PROTECTION BY ASSOCIATION: IMPLICATIONS OF SCENT TRAILING IN NEONATE EASTERN MASSASAUGAS (SISTRURUS CATENATUS)

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Abstract.—A driving force in the evolution of parental care is predation on developing young. However, little is known about the role of offspring in maintaining mother-offspring associations attributed to maternal attendance. Neonates that maintain close proximity to related or unrelated conspecific adults should experience reduced predation and thus be equally favored by natural selection. Yet, it is unknown whether neonates discriminate between maternal and unrelated conspecific scents. We investigated this subject and the role of neonates in maintaining mother-offspring associations using chemical cue-based Y-maze experiments and 76 Eastern Massasauga (Sistrurus catenatus) neonates born from 12 wild caught females. Sixty-seven neonates (88%) made a choice by selecting an experimental arm within the allotted 60 min. Neonates showed a two-fold preference to the maternal experimental arm versus the control (scent free) arm (P = 0.048, n = 31) but did not discriminate between maternal and unrelated conspecific adult female scents (P = 0.182, n = 36). Individuals that failed to make a choice (n = 9) exhibited significantly lower tongue flick rates (3.42/min ± 2.98 SD) than those that made a choice (16.38/min ± 9.16 SD, P < 0.001, n = 67). Neonates did not experience a concomitant increase in selection of maternal scent with increased exposure times to mother and littermates (P = 0.437, n = 26). Our results show that neonates play an active role in maintaining mother-offspring associations attributed to maternal attendance. Our findings also suggest that previous reports of neonate scent trailing behaviors postulated for locating overwintering sites are functionally identical to those used during maternal attendance.

Key Words.—behavior; chemical cues; life-history evolution; maternal attendance; parental care; pitvipers

INTRODUCTION

Parental care is broadly defined to include adaptive behavioral and non-behavioral parental traits that improve the fitness of offspring (Smiseth et al. 2012). Theory predicts that parental care will arise whenever indirect benefits (e.g., increased offspring survival) outweigh direct costs (e.g., increased parental mortality; Clutton-Brock 1991). Thus, one driving force in the evolution of parental care is predation on developing young (Klug and Bonsall 2010).

Behaviorally expressed parental care, or parental behavior, is widespread among endotherms (Clutton-Brock 1991), but it is rare in ectotherms (Shine 1988; Scott 1990; Crump 1996; Gross 2005). Still, the incredible diversity in ectotherm reproduction has led to the evolution of a multitude of parental behaviors (Shine 1988; Scott 1990; Crump 1996; Gross 2005). Consequently, these taxa provide excellent opportunities to elucidate the evolution of parental behavior through exploration of the mechanisms and pathways that lead to and maintain these traits.

In non-avian reptiles such as snakes, parental behavior has been documented in the form of egg and postnatal attendance (Shine 1988; Greene et al. 2002). Postnatal maternal attendance is a common parental behavior among viperids (Butler et al. 1995; Greene et al. 2002). Active defense of neonates, as indicated by a postpartum increase in aggressive behavior, occurs in the Prairie Rattlesnake (Crotalus viridis; Graves 1989) and Pygmy Rattlesnake (Sistrurus miliarius; Greene et al. 2002). Conversely, Hess and Clark (2014) found that postpartum Cottonmouths (Agkistrodon piscivorus) became more reluctant to engage predators when offspring were present. Maternal attendance sans aggressive behavior has also been reported (summarized by Butler et al. 1995). However, there is disagreement as to whether these observations represent parental behavior or if they are merely the result of mothers too fatigued to abandon their young after exhausting energy stores during gestation and parturition (for support of maternal attendance see Greene et al. 2002; Hess and Clark 2014; for opposing views see Klauber 1956; Martin 1992; Butler et al. 1995). Moreover, within the context of parental care theory, maternal attendance is only considered a form of parental care if it results in increased neonate survival (Clutton-Brock 1991; Smiseth et al. 2012) but direct proof of this is rare (for an example in lizards see Huang 2006). Using the Pygmy Rattlesnake (Sistrurus miliarius) as a model, Greene et al. (2002) provided the first empirical evidence...
that postnatal maternal attendance is adaptive in snakes and the only empirical evidence that postnatal maternal attendance is bidirectional between mother and offspring. Greene et al. (2002) hypothesized that maternal attendance arose because neonates may suffer from visual and heat pit impairment during their first postnatal ecdysis cycle (PEC) and therefore may be especially vulnerable to predation. In pitvipers this cycle lasts for approximately 1–2 weeks (Means 1985; Butler et al. 1995; Greene et al. 2002), and mothers remain with offspring until the cycle has completed (Butler et al. 1995; Greene et al. 2002).

Postnatal-ecdysis-cycle neonates that maintain close proximity to either related or unrelated conspecific adults should be equally favored by natural selection in species where conspecific aggression and cannibalism are rare or absent. For these species, association with any adult conspecific is likely to confer a survival advantage through lowered predation risk. However, it is unknown whether PEC offspring discriminate between maternal and unrelated conspecific scents.

In this study, we investigated the role of PEC neonates in maintaining mother-offspring associations attributed to maternal attendance using lab experiments with captive born Eastern Massasauga (Sistrurus catenatus) neonates. Sistrurus catenatus is a small North American rattlesnake endemic to Great Lakes region (Ernst and Ernst 2003). It is a federal candidate for listing under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service 2010) and is listed as endangered or of special concern (Michigan) in the United States and as threatened in Canada (Szymanski 1998; Nanjappa and Conrad 2011).

Accounts of S. catenatus mother-offspring associations have been reported up to several days after parturition (Swanson 1930; Reinert and Kodrich 1982; Johnson 2000; Eric Hileman, pers. obs.). To provide further insight into these field observations, we conducted Y-maze experiments using chemical cues to first test the hypothesis that neonates would select maternal scent over no scent (control). We then tested the hypothesis that offspring do not discriminate between maternal and unrelated conspecific adult female scents.

**Materials and Methods**

We collected 12 pregnant S. catenatus for captive parturition 1–7 July 2013 and 23 July to 19 August 2014 from a field site in Cass County, Michigan. Females were maintained in a thermostatically controlled room and housed individually in 10-gallon glass aquaria (LxWxH, 50.8 x 27.9 x 33.0 cm). We provided cardboard boxes for refuge and a location for parturition. We provided snakes natural light/dark photo periods and a thermal gradient (about 20–35 °C) using nursery heat tape and ambient room temperature for the upper and lower thermal limits, respectively. Snakes were offered one dead mouse weekly and water was provided ad libitum. We inspected cages daily for the presence of neonates and cleaned cages only as necessary to minimize disturbance. We released mother and offspring at the capture site of each mother within five days of parturition.

We constructed the Y-maze using a design similar to previous snake behavior studies (Fig. 1; Ford 1982; Brown and MacLean 1983; Burger 1989). The top of the structure was open so observers could record behaviors elicited during trials. We lined the interior of the maze with chemical-free butcher paper, which was replaced after each trial using latex gloves to prevent scent contamination. Parturition occurred 10–31 August 2013 and 10–13 August 2014. We conducted all trials within three days of parturition to ensure test subjects experienced similar stages of postnatal ecdysis cycles. We maintained ambient temperature during trials between 20.7 and 27.0°C (mean = 23.8° ± 1.09 standard deviation, SD). Treatment scents were procured from adults by swabbing cotton balls along the posterior two-thirds of the ventral, dorsal, and dorsal-lateral body surface while wearing latex gloves to prevent human scent contamination. Prior to use, we froze treatment scents up to five days. We randomly assigned which experimental arm received maternal scent. Depending on the experiment, the opposing experimental arm received either a control scent (scent-free cotton ball) or the scent of a presumably unrelated pregnant female. We applied control and treatment scents where the arms bifurcate by depressing a cotton ball onto the butcher paper at the proximal end of each arm and rolling it distally to provide a scent trail (Fig. 1). We left the cotton ball at the distal end of the arm for the duration of each trial. We used new treatment and control cotton balls for each trial.

Neonates remained with their mothers until selected for a trial, and we used each neonate only once. Using a snake hook, we placed neonates at the starting point of the Y-maze base (Fig. 1), and we allowed up to 60 min for snakes to travel through the maze. We considered that a neonate made a choice when the entire snake entered either experimental arm (Fig. 1). The trial ended if a choice was not made within 60 min. We excluded individuals that did not select an experimental arm from choice analyses. Observers monitored each trial from 1 to 31 August 2013 and 10–13 August 2014. We conducted all trials within three days of parturition to ensure test subjects experienced similar stages of postnatal ecdysis cycles. We maintained ambient temperature during trials between 20.7 and 27.0°C (mean = 23.8° ± 1.09 standard deviation, SD). Treatment scents were procured from adults by swabbing cotton balls along the posterior two-thirds of the ventral, dorsal, and dorsal-lateral body surface while wearing latex gloves to prevent human scent contamination. Prior to use, we froze treatment scents up to five days. We randomly assigned which experimental arm received maternal scent. Depending on the experiment, the opposing experimental arm received either a control scent (scent-free cotton ball) or the scent of a presumably unrelated pregnant female. We applied control and treatment scents where the arms bifurcate by depressing a cotton ball onto the butcher paper at the proximal end of each arm and rolling it distally to provide a scent trail (Fig. 1). We left the cotton ball at the distal end of the arm for the duration of each trial. We used new treatment and control cotton balls for each trial.

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To assess motivation, we recorded the number of individual tongue flicks per trial. As snakes near the base of the maze were not always visible, we used the minimum number of tongue flicks (MNTF) observed and trial run time to calculate a MNTF rate. We used
RESULTS

The 12 pregnant females held for captive parturition produced 99 neonates, including four stillborn and three individuals that died postpartum. We included 76 individuals in Y-maze trials: 28 males (snout-vent length, SVL = 18.1 ± 0.97 cm; mass = 10.5 ± 1.52 g) and 48 females (SVL = 18.6 ± 1.29 cm; mass = 10.8 ± 1.86 g). On average, neonates took 19.11 ± 18.698 min to complete a trial. Of the 76 neonates included in Y-maze trials, 67 (88%) made a choice by selecting an experimental arm within the allotted 60 min. Data for the minimum number of tongue flicks per minute adequately met the assumptions of normality for neonates that made a choice (P = 0.73, n = 67) and those that did not (P = 0.89, n = 9). However, there were unequal variances for MNTF rates between these groups (P = 0.01). Neonates that failed to make a choice (n = 9) within 60 min exhibited significantly lower tongue flick rates (3.42 ±
2.978 flicks/min) than those that made a choice (16.38 ± 9.156 flicks/min, n = 67; t = 8.66, df = 34, P < 0.001).

Offspring showed a two-fold preference to the maternal experimental arm (observed = 21; expected = 15.5) versus the control arm (observed = 10; expected = 15.5; total = 31, X² = 3.90 df = 1, P = 0.048). However, neonates did not experience a concomitant increase in selecting maternal scent with increased exposure times to mother and littermates (P = 0.437). Neonates exposed 0.1–1 d to mother and littermates selected the scent of the mother nine of 12 times. Neonates exposed for 2–3 d to mother and littermates selected the scent of the mother nine of 14 times. As predicted, we detected no preference by neonates presented with a maternal scent (observed = 14; expected = 18) and the scent of an unrelated pregnant female (observed = 22; expected = 18; total = 36, X² = 1.80, df = 1, P = 0.182).

**DISCUSSION**

Maternal attendance clearly is not the sole mechanism underpinning prolonged mother-offspring associations observed among *S. catenatus*. Rather, our results demonstrate that neonates are active participants in these associations. Previous studies on neonate conspecific scent trailing have targeted post-maternal attendance young, suggesting that these naïve individuals use this behavior to locate overwintering sites (Brown and MacLean 1983; Graves et al. 1986; Reinert and Zappalorti 1988; Costanzo 1989; Cobb et al. 2005). Although there has been limited empirical research on the role of offspring in maternal attendance associations, Greene et al. (2002) demonstrated that newborn *S. miliaris* tended to re-aggregate with their mother after separation. Our results agree with those of Greene et al. (2002) and confirm the predisposition of newborn *S. catenatus* to trail adult females within days of parturition. Our results are also consistent with Jellen and Kowalski (2007) who observed apparent maternal scent trailing behavior by neonate *S. catenatus* once mothers had left their field parturition sites. Cobb et al. (2005) observed similar behavior in Timber Rattlesnake (*Crotalus horridus*) neonates.

Our study is unique in demonstrating that *S. catenatus* neonates do not discriminate between the scents of maternal and unrelated adult females. *Sistrurus catenatus* are among dozens of reptile species known to communally aggregate during gestation and parturition (summarized in Graves and Duvall 1995; Eric Hileman, pers. obs.). Consequently, during the postnatal ecdysis cycle *S. catenatus* neonates may frequently be within close proximity of unrelated gestating or postpartum adult female conspecifics. Neonates may gain similar benefits in the form of reduced predation by associating with related or unrelated adult conspecifics. Furthermore, associations with unrelated conspecific adults may serve as an early dispersal mechanism without forfeiting the anti-predatory benefits associated with maternal attendance. Y-maze experiments conducted with Timber Rattlesnakes (*Crotalus horridus*) 8–23 d after the first neonate ecdysis support the tendency of neonates to follow unrelated conspecific chemical cues (Brown and MacLean 1983). Additionally, Reinert and Zappalorti (1988) observed a neonate *C. horridus* trailing an adult male from its active season habitat to a hibernaculum, supporting the notion that neonates may benefit from following adult conspecifics regardless of relatedness. In demonstrating that neonates do not discriminate between maternal and unrelated conspecific scents, we have shown for the first time that neonate scent trailing behaviors associated with maternal attendance and those used to locate overwintering sites are functionally identical. Moreover, it is likely that this generalized behavior arose from selective pressures imposed by both of these critical phenological events. This finding could have important implications for Eastern Massasauga conservation, especially if repatriation is considered as a management strategy.

Outside the anti-predatory benefits of maternal attendance, additional explanations have been proposed for the persistence of aggregations during the initial postnatal ecysis cycle. Butler et al. (1995) found that aggregations of Eastern Diamond-backed Rattlesnake (*Crotalus adamanteus*) neonates were more likely than lone individuals to flee from researchers and suggested that this group flight response may reduce predation. Reiser et al. (2008) demonstrated that the core temperature of neonate aggregations of Mohave Desert Sidewinders (*C. cerastes cerastes*) showed stability in spite of fluctuating environmental temperatures. Duvall et al. (1985) stated that neonate cutaneous water loss is higher during PEC than it is after the cycle is completed. Tu et al. (2002) substantiated these findings and reported that the high water loss occurs because PEC neonates are transiently endothermic (about 0.6° C above ambient air temperature) and their skin permeability barrier (i.e., lamellar lipids in the mesos layer) is roughly half the thickness and deposition of post-ecdysis neonates. Thus, PEC aggregations reduce cutaneous water loss by decreasing individual neonate surface area exposed to air (Graves and Duvall 1995; Tu et al. 2002). Additionally, Butler et al. (1995) suggested that aggregations may be necessary in order for neonates to gain scent recognition abilities needed to trail conspecifics. Our results suggest that scent recognition of conspecifics and scent trailing behavior is innate and does not require prolonged exposure to mother and littermates. However, Hoss (2013) found evidence that maternal attendance-based aggregations may be important in the development of kin discrimination in *Agkistrodon piscivorus*. Evidence of kin-based associations has been reported in only one other pitviper species (*C. horridus*; Bushar et al. 1998; Clark...
2004; Clark et al. 2012) but whether aggregations during maternal attendance influenced these associations is unknown. It is also unknown how widespread kin-based associations are among pitvipers but Hoss (2013) suggested this phenomenon may be common.

We recommend further research into the effect of aggregation exposure time on scent recognition of conspecifics, incorporating increased sample sizes and larger differences in exposure times between groups, to confirm our conclusion. We also recommend investigating whether S. catenatus exhibit kin discrimination with increased exposure time during maternal attendance. Future studies should also investigate the ability of attending mothers to discriminate offspring from unrelated conspeccific neonates (for an example of offspring recognition by post-parturient Midland Water Snakes, Nerodia sipedon, see Himes 2002). While neonates may experience similar increases in survival through associations with either maternal or unrelated adults, the indirect benefits of mothers can only be realized if the neonates she is attending are her own.

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