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Using Morphological, Genetic, and Venom Analyses to Present Current and Historic Evidence of *Crotalus horridus* x *adamanteus* Hybridization on Jekyll Island, Georgia

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Abstract - On 17 June 2019, we collected a unique juvenile rattlesnake from a wildlife response call on Jekyll Island State Park, GA. The snake exhibited intermediate color patterns and gross anatomical features suggesting potential hybridization between *Crotalus horridus* (Canebrake/Timber Rattlesnake) and *Crotalus adamanteus* (Eastern Diamondback Rattlesnake). Using mitochondrial and nuclear genetic sequencing, venom analyses, and morphological characteristics to test that hypothesis, we were able to verify that this specimen represents only the second documented observation of natural hybridization between *C. adamanteus* and *C. horridus* and the first reported with multiple lines of evidence sufficient for confirmation. Surprisingly, genetic analyses found evidence of previous introgression between these species, suggesting hybridization may not be a rare occurrence in the area (and perhaps specifically on Jekyll Island). We will continue to monitor the hybrid individual via radio-telemetry to assess its survival and any subsequent F2 hybridization reproduction events.

Introduction

Natural hybridization occurs when genetically distinct taxa produce offspring following reproduction due to natural contact (Mallet 2005). Willis (2013) recognized hybridization as a behavioral response to the conditions of mate choice and, as such, the influences on mate choice that give rise to natural hybridization can come in several forms. Mallet (2005) reported that natural hybrids are rare in terms of individual occurrence, but natural hybridization as a phenomenon may be common in terms of the number of species that have been known to hybridize (Gerhardt et al. 1980, Keck and Near 2009, Schultz 1969, Wayne and Jenks 1991). Although over 10% of animal species and 25% of plant species have been known to naturally hybridize (Mallet 2005), the current understanding of wild hybridization events and the behavioral, environmental, and anthropogenic factors that drive them is poor. Among the most common causes of hybridization in animals is the secondary contact of 2 previously isolated species followed by restricted mate access (Hinton

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et al. 2018, Hubbs 1955, Willis 2013). Secondary contact and restricted mate access can both be the result of natural or anthropogenically driven environmental changes (Rhymer and Simberloff 1996). In some cases, access restriction can be an issue of preference; for instance, Wyman et al. (2011) recognized that individuals tend to exhibit a gradient of preference in terms of mate recognition. In such circumstances, the heterospecific may not be the preferred choice in selection, but a potential choice if no better option is presented.

Consequences of hybridization are well documented. Darwin (1859) was among the first to recognize that putative hybrids often exhibited lesser fitness compared to that of either parental species. Perhaps the most deleterious consequence of hybridization would be the inviability and sterility of offspring due to genetic incompatibility (Orr 1996). On a broad scale, hybridization and introgression have been known to cause population-scale species collapse through gene swamping, leading to population decline and extirpation in several taxa (Orr 1996, Rhymer and Simberloff 1996, Schierenbeck 2011, Schilthuizen et al. 2011). For example, endangerment by natural hybridization and introgression is well-documented in North Carolina's reintroduced endangered *Canis lupus rufus* Audobon and Bachman (Red Wolf) population where hybridization with *Canis latrans* Say (Coyote) inhibits their recovery (Fredrickson and Hedrick 2006).

In rattlesnakes, hybridization has been documented for numerous species (Bailey 1942, Bogert and Oliver 1945, Campbell and Lamar 2004, Campbell et al. 1989, Klauber 1984, Murphy and Crabtree 1988), but the frequency of hybridization in the wild remains poorly understood. Campbell and Lamar (2004) noted ≤10 verified reports, and we were able to find 13 documentations with our literature search (Table 1), although other instances of both captive and natural hybridization have been discussed informally in literature and circulated on social media platforms. *Crotalus adamanteus* Palisot De Beauvois (Eastern Diamondback Rattlesnake [EDR]) occurs throughout the southeastern Coastal Plain of the United States where it is generally associated with open-canopy habitats such as *Pinus palustris* Mill. (Longleaf Pine) savannah, coastal sea islands, upland pine forest, and xeric sandhills (Campbell and Lamar 2004, Howze and Smith 2021, Waldron et al. 2006). Throughout much of its range, the EDR is sympatric with *Crotalus horridus* L. (Canebrake Rattlesnake [CBR]). Otherwise known as the Timber Rattlesnake in populations outside the Coastal Plain, the CBR has a far greater distribution across the United States, occurring from east Texas to New Hampshire, as far northwest as Wisconsin, and as far south as north Florida (Campbell and Lamar 2004). Habitat preference in the CBR is regionally dynamic, with snakes in the coastal plain utilizing pine forests and uplands adjacent to swamps and floodplains, and snakes in the northeast and midwest showing a greater association with open rocky uplands with deep rock access (Campbell and Lamar 2004). Of importance, there are only 3 hypothesized occurrences of EDR x CBR hybrids in the wild (D. Bartlett, Gainesville, FL, pers. comm.; Campbell and Lamar 2004; Klauber 1984). Additionally, at least 3 other species in the subfamily Crotalinae, including *Crotalus atrox* Baird and Girard (Western Diamondback Rattlesnake), *Sistrurus catenatus*

Table 1. Natural occurrences of hybridization events with Crotalid species documented in the literature.

Hybrid rattlesnake species	Location and date	Source
Crotalus ruber Cope (Red Diamond Rattlesnake) x *Crotalus oreganus helleri* Meek (Southern Pacific Rattlesnake)	San Diego County, CA, April 1954	Klauber 1972
Crotalus scutulatus (Kennicott) (Mojave Rattlesnake) x *Crotalus viridis* (Rafinesque) (Prairie Rattlesnake)	Hudspeth County, TX, 1985; Hidalgo County, NM, 2016	Murphy and Crabtree 1988, Zancolli et al. 2016
Crotalus adamanteus x *Crotalus horridus*	Jasper County, SC, 1972; Barbour County, AL, 1947	Campbell and Lamar 2004, Klauber 1972
Sistrurus catenatus x *Crotalus horridus*	Lee County, IA, 1942	Bailey 1942
Crotalus horridus x *Crotalus atrox*	Wise County, TX, September 2003; Lee County, TX, May 2007	Meik et al. 2008, Montgomery et al 2013
Crotalus willardi obscurus Harris and Simmons (New Mexico Ridgenose Rattlesnake) x *Crotalus lepidus klauberi* Gloyd (Banded Rock Rattlesnake)	Hidalgo County, NM, September 1981	Campbell et al. 1989
Crotalus scutulatus x *Crotalus molossus* Baird and Girard (Black-tailed Rattlesnake)	Texas and Arizona	Campbell and Lamar 2004
Crotalus atrox x *Crotalus scutulatus*	Texas	Campbell and Lamar 2004
Crotalus viridis x *Crotalus atrox*	Texas	Campbell and Lamar 2004
Crotalus basiliscus (Cope) (Mexican West-Coast Rattlesnake) x *Crotalus molossus*	Sonora, Mexico	Bogert and Oliver 1945
Crotalus oreganus helleri x *Crotalus scutulatus*	Los Angeles County, CA	Stebbins 2003
Crotalus atrox x *Crotalus mictlantecuhtli* Carbajal-Márquez (Veracruz Neotropical Rattlesnake)	Puente Nacional, Mexico	Neri-Castro et al. 2022
Crotalus lepidus x *Crotalus aquilus* Klauber (Queretaran Dusky Rattlesnake)	Aguascalientes and Zacatecas, Mexico	Rivas et al. 2017

(Rafinesque–Schmaltz) (Massasauga Rattlesnake), and *Agkistrodon contortrix* (L.) (Copperhead), have been known to naturally hybridize with CBRs (Bailey 1942, Meik et al. 2008, Montgomery et al. 2013); no other additional species have been known to hybridize with EDRs.

On 17 June 2019, we collected an unusual rattlesnake on Jekyll Island, GA. Upon collection and observation, we immediately recognized the specimen as aberrant, exhibiting intermediate morphological characteristics that suggested this snake was potentially a hybrid between an EDR and a CBR. Here, we describe this putative hybrid using morphological, genetic, and venom analyses. Our objectives in this study were to: (1) morphologically describe the putative hybrid according to the methods of Meik et al. (2008), and (2) incorporate genetic and venom analyses to support or refute our morphological findings.

Field Site Description

Jekyll Island State Park is a 2237.77-ha (5529.64-ac) landmass off the coast of Georgia, disjunct from the mainland by a system of sounds and saltmarsh (Fig. 1; Jekyll Island Authority 2021). Of the 14 barrier islands along Georgia's coast, only 4, including Jekyll Island, are accessible by causeway, allowing the island's >2,000,000 annual visitors easy passage on and off the island. Despite Jekyll Island's status as a tourist destination, state law dictates that no more than 668 ha (1675 ac) are available for development. This requirement has allowed the island to maintain a unique intermingling of business, tourist-focused recreation, and wildlife habitat. Jekyll Island's uplands consist of maritime oak and pine forest, coastal dune habitat, and high marsh hammocks. These upland ecosystems support a genetically distinct EDR population (Margres et al. 2019) and at least 1 documented CBR (this study).

Methods

Hybrid morphological analysis

The morphological description of a naturally occurring hybrid rattlesnake (*C. atrox x C. horridus)* by Meik et al. (2008) served as the basis for our strategy to analyze morphological characters for the putative hybrid specimen. We compared observable characteristics from the putative hybrid to those of all known representatives of the subfamily Crotalinae native to coastal Georgia—*Sistrurus miliarius* (L.) (Pigmy Rattlesnake), *Agkistrodon piscivorus* (Lacépède) (Cottonmouth), the Copperhead, EDR, and CBR—to discern the most likely heterospecific candidates. We hypothesized that the most likely parental species were both within the genus *Crotalus*. EDRs and CBRs are sympatric on Jekyll Island, and the biogeography of the area is such that, to our knowledge, no physical barriers exist to prevent hybridization. Herein we follow the methodology detailed by Powell et al. (1998) for scalation analyses and describe the subject's aberrant color pattern in detail. Because many of the recognized scale row counts and scalation patterns for EDRs and CBRs overlap, we selected specific trait patterns and counts to reduce overlap

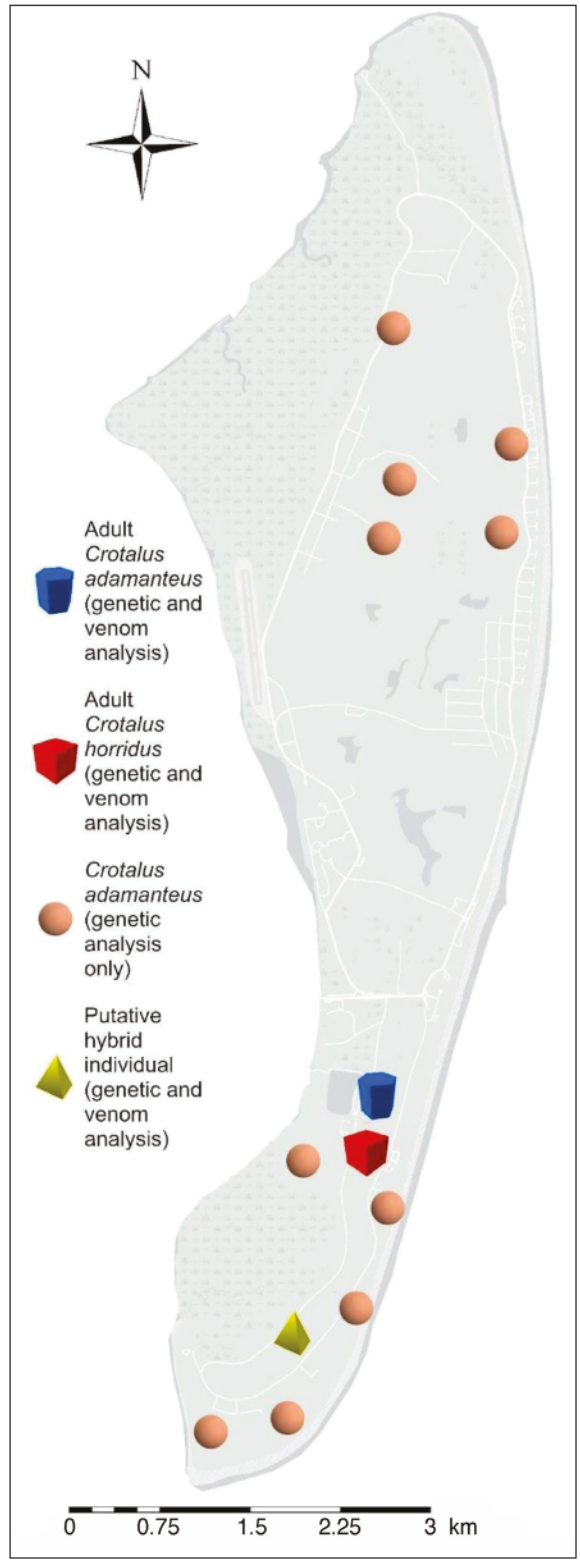

Figure 1. Sampling of *Crotalus adamanteus* (Eastern Diamondback Rattlesnake [EDR]), *Crotalus horridus* (Canebrake Rattlesnake [CBR]), and the putative hybrid individual from Jekyll Island State Park, GA. One adult CBR, 1 adult EDR, and the hybrid individual were included in genetic and venom analyses. Ten additional EDRs were included in genetic analyses.

as much as possible (Campbell and Lamar 2004, Powell et al. 1998). By evaluating the loreals, interoculabials, dorsal body pattern, tail coloration, vertebral stripe, white post-ocular stripe borders, blotch/band count, infralabials, dorsal scale rows, ventral scales, tail bands, subcaudals, and intersupraoculars, we provided a detailed comparison of the putative hybrid traits to those of the parental species. We chose to abstain from a standard full description of the subject because this specimen was implanted with a radio-transmitter (SI-2, Holohil Systems Ltd.) and released back into the wild for monitoring (i.e., all assessments were done while the snake was alive). To avoid overstressing the animal, we assessed only what we felt was necessary for comparison with the putative parental species.

DNA Extractions

We extracted DNA of EDR (n = 11), CBR (n = 1), and putative hybrid (n = 1) from blood samples using the Omega Bio-tek E.Z.N.A Tissue DNA Kit (Norcross, GA) according to the manufacturer's protocol. DNA was visualized on a 2% agarose gel to ensure the presence of high-quality DNA. All individual snakes were collected from Jekyll Island as part of a radio-telemetry study with the Jekyll Island Conservation Department (Fig. 1). Note that not all individuals were sequenced in all analyses described below.

DNA Sequencing

First, to assess maternity of the putative hybrid individual, we amplified a ~1-kb fragment of *cytochrome b* in 25-uL PCR runs using the H16064 (5'-CTT TGG TTT ACA AGA ACA ATG CTT TA-3') and L14910 (5'-GAC CTG TGA TMT GAA AAC CAY CGT TGT-3') primers and thermal cycling protocol described by Burbrink et al. (2000) for 4 individuals: 2 EDRs, 1 CBR, and the putative hybrid individual; we ran this analysis in duplicate to confirm results. Second, to assess admixture across the nuclear genome, we amplified the nuclear locus *NT3* in 25-uL PCR runs using the sense (5' ATG TCC ATC TTG TTT TAT GTG ATA TTT 3') and antisense (5' ACR AGT TTR TTG TTY TCT GAA GTC 3') primers and thermal cycling protocol of Townsend et al. (2008) for the same 4 individuals included in the *cytochrome b* runs described above. *NT3* was shown to possess several variable sites across EDRs and CBRs previously sequenced. We purified PCR products for both loci using the Qiagen QIAquick PCR Purification Kit (Gemantown, MD). We sequenced loci on a Applied Biosystems 3730 Genetic Analyzer (Bedford, MA). We aligned and edited all sequences using GENEIOUS (Biomatters Ltd, Auckland, New Zealand). Sequences were deposited on NCBI under accessions MZ345282–MZ345289.

Venom genetic analysis

Rattlesnake venoms are often classified into 2 categories (Mackessy 2008): type A neurotoxic venoms, and type B hemorrhagic venoms (Mackessy 2010, Straight and Glenn 1989). Type A venoms are characterized by high toxicity due to the presence of crotoxin, a heterodimeric phospholipase A_2 enzyme, and low levels of tissue-damaging metalloproteinase enzymes. Type B venoms, on the other hand,

lack crotoxin (Dowell et al. 2016, Rokyta et al. 2015) but possess high levels of metalloproteinase activity. Although most rattlesnake species have type B venoms, type A venoms are known from at least 10 species (Mackessy 2008), including CBR (Rokyta et al. 2015, Straight and Glenn 1989). CBR, like most species that possess type A venoms, is polymorphic for both venom types (Margres et al. 2021). To determine whether the CBR and the putative hybrid possessed type A (i.e., positive for crotoxin) or type B (i.e., negative for crotoxin) venoms, we followed the approach of Margres et al. (2021; modified from Rokyta et al. 2015 and Wooldridge et al. 2001) and used a PCR assay to determine whether the 2 crotoxin subunits were present or absent from the genome of each individual. Briefly, we amplified the acidic and basic subunits of crotoxin from template DNA in 25-uL PCR reactions using the acidic subunit-sense (5' GGT ATT TCG TAC TAC AGC TCT TAC GGA 3'), acidic subunit-antisense (5' TGA TTC CCC CTG GCA ATT 3'), basic

Figure 2. Comparison of the heterospecific parental species (Eastern Diamondback Rattlesnake [EDR] top left, Canebrake Rattlesnake [CBR] top right) with the putative hybrid individual (bottom).

subunit-sense (5' AAC GCT ATT CCC TTC TAT GCC TTT TAC 3'), and basic subunit-antisense (5' CCT GTC GCA CTC ACA AAT CTG TTC C 3') primers, respectively, under the following thermal cycling protocol: 95 °C for 5 minutes; then 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 5 minutes; and finishing with 72 °C for 10 minutes. Evidence for amplification was visualized by means of a 0.7% agarose gel and SYBR Safe DNA gel stain (Thermo Fisher Scientific, Bedford, MA) and photographed on a dual LED blue/white light transilluminator. We classified individuals as possessing type A venom if both crotoxin subunits amplified or type B venom if neither subunit amplified; given that each individual amplified both *cytochrome b* and *NT3* as described above, we were confident a lack of amplification was due to a lack of the genes themselves rather than low-quality DNA preventing amplification. Because the putative hybridization event involved EDRs, we also tested 11 EDRs from Jekyll Island for the presence of both crotoxin subunits as described above.

Venom proteomic analysis

We used reversed-phase, high-performance liquid chromatography (RP-HPLC) to analyze the venoms of an adult CBR from Jekyll Island, an adult EDR from Jekyll Island, the putative hybrid individual from Jekyll Island, a juvenile EDR from Levy County, FL, a juvenile type B CBR from Mitchell County, GA, and a juvenile type A CBR from Baker County, FL . Because (1) the putative hybrid was a juvenile, and (2) both species have been shown to undergo ontogenetic shifts in venom expression (Margres et al. 2015a; Schonour et al. 2020), including juvenile venom in our comparisons was necessary. Unfortunately, no juvenile EDR or CBR venom samples have been collected on Jekyll Island to date, hence the inclusion of mainland juveniles in our analyses. We collected venoms by allowing animals to bite a parafilm-covered collection dish. Venoms were then flash-frozen in liquid nitrogen, lyophilized, and stored at -80 °C prior to analysis. We performed RP-HPLC on a Beckman System Gold HPLC (Beckman Coulter, Fullerton, CA). For each sample, we injected 100 ug of total venom protein onto a Jupiter C18 column (Phenomenex, Torrence, CA) using the solvent system of A = 0.1% trifluoroacetic acid (TFA) in water and B = 0.075% TFA in acetonitrile. After 5 min at 5% B, a 1% per min linear gradient of A and B was run to 25% B, followed by a 0.25% per min gradient from 25% to 65% B at a flow rate of 1 mL per min. We monitored column effluent at 220 nm as previously described (Margres et al. 2015b). All procedures were approved by the University of South Florida IACUC under protocol #IS00008815.

Results

Morphological analysis

The focal subject was a juvenile female with a snout–vent length of 770 mm, total length of 825 mm, and weight of 300 g at time of capture. As shown in Figure 2, the post-ocular stripe was present, but the white post-ocular stripe borders were absent. The full list of morphological characteristic descriptions is provided in Table 2. The dorsal body pattern consisted of 26 broad blotches that tapered

dramatically as they extended laterally to the venter, and a rust-colored vertebral stripe that was very pronounced extended to the base of the tail. The blotches had a prominent white border and narrowed into wide bands as they proceeded caudally to the tail. The tail was solid black, with a velvet-like appearance and no discernible pattern. The venter was cream with dark flecking, and dark, alternating trapezoids on nearly every ventral scale. The individual had 3 interoculabials, 9 intersupraoculars, and 23 subcaudals anterior to the vent. We counted 175 ventral scales and 25 dorsal scale rows. Loreals were paired, and infralabials were not divided (Table 2).

DNA Sequencing

To assess maternity of the putative hybrid individual, we amplified the mitochondrial locus *cytochrome b* for 2 EDRs, 1 CBR, and the putative hybrid individual; all were collected on Jekyll Island, GA (Fig. 1). Because mitochondrial DNA is maternally inherited (Hutchinson et al. 1974), the expectation was for the EDRs to amplify EDR *cytochrome b* sequence, the CBR individual to amplify CBR *cytochrome b* sequence, and the hybrid individual to amplify *cytochrome b* sequence from either species, indicating the maternal species of the hybrid. Surprisingly, all 4 amplified sequences blasted to EDR *cytochrome b* sequence with >99% sequence similarity. These results were confirmed with an additional replicate. To rule out possible contamination, we next sequenced the nuclear locus *NT3* using

Table 2. Comparing morphological characters for the putative F1 hybrid and the putative parental species. * indicated traits that are EDR specific, ** indicate traits that are CBR specific , and † indicate intermediate traits. All other traits express by the hybrid individual cannot be classified in any category due to overlap in the count range between EDRs and CBRs

Characteristic	EDR	CBR	Hybrid subject
Loreals	1–2	2	2
Interoculabials	1–2	1–3	3**
Dorsal body pattern	Broad blotches/ diamonds	Narrow bands/ chevrons	Winged blotches†
Tail coloration	Black/brown and yellow	Black	Black**
Vertebral stripe	Absent	Present	Present**
White post-ocular stripe borders	Present	Absent	Absent**
Venter coloration	Cream, laterally mottled	Yellow-grey, dark flecking	Cream, dark flecking, alternating dark trapezoids on almost every scale†
Blotch/band count	24–35	15–34	26
Infralabials	Not divided	Not divided	Not divided
Dorsal scale rows	25–31	21–26	25
Ventral scales	170–187	163–183	175
Tail bands	5–10	None	None**
Subcaudals	20–26	15–26	23
Intersupraoculars	5–11	5–7	9*

the same DNA extractions as above. For the 627 bp for which all 4 individuals amplified, the CBR and 2 EDRs differed at 15 bp; the 2 EDRs possessed identical sequences. The putative hybrid, however, was heterozygous at each of the 15 variable sites, indicating it possessed 1 CBR allele and 1 EDR allele.

Venom genetic analysis

To determine whether the CBR and the hybrid individual possessed type A or type B venoms, we used a PCR-based assay (Margres et al. 2021; modified from Wooldridge et al. 2001) to detect the presence or absence of the 2 crotoxin subunits in the genome of each individual. The CBR was negative for both subunits, indicating it likely possessed type B (i.e., hemorrhagic) venom (see below), but the hybrid individual was positive for both subunits, indicating it was capable of expressing crotoxin and could possess type A (i.e., neurotoxic) venom. Given that the hybrid individual possessed both crotoxin subunits, we tested 11 EDRs from Jekyll Island to determine if such hybridization events were altering the venom composition of the EDR population. All 11 EDRs were type B; the acidic subunit did not amplify for any EDR. Although all 11 EDRs amplified a gene product when using the basic subunit primers, we confirmed that this amplification was of a basic PLA_2 pseudogene rather than a crotoxin subunit (*psd-PLA_2-gB1* described in Dowell et al. 2016; assembly accession KX211996).

Venom proteomic analysis

To determine if venom expression in the putative hybrid individual was intermediate between the 2 putative parental species, we used RP-HPLC to characterize venom composition for the putative hybrid, the CBR, and 1 of the 2 EDRs sequenced above. Because both species undergo an ontogenetic shift in venom expression (Margres et al. 2015a; Schonour et al. 2020), we also analyzed the venom of a juvenile EDR from Florida, a juvenile type B CBR from the Georgia mainland, and a juvenile type A CBR from Florida (see Methods). The venom of the hybrid individual showed expression levels consistent with both the CBR and juvenile EDR venoms, as expected (Fig. 3). To determine if the putative hybrid individual expressed crotoxin, we compared the retention times of the 2 crotoxin subunits from the juvenile type A CBR from Florida to peaks in the putative hybrid (Fig. 3). Unfortunately, 1 of the crotoxin peaks shared a retention time with a peak in EDRs, and, with the current data, it was not possible to determine whether this peak in the putative hybrid individual represented crotoxin or another toxic protein.

Discussion

Through genetic, taxonomic, and venom analyses, we identified 2 hybrid rattlesnakes on Jekyll Island, GA. One was the focal subject of this study, and upon analysis of mitochondrial DNA, we discovered evidence that our sole observed adult "CBR" possessed genetic evidence of historic hybridization with EDRs (i.e., "CBR" possessed EDR *cytochrome b* sequence). Overall, the genetic data showed that (1) the putative hybrid was the offspring of an EDR and a CBR although the maternal species of the hybrid could not be confirmed using mitochondrial DNA sequence,

(2) EDRs and CBRs have historically introgressed in the region, and (3) hybrid individuals can be viable and backcross with at least 1 of the parental species in the wild. Unfortunately, the historically hybridized adult individual was no longer in the telemetry study when we made that discovery, and we were not able to assess its morphological characteristics (although superficially the individual did not exhibit intermediate characteristics). Morphological traits of the juvenile hybrid individual that were diagnostic (strictly applicable to only 1 of the parent species) as CBR included interoculabial count, tail coloration, presence of vertebral stripe, absence of white post-ocular stripe borders, and absence of tail bands ($n = 5$). Only a single trait, intersupraocular count, was diagnostic as EDR. Traits that were non-diagnostic

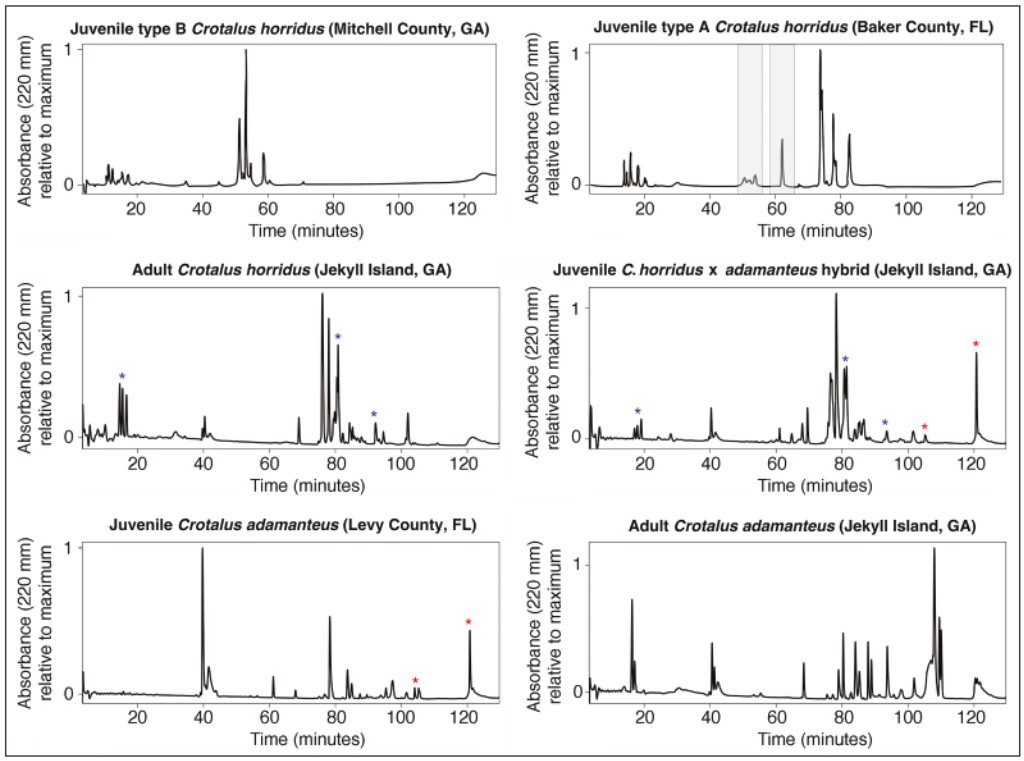

Figure 3. Reversed-phase high-performance liquid chromatography (RP-HPLC) of Canebrake Rattlesnake (CBR), Eastern Diamondback Rattlesnake (EDR), and putative hybrid EDR-CBR venoms shows the intermediate venom phenotype of the hybrid individual. Chromatographic peaks represent individual toxic proteins or sets of toxic proteins, and the area under each peak corresponds to the abundance of that toxin (set). Because EDR and CBR adult and juvenile venoms can differ in venom expression, multiple examples are shown. Examples of CBR toxins found in the putative hybrid are indicated with blue asterisks. Examples of EDR toxins found in the putative hybrid are indicated with red asterisks. Assignment was based on retention time. Crotoxin subunits are indicated in the top right venom profile (Juvenile type A CBR) in gray boxes. The presence of these toxins in the venom of the hybrid individual could not be confirmed using the above data due to overlapping retention times with EDR toxins (e.g., peak just after 60 minutes in the juvenile EDR from Levy County, FL).

(potentially applicable to either parent species due overlap) included loreal counts, blotch/band counts, non-divided infralabials, dorsal scale row counts, ventral scale counts, and subcaudal counts ($n = 6$); intermediate traits (applicable to both parent species; expressed as mixed traits that were both present in the F1 hybrid) included dorsal pattern coloration and venter coloration ($n = 2$; Fig. 2, Table 2).

Jekyll Island exhibits a unique set of environmental conditions that could produce behavioral abnormalities leading to heterospecific mate selection. Oceanic barrier island populations are often found on the periphery of species' ranges. Fringe populations, such as Jekyll Island's EDR and CBR populations, are often known to have different population dynamics, reproductive behaviors, and even entirely different natural histories than populations in the core of the range (Sjorgen et al. 1988, Snyder 2019). Jekyll's EDR population was shown by Margres et al. (2019) to be genetically distinct from mainland and nearby island populations. Although a similar body of data does not exist for CBRs on Jekyll Island, which are only known from 2 verified records (the historically introgressed individual mentioned in this study and a juvenile male putative CBR [SVL = 504 mm; weight = 106 g] that was captured 7 May 2013 in the water park near where the juvenile hybrid was collected; C.M. Harrison et al., unpubl. data), this isolation could apply to them as well. Given that we have captured 3 snakes with varying degrees of CBR genes (1 putative CBR and 2 EDR x CBR hybrids) over the same time-period we captured 218 EDRs (2011–2021), if a viable population of CBRs does exist on Jekyll, it is likely limited to an extremely low number of individuals. We acknowledge that the dense shrub layer caused by severe fire suppression in the maritime forests is difficult to survey, and detection probabilities for rattlesnakes that use these habitats are extremely low. Therefore, a viable population of CBRs could exist largely undetected, but with no road mortalities, incidental encounters through ongoing field work, or reports from citizen science campaigns, we consider this to be extremely unlikely. The collection locality for the described hybrid individual is the periphery of a popular water park located in a salt marsh interspersed with *Quercus virginiana* Mill. (Live Oak) hammocks and is surrounded by open-canopied marsh scrub ecotone. Both the putative CBR and the EDR x CBR hybrids collected on Jekyll Island have come from the same habitat corridor along the intracoastal waterway. Many EDRs in an ongoing radio telemetry study have been observed utilizing the same habitat around a wall of riprap and rock that runs the length of the water park's southern and eastern borders, and our resident historically introgressed CBR has been observed within 400 m of the area during her time in the radio-tracking study. EDRs have been found by the water park staff and are commonly observed along a recreational hiking trail just south of the water park. Although EDR x CBR hybrids are well known from the pet trade (Fig. 4; P. Moody, hobbyist captive snake breeder, pers. comm.) and other hybrids of various species have been captively produced (Klauber 1984), we believe each of the hybrids discussed in this manuscript occurred naturally. Considering the observed overlap in habitat use and seemingly low population number of CBRs, we hypothesize that our focal subject was the product of heterospecific mating in the

wild (rather than an escaped captive), produced as a result of restricted mate access for CBRs on Jekyll Island.

Given the evidence of historic introgression supported by the adult "CBR", we expect hybrids on Jekyll Island have (or had) the ability to backcross with a parental species and produce viable offspring, suggesting hybrids are viable and that hybrid venom is effective (at least to some degree). Because type A toxins were not identified in any of the EDRs tested, however, hybridization between these species may be biased from EDRs into CBRs, consistent with the differences in population size discussed above. Venom is a trophic trait, and venom variation within and between rattlesnake species is often attributed to dietary differences and/or coevolution with prey (Holding et al. 2016; Margres et al. 2015b, 2017). Once EDRs on Jekyll Island are > 1 m in length, they prey almost exclusively upon *Sylvilagus palustris* (Bachman) (Marsh Rabbit) (C.M. Harrison, unpubl. data), consistent with populations elsewhere (Means 2017). Adult CBRs, however, seem to prefer *Sciurus carolinensis* Gmelin (Eastern Gray Squirrel) on Jekyll Island (C.M. Harrison, unpubl. data). Given these putative dietary differences among EDRs and CBRs on Jekyll Island, an intermediate venom type may be expected to suffer from reduced toxicity in Marsh Rabbits and/or Eastern Gray Squirrels. Hybrid rattlesnake venoms, however, have been discussed as potentially adaptive, facilitating the spread of advantageous venom proteins such as crotoxin (Rokyta et al. 2015), although the generality of this assumption has been questioned (Zancolli et al. 2016). We have observed the focal hybrid individual consume small mammalian prey species during tracking

Figure 4. Variability in F1 *Crotalus adamanteus* (Eastern Diamondback Rattlesnake [EDR]) x *Crotalus horridus* (Canebrake Rattlesnake [CBR]) hybrids produced in captivity by Paul R. Moody.

and, at smaller sizes, EDRs and CBRs may have a much more similar diet due to gape-limitations and available prey species. Both parental species are known to undergo an ontogenetic shift in venom expression (Margres et al. 2015a, Schonour et al. 2020, Wray et al. 2015), suggesting hybrid individuals may also experience such a shift. Therefore, any fitness reductions due to the intermediate phenotype may not manifest until adulthood and venom/dietary specialization. The historically introgressed adult "CBR", to our knowledge, only consumed Eastern Gray Squirrels during its 17-month monitoring in our study; whether the juvenile hybrid individual goes on to successfully consume and potentially specialize on squirrels, rabbits, or neither remains unknown. Given that the hybrid juvenile possessed at least the ability to produce type A venom neurotoxins (i.e., although the individual was confirmed to possess both crotoxin subunit genes, we could not confirm crotoxin expression in the venom; Fig. 3) and the introgressed adult "CBR" lacked both crotoxin genes, dietary differences may be expected, although the ecological basis underlying these venom differences is unclear (e.g., Mackessy 2010, Margres et al. 2021, Strickland et al. 2018). The type A–B venom dichotomy has been shown to be maintained over small distances in CBRs (Margres et al. 2021, Rokyta et al. 2015) and other species (Strickland et al. 2018, Zancolli et al. 2019), but both venom types potentially being found on Jekyll Island was unexpected, especially with only sampling 2 individuals. Additional work is needed to better understand the ecological factors and evolutionary processes (1) maintaining type A and type B venoms in CBRs and other species (Margres et al. 2021), and (2) enabling (and perhaps facilitating) hybridization between species with extremely different venom phenotypes. We will continue to monitor this individual's diet and venom composition through our tracking study as well as any future hybrids that may be discovered to link hybrid diet to venom function.

Conclusion

By combining morphological analysis, venom comparisons, and genetics, we were able to confirm multiple, natural occurrences of hybridization between EDRs and CBRs in multiple individuals on Jekyll Island, GA; we recommend this thorough, robust approach for future hybrid venomous snake work when possible. Current and historical evidence of hybridization suggested that introgression may be biased from EDRs into CBRs on Jekyll Island due to restricted mate access for CBRs. We will continue to monitor the hybrid individual via radio-telemetry to assess its survival, performance, and any subsequent F2 hybridization reproduction events. Given that this study confirmed evidence of multiple hybridization events, it may occur again during our study.

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