



## Post-birth separation affects the affiliative behaviour of kin in a pitviper with maternal attendance

SHANNON K. HOSS<sup>1\*</sup>, DOUGLAS H. DEUTSCHMAN<sup>1</sup>, WARREN BOOTH<sup>2,3</sup> and RULON W. CLARK<sup>1</sup>

<sup>1</sup>Department of Biology, San Diego State University, 5500 Campanile Drive, San Diego, CA, 92182, USA

<sup>2</sup>Department of Biological Sciences, The University of Tulsa, 315 Oliphant Hall, Tulsa, OK, 74104, USA

<sup>3</sup>The Copperhead Institute, PO Box 6755, Spartanburg, SC, 29304, USA

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The relatedness of individuals can have pronounced effects on behavioural interactions, as engaging in mutually beneficial behaviours with kin can increase inclusive fitness. Parental care can be particularly important for kin discrimination in birds and mammals, but similar studies have not been conducted on species exhibiting more rudimentary forms of care. Maternal attendance of young is ubiquitous among viviparous temperate pitvipers, but the adaptive value of this behaviour has received little attention. We sought to determine if being deprived of a maternal attendance period as neonates altered how cottonmouths (*Agkistrodon piscivorus* Lacépède), a common North American pitviper, responded to kin vs. non-kin. We measured the affiliative behaviour of related and unrelated juvenile–juvenile and mother–juvenile pairs that had been allowed a maternal attendance period or had been separated since birth. We found that maternal attendance was not required for sibling or mother–offspring recognition, but did enhance female affiliative behaviour overall, and particularly that of sisters. In contrast, related juveniles that were separated at birth showed a reversal of the sex-specific affiliative behaviour observed in maternally attended juveniles. Post-birth separation had only a modest effect on mother–juvenile affiliative behaviour, and no effect on the strong affiliation between mothers and daughters. The patterns of affiliative behaviour observed in maternally attended snakes corresponded to patterns that have emerged from previous captive and field studies of pitvipers; however, the behaviour of juveniles separated at birth was atypical. Thus, it is possible that maternal attendance plays some role in the development of adaptive sex-specific and kin-directed affiliative behaviour in pitvipers. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, 116, 637–648.

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### INTRODUCTION

The ability to respond to conspecifics in a way that maximizes fitness is essential to all species, and particularly important for organisms that engage in social behaviour outside of the context of mating. In addition to recognizing and responding appropriately to potential mates, fitness gains can be enhanced by cooperating with conspecifics during activities such as predator defence (e.g. Krams *et al.*, 2010),

foraging (e.g. Kim, Krafft & Choe, 2005) and offspring care (e.g. Kingma, Hall & Peters, 2011). During conspecific interactions, individual responses are governed by the information that each individual has about the other. Simple traits such as sex, size and receptivity can be identified via sensory cues during a single interaction (Mason, 1993; Shine, Olsson & Mason, 2000; Shine *et al.*, 2003), but previous experience with a conspecific allows for more complex information to influence behavioural decisions (e.g. reciprocity: Axelrod & Hamilton, 1981). The ability to identify individuals based on their social significance

\*Corresponding author. E-mail: shannonkhoss@gmail.com

has been demonstrated in all major vertebrate groups, as well as some invertebrates (reviewed by Tibbetts & Dale, 2007), but exactly how these multi-component representations or memories of individuals are formed is still unclear (Johnston & Peng, 2008).

One piece of information that can affect conspecific interactions is the relatedness between individuals, as engaging in mutually beneficial behaviours with kin may theoretically increase an individual's inclusive fitness indirectly by increasing the fitness of relatives (i.e. kin selection) (Hamilton, 1964; Maynard, 1964; but see Griffin & West, 2002). Given the potential influence of kin selection on the evolution of social behaviour, a large amount of literature documenting kin discrimination in a wide range of species has accrued (reviewed by Hepper, 1991). Historically, the proximate mechanisms responsible for kin recognition were thought to be either genetically based ('recognition by phenotype matching') or familiarity-based ('recognition by association'), and thus innate and immediately assessed, or learned and retrieved from memory, respectively (reviewed by Penn & Frommen, 2010). However, the over-simplification of this dichotomy and the potential for organisms to integrate information gained from both phenotype matching and familiarity has been acknowledged (reviewed by Barnard, Hurst & Aldhous, 1991; Tang-Martinez, 2001; Penn & Frommen, 2010). For example, a recent study revealed that although male golden hamsters may be able to distinguish among multiple unfamiliar female hamsters using a variety of odour cues, association via physical contact with a female was required for memory formation, and thus future recognition (Johnston & Peng, 2008).

The extended post-birth association of parents and offspring in species with parental care provides related individuals an opportunity to collect information about one another that can be used during future interactions. Cross-fostering studies have found that the effect of rearing environment on kin discrimination varies considerably among species. Specifically, some species identify anyone with whom they were reared as 'kin', regardless of relatedness (e.g. prairie voles: Paz & Tang-Martinez, 1999), some are able to recognize unfamiliar kin, despite being reared with non-kin (e.g. zebra finches: Krause *et al.*, 2012), and still others appear to incorporate both phenotype matching and familiarity into 'kin templates', against which individuals are matched (e.g. golden hamsters: Mateo & Johnston, 2000). A limitation of these studies, however, is that they do not address how kin discrimination is affected by the complete lack of an early social environment (i.e., being reared in isolation). In fact, there is an absence

of studies in which hatchlings or newborns of species with parental care are immediately separated from all conspecifics and remain so for an extended period of time before kin discrimination is assessed. This is presumably due to the fact that the majority of species which engage in parental care do so obligatorily (i.e. young would not survive in the absence of care), preventing the immediate isolation of young from being a viable option – precocial avian species are an exception (Lickliter & Dyer, 1993). Fortunately, various forms of facultative parental care occur in the taxonomically diverse ectothermic vertebrates (i.e. fish: Crawford & Balon, 1996; amphibians: Crump, 1996; reptiles: Gans, 1996), making them ideal candidates for determining how the absence of care affects development.

In viviparous temperate-zone pitvipers, post-parturient females remain with their young for an extended period of time after birth ('maternal attendance'; reviewed by Greene *et al.*, 2002), but despite the apparent ubiquity of maternal attendance in this group of snakes, there have been few studies aimed at determining its adaptive value (Graves, 1989; Greene *et al.*, 2002; Reiserer, Schuett & Earley, 2008; Hoss & Clark, 2014). Anecdotal observations suggest that mother and offspring remain at the birth site until neonates complete their first skin shed, at which point they disperse (~10–14 days after birth, depending on species). During maternal attendance, mother and neonates form tight aggregations, and have been observed tongue-flicking and nudging each other's bodies and displaying behaviours thought to enhance the collection of chemosensory information by priming the vomeronasal organ (e.g. gaping and head-shaking; Graves & Duvall, 1983, 1985; Graves, Carpenter & Duvall, 1987). Although kin discrimination has been documented experimentally in only one pitviper species, the timber rattlesnake (*Crotalus horridus* Linnaeus; Clark, 2004a), this ability seems to be widespread in squamate reptiles (i.e. snakes and lizards; Main & Bull, 1996; Lena & de Fraipont, 1998; Bull *et al.*, 2001; Himes, 2002; Font & Desfilis, 2002; O'Connor & Shine, 2006; Head *et al.*, 2008; Pernetta, Reading & Allen, 2009). As such, it is likely that most, if not all, pitvipers are capable of kin discrimination, and an extended post-birth period of association between mother and offspring might play a role in its development (Greene *et al.*, 2002).

The current study focuses on whether maternal attendance is required for and/or enhances kin discrimination in the cottonmouth (*Agkistrodon piscivorus* Lacépède). The cottonmouth is a widespread semi-aquatic pitviper that is most commonly found in the floodplain forests and beaver marsh complexes of the south-eastern United States (Gloyd & Conant,

1990), and has been shown to exhibit maternal attendance of young (Wharton, 1966; Walters & Card, 1996). Our objective was to compare kin discrimination after 6–8 months of separation between cottonmouths that had experienced maternal attendance prior to separation and those that had been reared in isolation since birth. To do so, we quantified both sibling and mother–offspring discrimination using two measures of affiliative behaviour: (1) inter-individual distance and (2) time spent entwined.

## METHODS

Pregnant cottonmouths ( $N = 14$ ) were collected during July and August 2010 from Georgia (Clayton and Fayette Co.) and Alabama (Macon Co.), transported to San Diego State University (San Diego Co., CA), and maintained in a temperature- and lighting-controlled room ( $-22.7$ – $26.7$  °C and 12:12-h light–dark cycle). Snakes were housed in individual cages containing a hide-box, offered one mouse per week for food, provided water *ad libitum* and checked for parturition daily. Each pregnant female was randomly assigned to one of two treatment groups: maternal attendance (MA) or separated (SE). Upon parturition, MA females and their neonates were allowed a maternal attendance period, whereby the litter was left in the birth cage with the mother until all neonates in that litter completed ecdysis (8–11 days; mean  $\pm$  SD:  $8.67 \pm 1.21$ ), after which they were separated and maintained individually in cages for the duration of the study. Females assigned to the SE group were not allowed a maternal attendance period. Instead, neonates were separated from the mother and their littermates within 24 h of birth and maintained individually for the duration of the study. After neonate removal, the mother's cage was cleaned with a bleach solution to remove any chemical cues associated with her offspring. Although parturition checks were made on a 24-h interval, cottonmouths typically give birth in the early morning hours (S. Hoss, pers. observ.), so separations probably occurred within 8 h of birth. Litters contained 1–5 neonates ( $3.36 \pm 1.28$ ), excluding stillborns.

### JUVENILE–JUVENILE EXPERIMENT

This experiment was designed to assess the effects of a maternal attendance period on affiliative behaviour among juveniles, after individuals of both treatment groups had been separated for ~ 6 months. Behavioural trials were conducted during February and March 2011 on randomly assigned unique pairs of juveniles ( $N = 28$ ) that were matched according to

three factors: (1) treatment group (MA or SE), (2) sex (male or female) and (3) relatedness (kin or non-kin; i.e. siblings or non-siblings). Both members of the pair had the same designation for all three factors. Trials took place in a  $40 \times 40$ -cm arena fitted with an infrared time-lapse video camera. Each pair was placed in an arena and left undisturbed for 4 days; arenas were cleaned with a bleach solution between trials. From the videos, a still frame was taken every 3 h over the 4 days of the trial, and two measures of affiliative behaviour were recorded from each still: (1) the minimum distance between individuals (measured using Image J freeware; W. S. Rasband, National Institutes of Health, Bethesda, MD, USA) and (2) whether individuals were entwined. Entwining behaviour consisted of individuals wrapping parts of their bodies around each other or one individual coiling on top of the other, resulting in a single mass; this was distinct from instances where the coils of the two individuals did not overlap. Because snakes introduced to a novel area spend prolonged periods exploring before engaging in other behaviours (Clark, 2004b), we considered the first 30 h of the trial an acclimation period, so only measures made on the final 23 stills were analysed.

To minimize the potential effects of shedding and digestion on behaviour, individuals were not tried when they showed signs of entering a shed cycle (e.g. dull skin, opaque eyes) or within 1 week of being fed. Also, there is potential for male pitvipers to sire multiple litters and litters to be sired by multiple males (Uller & Olsson, 2008), so to ensure that our non-kin pairs did not contain individuals that were actually paternal half-sibs, we matched juveniles from geographically disparate populations. In addition, genetic analyses conducted as part of a forthcoming study (W. Booth, manuscript in preparation) identified multiple paternity in only one litter used in this study, and only siblings sired by the same father were used in kin pairs. Thus, we were able to confirm that individuals in all of our kin pairs were full-sibs.

### MOTHER–JUVENILE EXPERIMENT

This experiment was conducted during April and May 2011, after the completion of all juvenile–juvenile trials, and was focused on mother–juvenile affiliative behaviour. At the start of this experiment, all individuals had been separated for ~ 8 months. We established mother–juvenile pairs that differed according to three factors: (1) treatment group (MA or SE), (2) juvenile sex (male or female) and (3) relatedness (kin or non-kin; i.e. the direct progeny of the mother or an unrelated juvenile). Each mother ( $N = 14$ ) underwent two trials, the order of which

was random. In one trial, the mother was paired with one of her offspring, and in the other trial, she was paired with an unrelated juvenile of the same sex and treatment group. Trials using the same mother were separated by 2 weeks and juveniles were used in only one mother–juvenile pair. All other methods were identical to those of the juvenile–juvenile experiment, except that the arenas were larger (40 × 60 cm) to accommodate the adult female. All mothers and their offspring were released at the mother’s capture site upon completion of this study.

#### STATISTICAL ANALYSES

To avoid pseudoreplication, the average minimum distance between individuals during the final 3 days of the trial (i.e. the average of 23 distance measures taken from stills) was calculated for each juvenile–juvenile and mother–juvenile pair. The proportion of time entwined was calculated by dividing the number of stills in which individuals were entwined by the total number of stills. For juvenile–juvenile pairs, a general linear model was used to determine the effect of treatment group (MA or SE), sex (female or male) and relatedness (kin or non-kin) on average distance and proportion of time entwined, separately. For significant three-way interactions, we conducted post-hoc tests using Fisher’s least significant difference (LSD) on eight of the 28 possible pairwise comparisons. We chose these eight comparisons a priori, because we were interested only in comparing measures between MA and SE groups, for each sex\*relatedness combination (female kin, female non-kin, male kin, male non-kin), and between kin and non-kin groups, for each treatment\*sex combination (MA females, MA males, SE females, SE males). Dependent variables were square root transformed to meet

the assumptions of normality and homogeneity of variances.

Data from the mother–juvenile experiment were analysed using a linear mixed model design, in which treatment group, juvenile sex and relatedness were fixed factors, the individual ID of the mother was a random factor, and average distance or proportion of time entwined was the dependent variable. Mean distance for SE kin pairs had exceptionally large variances, causing the dataset to violate the assumption of homoscedasticity. We attempted numerous data transformations to remedy this problem, but were unable to obtain adequate homoscedasticity. As such, we conducted a linear mixed model on average distance using only data from MA pairs, with juvenile sex and relatedness as fixed effects, and the individual ID of the mother as a random effect. Also, we present and discuss the large variance observed in SE kin pairs. We did not encounter the same issue in the analysis of proportion of time entwined, so treatment group remained a fixed effect in that model. We did, however, square root transform proportion of time entwined to meet the assumptions of normality and homoscedasticity. For significant fixed effects, post-hoc tests were conducted using Fisher’s LSD. Significance was assessed at  $\alpha \leq 0.05$  and all analyses were conducted in SYSTAT 12 (Systat Corp.).

## RESULTS

### JUVENILE–JUVENILE EXPERIMENT

Untransformed values of mean distance and proportion of time entwined for each group are provided in Table 1. With respect to average distance, no significance was found for the main effects of treatment

**Table 1.** Mean ± standard error for measures of cottonmouth affiliative behaviour collected during two experiments

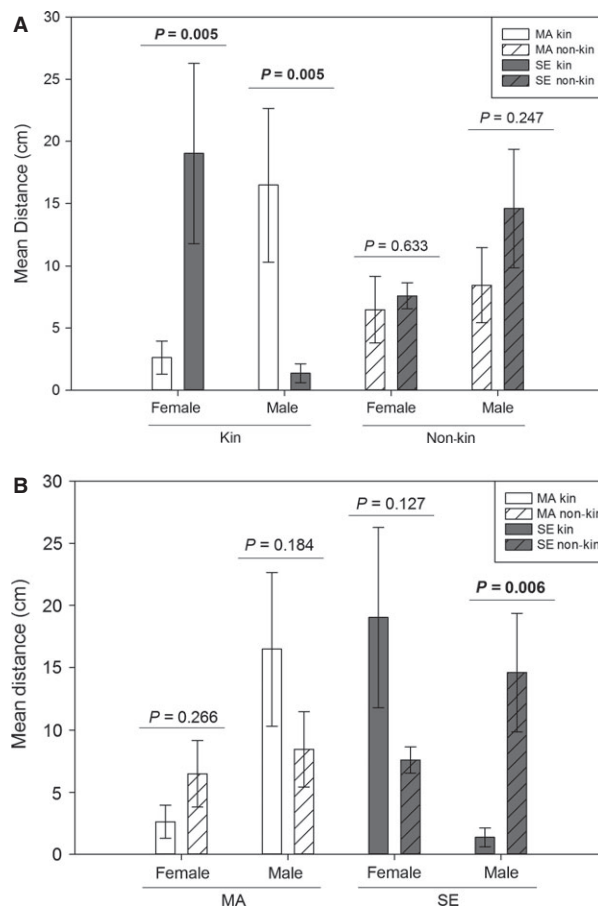
| Treatment           | Sex    | Relatedness | Juvenile–juvenile |             | Mother–juvenile |             |
|---------------------|--------|-------------|-------------------|-------------|-----------------|-------------|
|                     |        |             | Distance          | Entwined    | Distance        | Entwined    |
| Maternal Attendance | Female | Kin         | 2.63 ± 1.33       | 0.19 ± 0.10 | 2.69 ± 1.17     | 0.34 ± 0.09 |
|                     |        | Non-kin     | 6.49 ± 2.68       | 0.14 ± 0.08 | 8.96 ± 3.33     | 0.05 ± 0.02 |
|                     | Male   | Kin         | 16.49 ± 6.17      | 0.06 ± 0.04 | 4.97 ± 0.99     | 0.25 ± 0.14 |
|                     |        | Non-kin     | 8.45 ± 3.03       | 0.18 ± 0.09 | 9.31 ± 3.45     | 0.21 ± 0.11 |
| Separated           | Female | Kin         | 19.03 ± 7.23      | 0.00 ± 0.00 | 9.16 ± 7.27     | 0.31 ± 0.13 |
|                     |        | Non-kin     | 7.59 ± 1.05       | 0.19 ± 0.06 | 9.64 ± 2.48     | 0.03 ± 0.03 |
|                     | Male   | Kin         | 1.37 ± 0.76       | 0.43 ± 0.25 | 14.90 ± 10.61   | 0.38 ± 0.22 |
|                     |        | Non-kin     | 14.61 ± 4.75      | 0.08 ± 0.04 | 4.91 ± 2.45     | 0.28 ± 0.20 |

Juvenile–juvenile and mother–juvenile pairs were grouped by three factors: treatment, sex and relatedness. ‘Distance’ is the mean minimum distance (cm) between individuals in a pair, calculated over 23 time point. ‘Entwined’ is the mean proportion of time points during which individuals in a pair had sections of their bodies intertwined.

( $F_{1,20} = 0.58$ ,  $P = 0.454$ ), sex ( $F_{1,20} = 0.17$ ,  $P = 0.689$ ) or relatedness ( $F_{1,20} = 0.31$ ,  $P = 0.584$ ), nor the interactions between treatment and relatedness ( $F_{1,20} = 0.62$ ,  $P = 0.440$ ) or sex and relatedness ( $F_{1,20} = 1.38$ ,  $P = 0.255$ ). The interaction between treatment and sex was significant ( $F_{1,20} = 9.23$ ,  $P = 0.007$ ), as was the interaction between all three factors (treatment\*sex\*relatedness:  $F_{1,20} = 13.63$ ,  $P = 0.001$ ). Post-hoc tests revealed that the presence or absence of a maternal attendance period had a strong effect on how male and female juveniles responded to kin. If allowed a maternal attendance period, female kin remained significantly closer to each other than if they were separated at birth (Fig. 1A). Males showed the opposite pattern where maternally attended kin remained further from each other than kin that were separated at birth. No effect of maternal attendance was observed in male or female non-kin pairs. In the MA group, female kin remained closer to each other than non-kin, and male kin remained further from each other than non-kin, but these differences were not significant (Fig. 1B). This pattern was reversed in the SE group, where female kin remained further from each other than non-kin and male kin remained closer to each other than non-kin, but only the male comparison was significant.

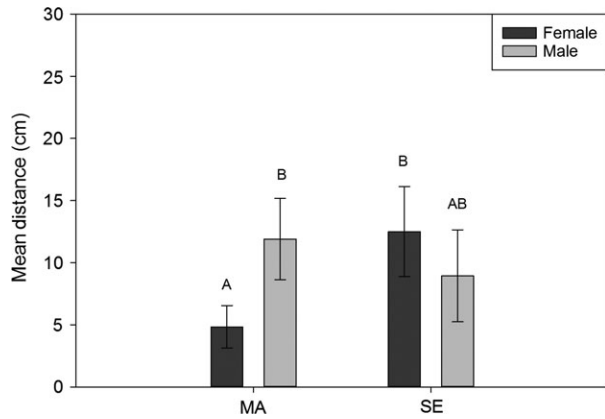
In addition to the significant interaction between all three factors, ANOVA revealed a strong two-way interaction between treatment group and sex, and we performed additional post-hoc tests (Fisher's LSD) examining the relationship between these two factors, while ignoring relatedness. Although it is not typical to interpret lower-order interactions in the presence of a significant higher-order interaction, we did so to allow comparison of our results with those of studies in which relatedness was unknown (see Discussion). The post-hoc tests revealed that females (related or unrelated) remained significantly closer to each other if they had been given a maternal attendance period (MA females vs. SE females:  $P = 0.014$ ; Fig. 2), but males did not show a significant effect of treatment group (MA males vs. SE males:  $P = 0.124$ ). Also, in the MA group, females remained significantly closer to each other than males (MA females vs. MA males:  $P = 0.024$ ), but this pattern was reversed in the SE group, although the difference was not significant (SE females vs. SE males:  $P = 0.078$ ).

With respect to proportion of time entwined, no significance was found for the main effects of treatment ( $F_{1,20} = 0.02$ ,  $P = 0.887$ ), sex ( $F_{1,20} = 0.72$ ,  $P = 0.406$ ) or relatedness ( $F_{1,20} = 0.29$ ,  $P = 0.594$ ), nor the interactions between treatment and sex ( $F_{1,20} = 2.54$ ,  $P = 0.127$ ), treatment and relatedness ( $F_{1,20} = 0.02$ ,  $P = 0.884$ ), or sex and relatedness ( $F_{1,20} = 2.67$ ,  $P = 0.118$ ). However, there was a significant



**Figure 1.** Mean ( $\pm$  standard error) distance between juvenile cottonmouths that experienced a maternal attendance period (MA) or were separated at birth (SE). Post-hoc tests were conducted to determine the effect of treatment, with respect to sex and relatedness (A), and the effect of relatedness, with respect to sex and treatment (B). The  $P$ -value associated with each pairwise comparison is provided above adjacent bars; statistically significant  $P$ -values are in bold. Note that graphs A and B display the same group means, but in a different orientation.

interaction between all three factors (treatment\*sex\*relatedness:  $F_{1,20} = 8.17$ ,  $P = 0.010$ ). Post-hoc tests revealed that female kin spent more time entwined if given a maternal attendance period than if separated at birth, but unrelated females were not affected by treatment (Fig. 3A). Maternal attendance had the opposite effect on male kin, where MA male kin spent significantly less time entwined than SE male kin, but again, treatment did not affect unrelated pairs. There was not a statistically significant effect of relatedness on entwining behaviour in males or females in the MA group, but female kin were entwined more often than female non-kin and male

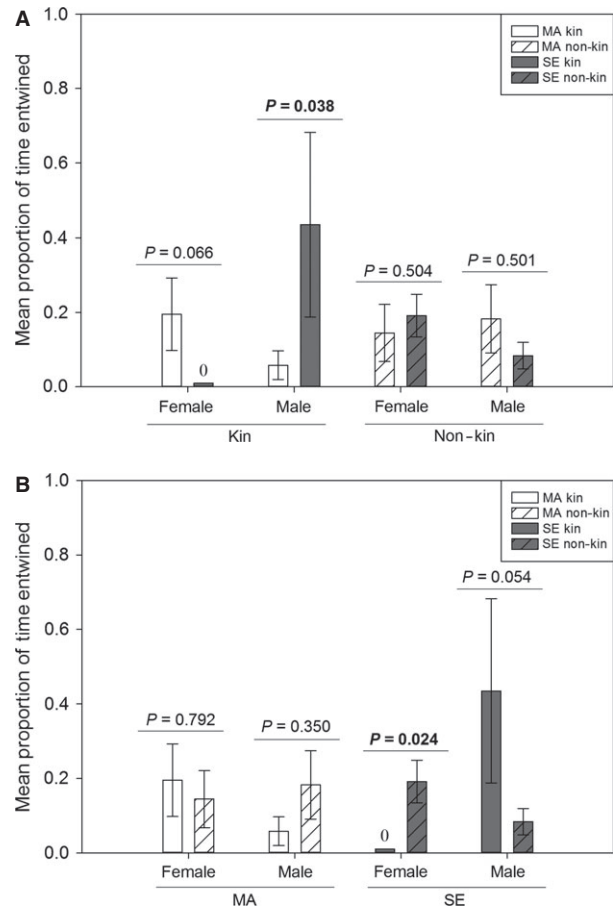


**Figure 2.** Mean ( $\pm$  standard error) distance between juvenile cottonmouths that experienced a maternal attendance period (MA) or were separated at birth (SE). Post-hoc tests were conducted to examine the significant interaction between treatment group and sex (kin and non-kin groups combined). Bars that share a letter were not significantly different.

kin were entwined less often than male non-kin (Fig. 3B). Thus, the pattern of entwining behaviour was reversed and stronger in juveniles that had been separated at birth, as was the case with average distance. Specifically, SE female kin were never observed in the entwined position, but SE female non-kin were entwined  $\sim 20\%$  of the time, SE male kin were entwined more often than SE male non-kin, but the large amount of variation in the SE male kin group prevented this difference from achieving statistical significance.

#### MOTHER–JUVENILE EXPERIMENT

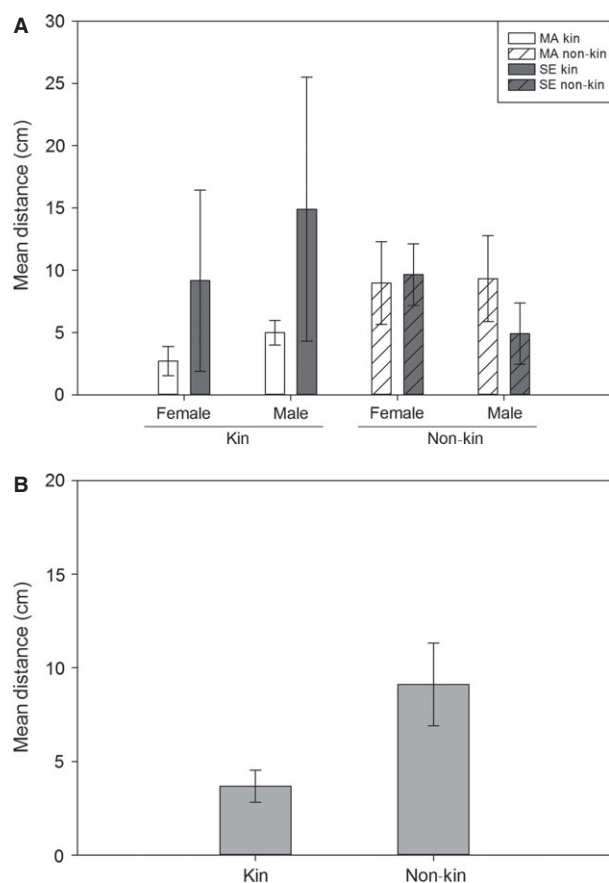
Untransformed values of mean distance and proportion of time entwined for each group are provided in Table 1. As discussed above, there was a large amount of variance in average distance data for SE pairs, which prevented their inclusion in the linear mixed model. This substantial variance was observed in related pairs, regardless of the sex of the juvenile, but not in unrelated pairs (Table 1; Fig. 4A). This indicates that related SE pairs did not show consistency in how close they remained to each other, but unrelated SE pairs did. In the linear mixed model using only data from MA pairs, there was no significant interaction between juvenile sex and relatedness ( $F_{1,9} = 0.14$ ,  $P = 0.717$ ), so it was removed from the model to conserve degrees of freedom. The reduced model did not find a significant main effect of juvenile sex ( $F_{1,10} = 0.29$ ,  $P = 0.603$ ), but the main effect of relatedness was significant ( $F_{1,10} = 4.97$ ,  $P = 0.050$ ), where related pairs remained



**Figure 3.** Mean ( $\pm$  standard error) proportion of time that pairs of juvenile cottonmouths spent entwined, relative to whether they experienced a maternal attendance period (MA) or were separated at birth (SE). Post-hoc tests were conducted to determine the effect of treatment, with respect to sex and relatedness (A), and the effect of relatedness, with respect to sex and treatment (B). The  $P$ -value associated with each pairwise comparison is provided above adjacent bars; statistically significant  $P$ -values are in bold. Note that graphs A and B display the same group means, but in a different orientation. The bar for 'SE kin female' is a placeholder and the '0' above it denotes that no pairs were observed entwined at any time point.

significantly closer to each other than unrelated pairs (Fig. 4B).

In the full linear mixed model on proportion of time entwined, there was no significant interaction between treatment, juvenile sex and relatedness ( $F_{1,19} = 0.03$ ,  $P = 0.876$ ), treatment and juvenile sex ( $F_{1,19} = 0.24$ ,  $P = 0.630$ ), or treatment and relatedness ( $F_{1,19} = 0.002$ ,  $P = 0.963$ ), so we removed them from the model to conserve degrees of freedom. The reduced model showed a non-significant interaction between juvenile sex and relatedness ( $F_{1,22} = 3.14$ ,



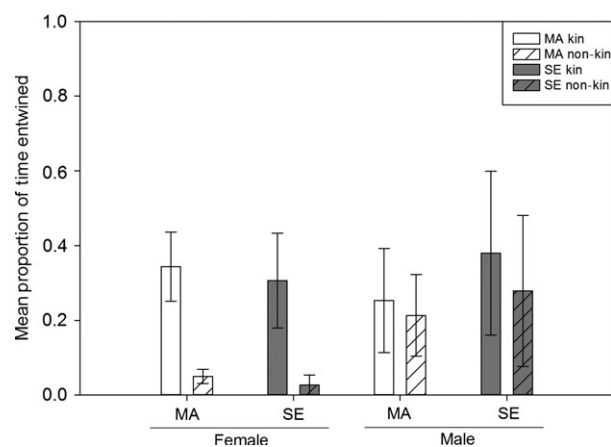
**Figure 4.** Mean ( $\pm$  standard error) distance between mother-juvenile pairs of cottonmouths that did or did not experience a maternal attendance period (MA or SE), and differed in terms of relatedness and the sex of the juvenile. Means for each group are presented in A, and means for MA pairs only (juvenile sex combined) are presented in B.

$P = 0.090$ ), no significant main effect of treatment ( $F_{1,22} = 0.02$ ,  $P = 0.888$ ) or juvenile sex ( $F_{1,22} = 0.38$ ,  $P = 0.546$ ), and a marginally significant main effect of relatedness ( $F_{1,22} = 3.69$ ,  $P = 0.068$ ). Although the interaction between juvenile sex and relatedness was not statistically significant, there was a tendency for related female pairs to be entwined more often than unrelated female pairs, regardless of treatment group, but this difference was not as strong when the juvenile of the mother-juvenile pairs was male (Fig. 5).

## DISCUSSION

### JUVENILE-JUVENILE EXPERIMENT

Our study provides several lines of evidence that maternal attendance plays a role in the development



**Figure 5.** Mean ( $\pm$  standard error) proportion of time that mother-juvenile pairs of cottonmouths spent entwined, with respect to whether they had experienced a maternal attendance period (MA or SE) and the sex of the juvenile.

of sex-specific affiliative behaviour and kin discrimination in juvenile cottonmouths. Maternally attended sisters showed the highest levels of affiliative behaviour (i.e. they remained closer to and were entwined more often with each other), but sisters that had been separated at birth were even less affiliative than unrelated females, and in fact had the largest mean distance of any group (including males). Furthermore, they were the only group in which no pairs were ever entwined, indicating possible avoidance behaviour. Males exhibited patterns of affiliative behaviour opposite to that of females, although the effects of relatedness were not as pronounced in males. Maternally attended brothers were less affiliative than unrelated males, as well as brothers that had been separated at birth. Interestingly, post-birth separation resulted in the same extreme reversal of affiliative behaviour in brothers as was seen in sisters. In other words, while maternally attended brothers showed relatively low levels of affiliative behaviour, separated brothers showed the highest levels of affiliative behaviour across all groups, including females. Finally, when data for related and unrelated pairs were combined, affiliative behaviour was female-biased in the MA group, but male-biased in the SE group.

The female-biased affiliative behaviour observed in maternally attended juveniles, especially with respect to relatedness, corresponds to what has been seen in both captive and field studies of pitviper social behavior. Clark (2004a) conducted a similarly designed study, in which he paired sibling and non-sibling timber rattlesnakes after 2.5 years of separation. Although timber rattlesnakes exhibit maternal

attendance, this behaviour was not incorporated into the design; instead, neonates of all litters were kept in a communal cage until they completed their post-natal shed, to mimic the birthing rookeries observed in the source population. It was found that sisters remained closer to and were entwined more often with each other than unrelated females, and both groups of females (kin and non-kin) were more affiliative than males. In a follow-up field study of timber rattlesnakes, Clark *et al.* (2012) used genetic methods to calculate the relatedness of individuals found in natural aggregations in order to determine if they were more closely related than would be expected if snakes formed aggregations with random individuals. They found that aggregations of pregnant females consisted of closely related individuals, sometimes from different generations, and same-aged juvenile pairs found together at dens were probably siblings, although juvenile sex did not appear to be a significant factor. The only work concerning sex differences in affiliative behaviour in cottonmouths combined data from an observational field study and an experimental captive study to show that adult females were much more gregarious than adult males, and that males actively avoided each other (Roth & Lutterschmidt, 2011); however, the relatedness among individuals was not considered.

The general pattern of sex-specific affiliative behaviour toward kin and non-kin observed in pitvipers can be explained in terms of the fitness costs and benefits of associating with relatives (Hamilton, 1964). For example, pregnant females of many pitviper species, including cottonmouths, have been known to form aggregations during gestation and parturition, which could benefit females and their neonates through enhanced thermoregulation and a reduction in predation risk (reviewed by Graves & Duvall, 1995; Reiserer *et al.*, 2008). In addition to benefiting directly from aggregations, females could increase their inclusive fitness by preferentially aggregating with relatives. As mentioned previously, Clark *et al.* (2012) provided evidence that this phenomenon probably occurs in wild timber rattlesnakes. Male affiliative behaviour, on the other hand, might be costly in pitvipers. For example, adult males compete for access to mates and engage in ritualized combat upon encountering a rival male (Gillingham, 1987), which may explain their apparent intolerance of other males (Clark, 2004a; Roth & Lutterschmidt, 2011). The male cottonmouths in the current study were not sexually mature, however, and were significantly less affiliative with kin than non-kin if they had experienced maternal attendance. This might be expected if cottonmouths exhibit male-biased juvenile dispersal and avoid related males during this time to reduce kin competition and

inbreeding (Johnson & Gaines, 1990), as well as the potential for cannibalizing siblings. While cannibalism in cottonmouths has been documented (reviewed by Campbell & Lamar, 2004), information on dispersal patterns of this species is not available; however, recent genetic studies have found evidence for male-biased dispersal in several snake species (Rivera, Gardenal & Chiaraviglio, 2006; Keogh, Web & Shine, 2007; Clark *et al.*, 2008; Dubey *et al.*, 2008; Pernetta *et al.*, 2011).

While the absence of a maternal attendance period did not have strong effects on the affiliative behaviour of unrelated juveniles, it drastically changed how males and females responded to kin (see Fig. 1A); this result was entirely unexpected. If maternal attendance functions to enhance species-typical kin discrimination, we would expect to see a smaller or no effect of relatedness on affiliative behaviour in juveniles separated at birth. Not only did we see a much larger effect (i.e. increased kin discrimination), but the sex-specific differences were reversed in kin groups, where separated brothers behaved like maternally attended sisters and separated sisters behaved like maternally attended brothers. If maternal attendance is essential for kin recognition, we would expect to see evidence for kin recognition (i.e. kin pairs behaving differently than non-kin pairs) in maternally attended juveniles, but not separated juveniles. Again, not only did we find evidence for kin recognition in both groups, but discrimination was even more pronounced in juveniles that were separated at birth.

Although we do not have an explanation for why separated siblings showed a reversal, rather than a lack of behavioural patterns, we can conclude that the behavior of maternally attended juveniles was closely aligned to what has been documented in captive and wild snakes, and thus appeared to be species-typical behaviour, but the behaviour of siblings that were separated at birth was atypical, in comparison. Similar disruptive effects on the development of normal filial imprinting have been documented in hatchlings of precocial avian species that were reared in isolation or abnormal post-hatching social environments (reviewed by Lickliter & Dyer, 1993).

#### MOTHER–JUVENILE EXPERIMENT

Maternal attendance also affected mother–juvenile affiliative behaviour, but because average distance and proportion of time entwined did not always vary in the same manner, we will discuss the two measures independently, instead of combining them under the term ‘affiliative behaviour’. Regardless of juvenile sex, mother–juvenile pairs that had a maternal attendance period remained significantly closer



to each other if they were related than if they were unrelated. In contrast, there was a substantial amount of variation in average distance of related mother–juvenile pairs in the SE group (see Fig. 4A), which suggests that being separated at birth results in inconsistent behaviour toward kin. Entwining behaviour showed the same non-significant pattern in both treatment groups, with mother–juvenile pairs being entwined more often if they were related than if they were unrelated, but the effect of relatedness was much stronger if the juvenile was female; indeed, mothers from both treatment groups rarely entwined with unrelated females.

Similar to what was observed in the juvenile–juvenile experiment, related females (i.e. mother–daughter pairs) that had been given a maternal attendance period showed high levels of affiliation; however, treatment group did not affect entwining behaviour. This suggests that the affiliative behaviour of mothers and daughters is more robust than that of sisters and not dependent upon a post-birth period of association (i.e. maternal attendance). The potential benefits of increased affiliation between sisters (discussed above) also apply to mother–daughter affiliations, and the multi-generational aggregations of related females discovered in free-ranging timber rattlesnakes (Clark *et al.*, 2012) suggests that mother–daughter interactions do occur outside of the maternal attendance period. However, male juveniles can also benefit from affiliating with their mother. There is evidence that neonatal rattlesnakes navigate to den sites by following adult conspecific scent trails (Brown & MacLean, 1983; Graves *et al.*, 1986; Reinert & Zappalorti, 1988; Cobb *et al.*, 2005), and limited evidence that some neonates trail their mother (but not necessarily interact with her) for weeks after birth-site dispersal (Cobb *et al.*, 2005; Jellen & Kowalski, 2007). In support of this limited evidence, 91% of timber rattlesnakes genetically identified as mother–offspring pairs were found at the same hibernaculum, despite the availability of other nearby hibernacula (Clark *et al.*, 2008).

If separated at birth, mother–juvenile pairs did not show the drastic reversals in kin-associated affiliative behaviour seen in the juvenile–juvenile experiment and did not differ from MA pairs in entwining behaviour; rather, mean distance of kin pairs in the SE group was highly variable. This suggests that maternal attendance is not required for mother–offspring recognition (e.g. both treatment groups showed a strong relatedness effect on their propensity to entwine with female juveniles), but that it possibly enhances kin-associated affiliative behaviour overall. Greene *et al.* (2002) found evidence for mother–offspring attraction in another pitviper species with maternal attendance, the pygmy rattlesnake

(*Sistrurus miliarius* Linnaeus). In a choice-experiment conducted in captivity, Greene and colleagues found that mothers preferred to remain with their neonates and neonates preferred to remain with their mother, vs. remaining solitary. These results provide some evidence of mutual attraction between mothers and offspring; however, subjects did not have the choice of associating with non-related individuals, so if they did not want to remain solitary, their only option was to associate with a related individual. Thus, it is unclear whether these results reflect true mother–offspring recognition and attraction or simply the gregarious nature of most snakes when in captive situations (Aubret & Shine, 2009).

To the best of our knowledge, there is only one empirical study documenting mother–offspring recognition in a snake, in which post-parturient water snakes (*Nerodia sipedon* Linnaeus; a viviparous North American colubrid species lacking maternal attendance) preferentially cannibalized unrelated vs. related neonates (Himes, 2002). Other studies have determined that several viviparous lizard species are capable of mother–offspring recognition and that this ability is not entirely dependent upon post-birth associations between relatives (Main & Bull, 1996; Lena & de Fraipont, 1998; Head *et al.*, 2008). However, Lena & de Fraipont (1998) found that juvenile common lizards (*Lacerta vivipara* Jaquin) that had been previously housed with relatives preferred shelters containing mother or sibling scents, but if they had been separated at birth, they were still attracted to the scent of their mother but no longer attracted to the scent of a sibling. These results corroborate our finding that familiarity appears to be more important for future interactions with siblings than with mothers. It is important to note, however, that common lizards do not engage in parental behaviour, but parents and offspring have overlapping home ranges (Clobert *et al.*, 1994), and consequently have the potential for continued interactions.

## CONCLUSIONS

Our results suggest that maternal attendance in cottonmouths is not required for sibling or mother–offspring recognition, but does generally enhance female affiliative behaviour and is critical for the affiliative behaviour of sisters. Furthermore, being separated at birth appears to specifically affect the affiliative behaviour of kin, as evidenced by a reversal in the sex-specific pattern of inter-individual distance and entwining of siblings, substantial variation in inter-individual distance of mother–offspring pairs, and the absence of such effects in non-kin pairs. It is important to note, however, that our

experimental design precluded our ability to separate the effects of early social interaction with an attending mother vs. siblings, as neonates were reared in isolation or with both an attending mother and littermates. It is possible that post-birth interaction with littermates alone would be sufficient for the development of species-typical behaviour, but it is unknown whether or to what extent litters aggregate in the absence of an attending mother. The lack of research concerning social behavior in snakes (Stahlschmidt, 2011; Doody, Burghardt & Dinets, 2013), particularly with regard to interactions among relatives (Clark *et al.*, 2012), makes it difficult to evaluate how important kin selection might be and whether it is disproportionately important in species exhibiting maternal attendance. It is doubtful, however, that kin selection is strong enough to have driven the evolution of maternal attendance in this group, and more likely that enhanced affiliative behaviour is only one of many benefits conveyed to offspring by an attending mother (Greene *et al.*, 2002; Reiserer *et al.*, 2008; Hoss & Clark, 2014).

Increased familiarity with relatives during a post-birth period of association might affect kin discrimination and affiliative behaviour in other species characterized by facultative parental care, but such studies are absent from the literature. Unfortunately, these are the species most likely to provide insight as to the selective factors responsible for the evolution of obligatory forms of parental care and other complex social behaviors (While, Uller & Wapstra, 2009). Indeed, there is quickly accruing evidence that some groups thought to be relatively asocial, such as squamate reptiles, exhibit various forms of cryptic sociality (e.g. monogamous pair-bonds and family groups: Chapple, 2003; social recognition: Yeager & Burghardt, 1991; non-random kin aggregations: Clark *et al.*, 2012; spatial and temporal social organization: Shine *et al.*, 2005; and kin discrimination: Clark, 2004a; Pernetta *et al.*, 2009). Given the impressive capacity of organisms to form complex representations of individuals, the importance of this ability to the evolution of social behaviour, and the recent discoveries of cryptic sociality in 'asocial' taxa, we encourage further research on the adaptive value of facultative parental care in ectothermic vertebrates.

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