see Results) caused us to question the ontogenetic distribution of orange coloration in juvenile males and the intruder sizes used, prompting us to collect size and color data from juvenile male lizards to determine the natural ontogenetic pattern of orange coloration. We recorded detailed data on male coloration from 3 May to 15 July in 1998–2001 at Arcadia Lake in central Oklahoma. We recorded 371 observations on 160 different yearling males for which we measured SVL and classified into one of the three following color categories: (1) juvenile orange color present, neither adult yellow or green present; (2) juvenile orange present, either adult yellow or green (almost always both) present; and (3) juvenile orange absent, either yellow or green (almost always both) present. Collection of these data required that multiple observations were recorded for some of the same males, but because only one observation per male at each size/age was recorded, pseudoreplication was avoided. We never recorded two datapoints for a particular male when the color category had not changed from that of its previous capture.

Our second set of staged, field intrusions was conducted at Sooner Lake (SL). Intruders were grouped into two size-matched pairs: small (SVL 61.5–62.0 mm) and large (SVL 76 mm). Again, one intruder from each size class was selected at random to serve as the intruder with orange coloration and the other as the intruder with no orange coloration. We followed the same protocol as in the WM experiment during intrusions at SL, except that we tethered intruders from the end of an extendable pole with approximately 5 cm of monofilament fishing line. Adult males at SL were also somewhat larger (SVL > 100.0 mm).

RESULTS

The juvenile orange coloration generally takes the form of diffuse, spotted coloration, often in the form of broken or complete vertical bars, on the lateral torso, and occasionally splotches in the occipital and neck region; yellow color first appears on the dewlap, and the first green appears on the forelimbs. There was substantial overlap of orange and yellow-green coloration between 65 mm and 84 mm SVL, with a little overlap up to 94 mm SVL (Fig. 1). Some juvenile lizards exhibited orange coloration until well beyond the average sexually mature size (72 mm SVL, Fig. 1), and some exhibited the adult male-typical yellow-green coloration before reaching the average sexually mature size (Fig. 1). Although the data in Figure 1 are from Arcadia Lake lizards, our extensive experience with the Wichita Mountains and Sooner Lake lizards confirms the trend in coloration overlap for different size classes. The large size range in intruders used in these experiments was appropriate because it spanned the possible natural color patterns and their potential associated threat.

There were statistically significant intercorrelations between latency, maximum aggression, aggressive acts per minute and graded agonism score (pairwise $r > 0.5$ for all, except pairwise $r < -0.5$ for relationships with latency) for WM and SL. Consequently, we analyzed graded agonism score instead of the other variables, because graded agonism score best summarizes the range of intensity of agonistic behavior of the lizards. For the WM data, no significant differences were found for graded agonism score based on the size of the intruder ($F = 0.006$, $P = 0.99$), presence/absence of orange coloration ($F = 0.393$, $P = 0.55$), or the interaction between the two factors ($F = 0.133$, $P = 0.88$). Likewise for the SL data, no significant differences were found for graded agonism score based on the size of the intruder ($F = 2.362$, $P = 0.16$), presence/absence of orange coloration ($F = 0.675$, $P = 0.43$), or the interaction between the two factors ($F = 0.152$, $P = 0.71$).

Examination of the average response by all 10 subject lizards at each site showed several results contrary to our expectations, although they were not statistically significant. At WM, the average response was higher to the intruders with orange coloration than to those without it. The pattern of response to intruder size was also surprising, because the greatest aggression was directed toward the smallest intruders. Indeed, the intruders that should have evoked the least aggression, the small intruders with orange coloration, received the highest level of aggression. At SL, the average response was higher also toward juveniles with orange coloration, contrary to our expectations. However, the differ-

ence in response based on intruder size followed our predictions, and the highest aggression was directed toward the largest intruder, but none of these differences was statistically significant.

DISCUSSION

Similar agonistic responses by territorial adult males toward juveniles with and without orange coloration in two different populations does not support the hypothesis that the orange coloration of juvenile males serves as a form of female mimicry for reducing adult male aggression. Juvenile coloration has been shown to reduce aggression in *Tropidurus delanonis*, perhaps supporting a female mimicry hypothesis for this species, although the size of intruders used in the experiments was not mentioned (Werner, 1978). Cooper and Vitt (1985) tested the hypothesis that the bright blue tails of juvenile *Eumeces fasciatus* and *Eumeces laticeps* are used as an inhibitory signal to adult males for reducing aggression, but they rejected this hypothesis because of a lack of significant difference in responses to normal versus painted juveniles. We reject the female mimicry hypothesis in *C. collaris* for similar reasons.

The orange coloration of juvenile male Eastern Collared Lizards may be expressed together with subordinate behavior, but subordinate behavior alone may be responsible for reduced aggression from adult males. Juvenile males have been shown to behave as satellites, staying close to the edges of adult male territories and displaying subordinate behavior such as low rates of activity and display and greater wariness (Baird et al., 1996; Baird and Timanus, 1998). In this study, juveniles were tethered and could not flee upon challenge by an adult male, a tactic often used by subordinate, satellite males (Baird et al., 1996; Baird and Timanus, 1998).

When viewed from the perspective of the territorial adult males, it seems unlikely that the orange coloration of juvenile males would evolve as a form of female mimicry to reduce aggression toward juvenile males. There appears to be no pressure for adult males to recognize such a visual signal. Males that display orange coloration are often not sexually mature, and they are much smaller than adult males. Juvenile males, then, represent no significant threat to an adult male's fitness, and there would be no advantage for such a signal to evolve. Also, the orange bars on adult females are most likely not a signal to inhibit male courtship and/or aggression. They are more likely a stimulatory signal to males (Cooper and Greenberg, 1992; Baird, in press), with the orange bars becoming the brightest just before ovulation (Ferguson, 1976). A signal to stimulate courtship and subsequent copulation attempts certainly would not be advantageous to juvenile males. In addition, despite the fact that juvenile orange coloration is nearly identical to female orange coloration in spectral properties (J. Macedonia, unpubl. data), the way in which the color is displayed on the body is sometimes very different between gravid females and juvenile males. Adult females have a color pattern of vertical bars, whereas juvenile males are much more variable in the way orange is present; sometimes the orange is arranged in vertical bars, and sometimes it is not.

Further studies should address the hormonal mechanism for the development of orange coloration in

juvenile male Eastern Collared Lizards. The orange coloration is most likely the result of an undetermined hormone circulating prior to sexual maturity, but, unfortunately, it is unclear exactly what hormone is responsible. The color pattern displayed may be a product of some biochemical precursor or byproduct during the metabolic development of coloration from hatchling to adult (i.e., synthesis of the yellow that covers the head, ventral, and ventrolateral areas of the body). It would also be instructive to determine whether the presence of a juvenile conspecific with conspicuous orange coloration in the territory of an adult male increases the probability that a visually oriented predator (e.g., raptors, *Masticophis* snakes) is attracted to that male's territory. The results of this study represent a first step in determining the significance of orange coloration in juvenile *C. collaris*. Additional studies will shed more light on the complexity and subtlety involved in the role of coloration in social interactions of this species.

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