

# Fixed prey cue preferences among Dusky Pigmy Rattlesnakes (*Sistrurus miliarius barbouri*) raised on different long-term diets

Matthew L. Holding<sup>1</sup> · Edward H. Kern<sup>1</sup> · Robert D. Denton<sup>1</sup> ·  
H. Lisle Gibbs<sup>1</sup>

Received: 1 April 2015 / Accepted: 3 August 2015 / Published online: 8 August 2015  
© Springer International Publishing Switzerland 2015

**Abstract** Chemoreception is often crucial to the interaction between predators and their prey. Investigating the mechanisms controlling predator chemical preference gives insight into how selection molds traits directly involved in ecological interactions between species. In snakes, prey cue preferences are influenced by both direct genetic control and experience-based plasticity. We assessed prey preference in a group of Dusky Pigmy Rattlesnakes that had eaten only mice or lizards over a 5 year period to test whether genetics or plasticity primarily determine the preference phenotype. Our results provide evidence for genetic determination of preference for lizard chemical cues in pigmy rattlesnakes. Snakes preferred the scent of lizards, regardless of their initial diet, and the response to mouse scent did not differ from the water-only control. We discuss these findings in light of previous studies that manipulated snake diets over shorter timescales.

**Keywords** Prey preference · Tongue-flick · Pigmy rattlesnake · *Sistrurus miliarius* · Plasticity · Vomeronasal organ

## Introduction

The means by which predators locate and attack their victims can dictate key features of predator–prey dynamics, such as encounter rate, defensive strategies, and the likelihood of coevolution in antagonistic interactions (Brodie and Brodie 1999; Gilman et al. 2012). Instances of geographic or ontogenetic variation in the preference of predators for particular prey provide the opportunity to investigate the conditions under which selection

---

✉ Matthew L. Holding  
matthewholding28@gmail.com

<sup>1</sup> Department of Evolution, Ecology, and Organismal Biology, The Ohio State University, Columbus, OH, USA

favors phenotypic plasticity versus fixed traits with a direct genetic basis in such interactions.

Chemoreception is a common mechanism by which predators identify and locate suitable prey. Chemical cue preferences are highly tractable, as they can be assayed with choice experiments or the measurement of response intensity to a chemical stimulus. Squamate reptiles have been developed as a model system for studying the relative effects of genes and environment on chemical cue preference (Burghardt and Hess 1968; Burghardt 1993). The tongue of many squamate reptiles is paired with a specialized chemosensory surface, the vomeronasal organ, and exclusively used for chemosensory purposes. As such, the positive relationship between interest in prey chemical cues and the intensity of tongue-flicking behavior is well-established for many species (Burghardt and Hess 1968; Burghardt and Pruitt 1974; Cooper and Burghardt 1990). Tongue-flick studies in different snake species have revealed diverse sources of influence on prey preference including a heritable genetic basis for preference for certain prey chemical cues (Arnold 1981; Waters and Burghardt 2005), ontogenetic shifts in preference that parallel shifts in gut contents of wild specimens (Saviola et al. 2012), and experience-related modulation of preference that may be induced by a single meal (Burghardt and Krause 1999; Waters and Burghardt 2013).

The impacts of multi-year diet restriction on prey preference have not been explored in snakes, with the longest experimental study involving different diets lasting from birth to 1 year of age (Gove and Burghardt 1975). Further, most studies of snake chemical cue preference have been conducted on active foraging snakes in the family Colubridae, primarily *Thamnophis sirtalis*. Preference studies of sit-and-wait predators, such as viperid snakes, are comparatively rare, limiting the scope for inference about the importance of taxonomic and life-history differences in the evolution of preference (but see Clark 2004b; Bevelander et al. 2006; Saviola et al. 2012).

To investigate the impact of long term diet restriction on prey preference in an ambush-foraging pitviper, we studied a captive population of Dusky Pigmy Rattlesnakes (*Sistrurus miliarius barbouri*) fed from birth to the age of 5.5 years on either mice or lizards (see Gibbs et al. 2011). There is previous experimental evidence showing that this snake uses chemoreception when selecting ambush sites (Roth et al. 1999; Bevelander et al. 2006). Furthermore, our experimental diets represent both major and minor taxa present in the natural diet of the Dusky Pigmy Rattlesnake, which is largely composed of reptiles and frogs, with small mammals representing a minor diet component (Gibbs and Mackessy 2009). Under an experience-based model where foraging success effects future preference, we would expect to see elevated preference for chemical cues of a snake's long-term prey type. Alternatively, if snakes from both diet groups preferred a single prey scent over the other, this would be evidence for strong genetic effects on preference. We assessed these alternatives using the classical method of tongue flick counts during presentation of aqueous prey extracts.

## Materials and methods

### Test subjects

The Dusky Pigmy Rattlesnakes used in this experiment were captive born from four pregnant females collected near Deland, Florida, in 2008. At birth, we placed snakes into

experimental diet groups as part of the study of Gibbs et al. (2011) on the effects of snake diet on variation in their venom. The snakes used in the current study had been fed exclusively on either Cuban brown anoles (*Anolis sagrei*; hereafter referred to as lizards,  $n = 3$  snakes) or white mice (*Mus musculus*; hereafter referred to as mice,  $n = 6$  snakes) for 5.5 years. Two snakes were the single offspring of two other mothers. A group of four-siblings was equally divided among the diet treatments, while a three-sibling group consisted of one mouse-fed and two lizard-fed individuals.

### Feeding and housing

We housed all snakes in one room and fed them every 2 weeks. We fed each snake a meal that equaled 12 percent of its body weight 22 days before beginning the scent trials to control for hunger levels in the snakes. We transferred all snakes to large-size Kritter Keepers<sup>®</sup> (Model 20025, 34 cm × 23 cm × 22 cm, Lee’s Aquarium and Pet Products, San Marcos, CA) that were laid on their longest side with the lid facing the middle of the room. The clear side of the enclosure facing up was covered with black paper into which a rectangular port was cut for a GoPro<sup>®</sup> video camera (Hero 3 model, GoPro, Inc., San Mateo, CA).

### Procedure for tongue-flick trials

We prepared scent extracts of lizard and mouse following the procedure of Clark (2004a) which involves suspending prey items in distilled water for 1 h, storing the aliquots of the prey-infused water at  $-80\text{ }^{\circ}\text{C}$  and then using thawed aliquots for tongue flick trials. Aliquots of pure distilled water served as controls. To prepare for a trial, we opened one thawed scent vial and dipped a single cotton swab into the extract. Next, we fixed the cotton swab to the end of a 750 cm long × 1 cm wide wooden rod. Then, we placed the GoPro<sup>®</sup> camera in the port atop the enclosure, opened the enclosure door, and introduced the scent to the snake.

The trial began when the swab reached 5 cm in front of the snake’s head where it was held motionless and the snake’s response assessed. Each trial lasted for 40 s, during which time the observer counted all tongue-flicks and notified a second observer sitting out of view of the time of strikes by the snake. We confirmed the final tongue flick counts and timing of strikes secondarily when both E.H.K. and a second, naïve observer viewed and scored all trial videos. The inter-rater reliability of tongue-flick counts between these observers was measured as 0.99 via Pearson’s correlation coefficient.

We observed all snakes in nine separate trials over a period of 9 days, with one trial per day. Three tests occurred with each scent extract (mouse, lizard, distilled water). Given the three experimental scents tested, there were three possible orders in which we could have presented them to the snakes. We randomly assigned two snakes on mouse diets and one snake on the lizard diet to each possible presentation order. We fixed this order across the 9 day trial period (e.g. a snake might have been presented mouse, lizard, water, mouse, lizard, water, mouse, lizard, water).

### Data analysis

We used our tongue flick counts and timing of strikes during trials to calculate the tongue-flick attack score (TFAS; Cooper and Burghardt 1990). The TFAS is a composite measure

that considers the number of tongue-flicks observed while providing additional weight to a strike by the snake at the cotton swab. The TFAS is calculated as  $TFAS = TF + (TL - \text{latency})$ , where TF is the number of tongue-flicks, TL is the trial length, and latency is the time at which a strike occurs. If there is no strike, latency is equal to trial length.

Trials without any tongue-flicks were omitted from analysis, since we cannot confirm awareness of the scent in these cases. We used SPSS Statistics v.22 (IBM Corp., Armonk, NY) during analysis. The TFAS data were log-transformed to meet assumptions of normally-distributed residuals and homoscedasticity. We fitted a *linear mixed-effects model* (LMM) to the transformed data, with experimental diet, scent, and scent\*diet interaction as fixed factors. Snake ID was specified as a random subject-variable with a scaled identity covariance structure to model the non-independent measurements done on a single snake, while trial day (1–9) was included as a repeated-measures variable with a first-order autoregressive covariance structure to model potential non-independence of all measures taken closer together in time. Degrees of freedom for the model were estimated with the Satterthwaite approximation. A significant main effect of scent would support genetic determination of scent preference, while a significant scent\*diet interaction would support diet-induced plasticity in chemical cue preference.

## Results

Snakes reacted with at least one tongue-flick in 56 of 81 trials. For both experimental diet groups, lizard scent elicited nearly twice the number of tongue-flicks, led to snakes striking the cotton swab about three times more frequently, and there were fewer tongue-flicks before the first strike, meaning that the decision to strike required less chemical investigation (Table 1).

The linear mixed-effects model revealed that the scent extract presented affected the TFAS ( $F_{2,34.3} = 4.65$ ,  $P = 0.016$ , Fig. 1), where the TFAS in response to lizard scent was approximately double that shown in response to mouse or water presentation. There was no main effect of experimental diet ( $F_{1,6.6} = 0.064$ ,  $P = 0.808$ ) or a scent \* diet interaction

**Table 1** The tongue-flick and strike response of Dusky Pigmy rattlesnakes to a 40 s presentation of lizard extract, mouse extract, or water on a cotton swab

Diet	Scent	# Trials with a tongue-flick	Proportion of trials with a tongue-flick	# Tongue flicks	Proportion of trials with a strike	# Tongue flicks before strike
	Lizard	8	0.89	11.9 ± 4.7	0.38	1.5 ± 0.5
Lizard	Mouse	6	0.67	5.5 ± 3.3	0.17	4.0 [n = 1]
	Water	7	0.77	6.8 ± 2.1	0.29	5.5 ± 3.5
	Lizard	9	0.5	8.4 ± 3.6	0.56	0.83 ± 0.2
Mouse	Mouse	14	0.77	5.4 ± 0.6	0.14	3.5 ± 2.5
	Water	12	0.67	6.7 ± 1.3	0.17	4.0 ± 2.0

Other than the proportion of trials with a tongue-flick, all values were calculated from only the trials during which a snake reacted to presentation with at least one tongue-flick. Data are proportions or means (±1 SE), and means were calculated by first averaging repeated trials within an individual snake

**Fig. 1** Mean ( $\pm$ SE) tongue-flick attack scores (TFAS) for lizard-fed ( $n = 3$ ) and mouse-fed ( $n = 6$ ) Dusky Pigmy rattlesnakes presented with each of three aqueous scent extracts (lizard extract, mouse extract, or water control). Means and SE were calculated by first averaging repeated trials on the same scent carried out on an individual snake



( $F_{2,34.3} = 0.047$ ,  $P = 0.954$ ), which suggests a lack of inducible plasticity in the chemical preferences of these snakes. Dunn–Šidák corrected post hoc comparisons of the model-estimated means for each prey scent to the control scent showed that Pigmy rattlesnakes preferred lizard scent over the water control ( $P = 0.017$ ; model-estimated 95 % CI for difference in TFAS: 1.21–9.38), while the response to mouse scent did not differ from water ( $P = 0.962$ ). This confirms an overall preference for lizard scent, regardless of whether snakes had been fed only lizards or only mice since their birth.

## Discussion

Our main result is that regardless of whether these rattlesnakes were fed only mice or only lizards from birth to five and a half years of age, they preferred the scent of lizards. This provides strong indirect evidence for a canalized genetic determination of preference for lizard chemical cues in these snakes. Furthermore, there is no evidence that 5 years of feeding on mice elevates a rattlesnake's response to mouse scent in any way, since the response to mouse scent did not differ from the water-only control. Given the strong evidence for a genetic basis for preference, we can expect chemoreception to be targeted by divergent selection among areas with varying availability of specific prey species given that pigmy rattlesnakes eat a variety of prey (Gibbs and Mackessy 2009).

Toxicity assays show that Pigmy rattlesnake venom is most toxic to *A. sagrei*, as compared to frog and mouse models (Gibbs and Mackessy 2009). This suggests that chemical cue preference of these predators is well-matched with both their capability to capture lizard prey over mice and the increased abundance of lizards in their diet. Margres et al. (2015) showed that fang morphology and venom variation are functionally-integrated phenotypes for feeding in the Eastern Diamondback Rattlesnake (*Crotalus adamanteus*). Our result suggests that chemical preference could be another component of the integrated feeding system of rattlesnakes, where the importance of particular prey species leads to the parallel evolution of both prey-specific venom and chemical cue targeting.

Our study is limited by both sample size and potential non-independence among the siblings in groups. Squamate chemoreception is a highly stereotyped response to certain scents based on chemoreceptor density and chemical concentration (Burghardt and Hess 1968; Halpern 1992; Burghardt and Chmura 1993), allowing studies like ours to measure chemical preference with a high ratio of signal to noise despite small samples (e.g. Gove

and Burghardt 1975; Mushinsky and Lotz 1980; Weaver and Kardong 2009). Reduced power would bias toward not detecting real effects of our treatments: in contrast we detected a clear pattern of preference for the lizard scent. Heritable variation in chemosensory responses among litters has been previously documented in garter snakes from a single site (Burghardt et al. 2000), as well as preference polymorphism within a single litter (Burghardt 1975). For our purposes, more homogeneity in genetic makeup should facilitate testing the alternatives of genetic and environmental control of phenotype.

Prey preference in snakes has been used as a trait to study both adaptive genetic divergence in behavior (Arnold 1981; Drummond and Burghardt 1983; Aubret et al. 2006) and the contexts under which experience results in a phenotypically plastic trait (Clark 2004a; Aubret et al. 2006; Waters and Burghardt 2013). Studies with diet exposure periods ranging from 1 week to 1 year in a range of species have shown evidence for both plasticity (Fuchs and Burghardt 1971) and a strong genetic basis for the trait (this study; Burghardt and Hess 1968; Arnold 1977; Arnold 1981). We see two future research directions as useful for understanding the ecological and evolutionary bases for the high degree of variation in the factors determining prey preference shown by this work. First, comparative phylogenetic analyses would establish how labile feeding preference is over evolutionary time and if there are broad ecological correlates with prey preference characteristics within lineages of snakes (Cooper 2008). Second, population-level comparisons of preference variation within a single species could yield insights into how geographic variation in selection shapes the evolution of plasticity, especially if paired with estimates of both local prey availability and levels of gene flow (Arnold 1981; Gomulkiewicz et al. 2007). These approaches complement each other by providing macro and microevolutionary perspectives on what determines variation in this behavioral phenotype.

**Acknowledgments** We thank A. Pomento, M. Saccucci, M. Parsley, and P. Hudson for help with viewing video trials and for discussions about study design. The work was funded by The Ohio State University and M.L.H. was supported by a National Science Foundation Graduate Research Fellowship. Methods were approved by The Ohio State University's IACUC under protocol 2008A0087-R2.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

- Arnold SJ (1977) Polymorphism and geographic variation in feeding behavior of the garter snake *Thamnophis elegans*. *Science* 197:676–678
- Arnold SJ (1981) Behavioral variation in natural populations. I. Phenotypic, genetic and environmental correlations between chemoreceptive responses to prey in the garter snake, *Thamnophis elegans*. *Evolution* 35:489–509
- Aubret F, Burghardt GM, Maumelat S, Bonnet X, Bradshaw D (2006) Feeding preferences in 2 disjunct populations of tiger snakes, *Notechis scutatus* (Elapidae). *Behav Ecol* 17:716–725
- Bevelander G, Smith TL, Kardong KV (2006) Microhabitat and prey odor selection in the foraging Pigmy Rattlesnake. *Herpetologica* 62:47–55
- Brodie ED, Brodie ED (1999) Predator-prey arms races. *Bioscience* 49:557–568
- Burghardt GM (1975) Chemical prey preference polymorphism in newborn garter snakes *Thamnophis sirtalis*. *Behaviour* 52:202–225
- Burghardt GM (1993) The comparative imperative: genetics and ontogeny of chemoreceptive prey responses in natricine snakes. *Brain Behav Evol* 41:138–146
- Burghardt GM, Chmura PJ (1993) Strike-induced chemosensory searching by ingestively naive garter snakes (*Thamnophis sirtalis*). *J Comp Psychol* 107:116–121

- Burghardt GM, Hess EH (1968) Factors influencing the chemical release of prey attack in newborn snakes. *J Comp Psychol* 66:289–295
- Burghardt GM, Krause MA (1999) Plasticity of foraging behavior in garter snakes (*Thamnophis sirtalis*) reared on different diets. *J Comp Psychol* 1999:277–285
- Burghardt GM, Pruitt CH (1974) Role of the tongue and senses in feeding of naive and experienced Garter Snakes. *Physiol Behav* 14:185–194
- Burghardt GM, Layne DG, Konigsberg L (2000) The genetics of dietary experience in a restricted natural population. *Psychol Sci* 11:69–72
- Clark RW (2004a) Feeding experience modifies the assessment of ambush sites by the timber rattlesnake, a sit-and-wait predator. *Ethology* 110:471–483
- Clark RW (2004b) Timber Rattlesnakes (*Crotalus horridus*) use chemical cues to select ambush sites. *J Chem Ecol* 30:607–617
- Cooper W (2008) Tandem evolution of diet and chemosensory responses in snakes. *Amphibia-Reptilia* 29:393–398
- Cooper W Jr, Burghardt G (1990) A comparative analysis of scoring methods for chemical discrimination of prey by squamate reptiles. *J Chem Ecol* 16:45–65
- Drummond H, Burghardt G (1983) Geographic variation in the foraging behavior of the garter snake, *Thamnophis elegans*. *Behav Ecol Sociobiol* 12:43–48
- Fuchs JL, Burghardt GM (1971) Effects of early feeding experience on the responses of garter snakes to food chemicals. *Learn Motiv* 2:271–279
- Gibbs HL, Mackessy SP (2009) Functional basis of a molecular adaptation: prey-specific toxic effects of venom from *Sistrurus rattlesnakes*. *Toxicon* 53:672–679
- Gibbs HL, Sanz L, Chiucchi JE, Farrell TM, Calvete JJ (2011) Proteomic analysis of ontogenetic and diet-related changes in venom composition of juvenile and adult Dusky Pigmy rattlesnakes (*Sistrurus miliarius barbouri*). *Journal of Proteomics* 74:2169–2179
- Gilman RT, Nuismer SL, Jhwueng D-C (2012) Coevolution in multidimensional trait space favours escape from parasites and pathogens. *Nature* 483:328–330
- Gomulkiewicz R, Drown DM, Dybdahl MF, Godsoe W, Nuismer SL, Pepin KM, Ridenhour BJ, Smith CI, Yoder JB (2007) Dos and don'ts of testing the geographic mosaic theory of coevolution. *Heredity* 98:249–258
- Gove D, Burghardt G (1975) Responses of ecologically dissimilar populations of the water snake *Natrix s. sipedon* to chemical cues from prey. *J Chem Ecol* 1:25–40
- Halpern M (1992) Nasal chemical senses in reptiles: structure and function. In: Gans C, Crews D (eds) *Biology of the reptilia. Physiology E, hormones, brain, and behavior*. University of Chicago Press, Chicago, Illinois, pp 423–523
- Margres MJ, Wray KP, Seavy M, McGivern JJ, Sanader D, Rokyta DR (2015) Phenotypic integration in the feeding system of the eastern diamondback rattlesnake (*Crotalus adamanteus*). *Mol Ecol* 24:3405–3420
- Mushinsky H, Lotz K (1980) Chemoreceptive responses of two sympatric water snakes to extracts of commonly ingested prey species. *J Chem Ecol* 6:523–535
- Roth ED, May PG, Farrell TM (1999) Pigmy Rattlesnakes use frog-derived chemical cues to select foraging sites. *Copeia* 1999:772–774
- Saviola AJ, Chiszar D, Mackessy SP (2012) Ontogenetic shift in response to prey-derived chemical cues in prairie rattlesnakes *Crotalus viridis viridis*. *Curr Zool* 58:549–555
- Waters MR, Burghardt GM (2005) The interaction of food motivation and experience in the ontogeny of chemoreception in crayfish snakes. *Anim Behav* 69:363–374
- Waters RM, Burghardt GM (2013) Prey availability influences the ontogeny and timing of chemoreception-based prey shifting in the striped crayfish snake, *Regina alleni*. *J Comp Psychol* 127:49–55
- Weaver RE, Kardong KV (2009) Microhabitat and prey odor selection in *Hypsiglena chlorophaea*. *Copeia* 2009:475–482