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The effects of hybridization on divergent venom phenotypes: Characterization of venom from *Crotalus scutulatus scutulatus* × *Crotalus oreganus helleri* hybrids

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ABSTRACT

Hybridization between divergent species can be analyzed to elucidate expression patterns of distinct parental characteristics, as well as to provide information about the extent of reproductive isolation between species. A known hybrid cross between two rattlesnakes with highly divergent venom phenotypes provided the opportunity to examine occurrence of parental venom characteristics in the F_1 hybrids as well as ontogenetic shifts in the expression of these characters as the hybrids aged. Although venom phenotypes of adult rattlesnake venoms are known for many species, the effect of hybridization on phenotype inheritance is not well understood, and effects of hybridization on venom ontogeny have not yet been investigated. The current study investigates both phenomena resulting from the hybridization of a male snake with type I degradative venom, *Crotalus oreganus helleri* (Southern Pacific Rattlesnake), and a female snake with type II highly toxic venom, *Crotalus scutulatus scutulatus* (Mojave Rattlesnake). SDS-PAGE, enzymology, Western blot and reversed phase HPLC (RP-HPLC) were used to characterize the venom of the *C. o. helleri* male, the *C. s. scutulatus* female and their two hybrid offspring as they aged. In general, *Crotalus o. helleri* × *C. s. scutulatus* hybrid venoms appeared to exhibit overlapping parental venom profiles, and several different enzyme activity patterns. Both hybrids expressed *C. o. helleri* father-specific myotoxins as well as *C. s. scutulatus* mother-specific Mojave toxin. Snake venom metalloprotease activity displayed apparent sex-influenced expression patterns, while hybrid serine protease activities were intermediate to parental activities. The *C. s. scutulatus* × *C. o. helleri* hybrid male's venom profile provided the strongest evidence that type I and type II venom characteristics are expressed simultaneously in hybrid venoms, as this snake contained distinctive characteristics of both parental species. However, the possibility of sex-influenced development of metalloprotease activity, as seen in the ontogenetic shifts of the hybrid female, may influence the levels of expression of both type I and type II characteristics in hybrid venoms. Ultimately, the chronological analysis of this known hybrid system reveals the most distinct characteristics that can be used in determining successful hybridization between snakes that follow the type I-type II trend in rattlesnake venom composition, namely the presence of metalloprotease activity and Mojave toxin.

1. Introduction

The effectiveness of venom plays a critical role in the success of prey capture throughout a rattlesnake's lifetime. Ontogenetic shifts in venom composition are rooted in changes in diet preference as a snake ages (Andrade and Abe, 1999; Daltry et al., 1996; Gutiérrez et al., 1991; Mackessy, 1988; Mackessy et al., 2003), and venom composition has even been shown to undergo subtle changes as early as following the first natal shed, likely before a snake takes its first meal (Wray et al., 2015). Younger rattlesnakes are gape-limited to taking smaller prey items, primarily ectotherms including invertebrates, amphibians, and lizards, while adults tend to feed on larger endothermic prey (Mackessy, 1988). Prey availability also shifts as snakes get larger and are able to consume larger prey items. These dietary shifts often correlate with a shift in venom composition from a highly toxic venom with low metalloprotease activity (type II) to a largely proteolytic, lower toxicity venom (type

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I) (Durban et al., 2013; Mackessy, 1988, 2008, 2010a,b).

Type I venoms typically have lower toxicity and are thought to aid in predigestion of larger mammalian prey, while type II venoms are likely optimized for rapid prey incapacitation, facilitating capture and handling (Mackessy, 1988, 2010a; Mackessy et al., 2003). Although the type I and type II venom compositional trend is widespread among adults of many rattlesnake species (Mackessy, 2008), and a type II to type I ontogenetic shift has been observed most frequently, a modified type I to type II ontogenetic shift in venom composition has been noted in *C. v. viridis* (Prairie Rattlesnake). This opposite trend involved a decrease in metalloprotease activity and an increase in myotoxin concentration, likely correlating with an ontogenetic shift in diet (Saviola et al., 2015). Because of the clear differentiation in venom components and activities observed in venoms of adult rattlesnakes, type I and type II venom characteristics are thought to be largely mutually exclusive (Mackessy, 2010b).

Some snakes retain a highly toxic juvenile venom phenotype through adulthood, likely as a result of paedomorphosis (Calvete et al., 2009, 2012; Gutiérrez et al., 1991; Mackessy, 2010b; Mackessy et al., 2003). In rattlesnakes, this paedomorphic venom profile is typically characterized by the presence of multi-subunit PLA_2-based neurotoxins, all homologs of crotoxin, which are largely responsible for the high toxicity of these venoms (Calvete et al., 2012; Mackessy et al., 2003; Saravia et al., 2002). Interestingly, this highly toxic phenotype is maintained through adulthood despite an age-related shift in diet (e.g., Mackessy et al., 2003).

Crotalus scutulatus (Mojave Rattlesnake) is a snake typically found in deserts of the southwestern United States through central Mexico. Its venom contains a potently toxic PLA_2-based neurotoxin (Mojave toxin), and adult *C. s. scutulatus* venom has been shown to contain very low protease activity (Cate and Bieber, 1978; Glenn and Straight, 1978; Glenn et al., 1983; Ho and Lee, 1981; Mackessy, 1988). However, geographic variation in *C. scutulatus* venom has also been observed previously in a population in Arizona, USA (Glenn and Straight, 1983; 1990), and more recently in Mexico (Borja et al., 2014). Individuals that contained Mojave toxin and low metalloprotease activity were designated A venoms, and those that lacked Mojave toxin but had hemorrhagic venom were designated B venoms. Despite this geographic variation in venom composition, the type II venom characteristics of high neurotoxicity coupled with low metalloprotease activity is the most common and widespread venom phenotype observed in *C. scutulatus*.

Crotalus o. helleri (Southern Pacific Rattlesnake) is found from southwestern California to Baja California, Mexico. Its venom has previously been reported to disrupt clotting mechanisms and affect smooth and striated muscle, among other activities (Metsch et al., 1983; Ruiz et al., 1980). *Crotalus o. helleri* venom shifts from a more toxic type II phenotype to a more proteolytic type I phenotype as snakes age, and diet changes from primarily lizards to a diet that consists of mammalian prey (Mackessy, 1988). Proteolytic activity continues to increase as its capacity for larger mammalian prey also increases, suggesting a link between venom phenotype and prey taken. Though in most cases it fits a classical type I phenotype, *C. o. helleri* has been shown to have a moderate level of intraspecific diversity (Sunagar et al., 2014). Moreover, a highly localized population of *C. o. helleri* in southern California was determined to contain Mojave toxin and have reduced proteolytic activities. However, because these populations of Mojave toxin-positive *C. o. helleri* were geographically isolated from *C. s. scutulatus*, it was concluded that this novel venom phenotype was most likely not the result of recent introgression between *C. o. helleri* and *C. s. scutulatus* (French et al., 2004).

Hybridization in rattlesnakes has been documented in scientific literature as early as 1942, when Bailey identified a hybrid between *Crotalus horridus horridus* (Timber Rattlesnake) and *Sistrurus catenatus catenatus* (Eastern Massasauga) by conducting a thorough examination of scalation, body proportions and color patterns (Bailey, 1942). Instances of both intergeneric and interspecific hybridization between various species of rattlesnakes have been investigated via morphology, venom characteristics and genetics. Morphologically, laboratory bred *Crotalus atrox* (Western Diamondback Rattlesnake) × *C. s. scutulatus* (Mojave Rattlesnake) F_1 and F_2 hybrids appeared to display overlapping parental characteristics (Aird et al., 1989). Characters such as the presence of dark flecks on dorsal scales, the number of scales between supraocular scales, position of the postocular light stripe, dorsal blotch patterning and light and dark tail rings were used to investigate inheritance patterns of morphological characteristics in both F_1 and F_2 generations. *Crotalus atrox* × *C. horridus* hybridization was investigated by Meik et al. using scalation, color pattern and scanning electron microscopy of scale features (2008). A hybrid between *C. s. scutulatus* and *C. atrox* was genetically confirmed using allozyme data (Murphy and Crabtree, 1988). The hybrid individual presented with morphological characteristics intermediate to both parents, and allozyme data revealed that the hybrid contained species-specific markers of both *C. s. scutulatus* and *C. atrox*.

Hybridization between rattlesnake species with highly divergent adult venoms provides the opportunity to characterize the venom variation that results from hybridization, to contextualize this variation within the type I-type II dichotomy of venom composition, and to study the venom gene inheritance patterns that result in a hybridized venom phenotype. Previously, only four studies have investigated rattlesnake hybridization using venom profiles. Glenn and Straight determined that hybridization was occurring between two species based on the presence of a type II neurotoxin in a type I species (1990). Snakes captured from an intergrade zone in southwestern New Mexico involving *Crotalus viridis viridis* (Prairie Rattlesnake) and *C. s. scutulatus* showed a typical *C. v. viridis* morphology, but venom contained Mojave toxin, a distinct component of *C. s. scutulatus* that is not found in the venom of *C. v. viridis*. The authors concluded this was the result of historical hybridization and backcrossing of hybrids into *C. v. viridis* populations. However, this study assumed hybridization based on the presence of Mojave toxin in *C. v. viridis* populations without investigating metalloprotease activities as well. As such, it was not confirmed if hybrids displayed both type I and type II characteristics. Further characterizing hybridization between *C. v. viridis* and *C. s. scutulatus* in southwestern New Mexico, Zancolli et al. used both venomic and genomic data to determine that in this case, hybridization between snakes with type I and type II venoms did not result in the adaptive radiation of Mojave toxin into *C. v. viridis* populations, and ultimately these hybridized venoms remained confined to the hybrid zone (2016).

Another study investigated hybrid morphology and venom profiles in a known hybrid system between one *C. atrox* and one *C. s. scutulatus* (Aird et al., 1989). Hybrids retained characteristics of both parents, but the *C. s. scutulatus* parent was described as having a type B venom (corresponding to type I venom), and once again, the presence of type I and type II characteristics resulting from hybridization was not confirmed. Aird et al. (2015) used venom gland transcriptomics and venomics to determine that *Protobothrops flavoviridis* × *Protobothrops elegans* hybrids, the result of species invasion (by *P. elegans*) due to human action on the Ryukya Islands of Japan, express overlapping parental venom profiles; however, *P. flavoviridis* and *P. elegans* have similar venom components and do not follow the type I-type II trend in venom composition seen in American rattlesnakes.

Venom ontogeny in a number of rattlesnake species has been