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SHORT COMMUNICATION

Like mother, like offspring: maternal and offspring wound healing correlate in snakes

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SUMMARY

Immune function early in life can be influenced by parental effects and the environment, but it remains unclear how these two factors may interact to influence immunocompetence. We evaluated maternal and environmental contributions to offspring healing ability in a viviparous reptile, the northern watersnake (*Nerodia sipedon*). We measured wound healing rates, a highly integrative and biologically relevant measure of innate immunity, of females and their offspring collected from sites contaminated with a toxic heavy metal and compared them with those of individuals from reference sites. We found that female watersnakes that healed the fastest produced offspring that also exhibited faster healing rates. However, we detected no influence of environmental pollution on maternal or offspring healing rates. To our knowledge, our study is the first to correlate maternal and offspring wound healing ability in a wild vertebrate.

Key words: parental effects, immunocompetence, maternal transfer, mercury.

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INTRODUCTION

Immunocompetence is a critical determinant of individual fitness (Graham et al., 2010) that is shaped by both parental and environmental factors (Grindstaff et al., 2003; Lazzaro and Little, 2009). Previous studies have shown that parental effects, including genetic and non-genetic components, and environmental effects (i.e. resource availability, abiotic conditions, anthropogenic stressors) can influence initial offspring immune function (Brinkhof et al., 1999; Grindstaff et al., 2003; Rubolini et al., 2006; Pölkki et al., 2012). In a cross-fostering experiment with great tit (*Parus major*) nestlings, maternal origin accounted for 15% of the variation while rearing environment explained 31% of the variation in offspring immunity, suggesting both parental and environmental sources can contribute to initial immunocompetence (Brinkhof et al., 1999). However, few studies have examined the potential repercussions of environmental factors on offspring immunity that are mediated through parental pathways. For example, many immunosuppressive contaminants can be maternally transferred from a female to her offspring during sensitive stages of development (Eisenreich et al., 2009; Bergeron et al., 2010). Exposure to contaminants could provide a source of variation in female and offspring immunocompetence, resulting in adverse effects such as reduced antibody responses and impaired T-cell function (Hawley et al., 2009; Pölkki et al., 2012). Because both parental and environmental sources can have multi-generational effects on phenotype, it is important to understand the contribution that each of these factors has on immunity.

We evaluated maternal and environmental contributions to offspring healing ability in a viviparous reptile, the northern watersnake (*Nerodia sipedon* Linnaeus 1758). We sought to understand the influence of mercury (Hg), a common pollutant thought to have immunosuppressive properties (Spalding et al., 2000; Finkelstein et al., 2007; Hawley et al., 2009), on maternal and offspring healing rates. We collected gravid female watersnakes along reference river sites located in central Virginia, USA, and at the South River, a site historically contaminated with Hg. We measured wound healing rate, a highly integrative and biologically relevant measure of innate immunity (Demas et al., 2011), of females and their offspring collected from sites polluted with Hg and compared them with those of individuals from reference sites.

MATERIALS AND METHODS

Upon capture, we measured snout-to-vent length (SVL; cm) and mass (g), and took a small tail biopsy as an indicator of Hg accumulation for all gravid females (Burger et al., 2005; Drewett et al., 2013). We transported females (N=20) to the laboratory and maintained them individually within a walk-in environmental chamber (25°C), but provided heat lamps that allowed them to thermoregulate naturally. Following parturition (9 August–4 September 2011), we randomly selected a single male neonate from each litter to be used in the experiment. To assess maternal transfer of Hg, we euthanized, lyophilized and homogenized three randomly selected neonates per litter to be analyzed for Hg. Samples were analyzed for total mercury at the College of William and Mary, Williamsburg, VA [see Drewett et al. (Drewett et al., 2013) and Chin et al. (Chin et al., 2013a) for details about snake collection, housing and sample analysis].

We used a 4 mm (adult females) or 3 mm (neonates) diameter sterile biopsy punch to administer a circular, superficial wound to the skin of the posterior right lateral side of females 8 days after their first post-parturition shed and of neonates 14 days post-birth. We injected a local anesthetic (2% lidocaine HCL; neonates: 10 µl, adults: 30 µl) subcutaneously to a sterilized area prior to wounding. Only the top layer of skin was removed by the punch and no muscle tissue was disturbed. In some cases, a small amount of bleeding occurred around the excised area and we applied pressure to the area until bleeding had completely stopped. We assessed wound

healing by photographing the wound daily using a dissecting scope interfaced to a laptop computer. Photographs were not taken on the day after feeding to prevent regurgitation. A single reviewer, blind to snake identities, analyzed photographs using Micron imaging software (Westover Scientific, Mill Creek, WA, USA) to determine wound area (mm^2). We considered wound area to be any area encompassed by the epidermal margin, and once re-epithelialization was complete across the entire wound area (i.e. epidermal margins joined), the individual was considered 'healed'. During healing, we housed neonates individually within the environmental chamber (25°C) in 51 plastic shoeboxes and females in 751 aquaria, both with a paper towel substrate and a water bowl small enough to prevent submergence [see Chin et al. (Chin et al., 2013a) for husbandry details]. Following completion of wound healing, neonates and adult females were released at the female's capture location.

We conducted all statistical analyses using SAS (version 9.2, SAS Institute, Cary, NC, USA) and assessed significance at $\alpha=0.05$. All data met parametric assumptions of normality and homoscedasticity. Both SVL and body mass were included in all initial models but were subsequently dropped due to non-significance ($P\geq0.33$). Due to technical difficulties with the camera or shedding during the healing phase, four individuals (two adults and two neonates, unrelated to one another) were unable to be used in statistical analyses, yielding final sample sizes of 18 females, 18 neonates and 16 female–offspring pairs.

We tested for differences between healing rate ($mm^2\,day^{-1}$) of females and offspring using a generalized linear mixed model (PROC GLIMMIX) that corrected for initial wound size. We assessed the relationship between days to full recovery of females and their respective offspring using a linear regression. We examined the influence of Hg on wound healing progression (% of wound healed over time) of females and their offspring using two repeated-measures ANOVA models with site (contaminated *versus* reference) as the main effect. Finally, we evaluated the influence of tissue Hg concentrations on days to full recovery (wound 100% healed) for both females (tail tissue) and neonates (whole-body tissue) using two linear regression models.

All work was conducted in accordance with the animal care and use committee at Virginia Tech (IACUC protocol 11-131).

RESULTS

Initial wound area averaged $12.56\pm0.24\,mm^2$ for adult females ($N=18$) and $8.42\pm0.26\,mm^2$ for neonates ($N=18$). Days to full recovery varied considerably among individuals (6–28 days for adult females; 4–17 days for neonates). Although mean time required to fully heal was shorter for neonates (9.33 ± 0.84 days) than females (16.83 ± 1.81 days), healing rates did not differ (females: $0.95\pm0.12\,mm^2\,day^{-1}$; neonates: $1.07\pm0.13\,mm^2\,day^{-1}$; $F_{1,34}=0.52$, $P=0.47$) after correcting for differences in initial wound area. We detected a significant positive correlation between days to full recovery for adult females and their offspring ($N=16$, $r^2=0.34$, $P=0.02$; Fig. 1).

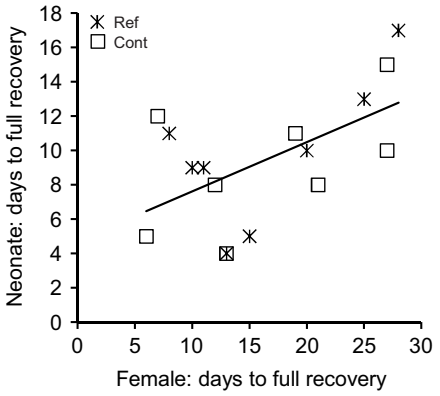

Fig. 1. Relationship between days to full recovery of female *Nerodia sipedon* and their offspring ($N=16$, $r^2=0.34$, $P=0.02$). Litters from reference (Ref) and contaminated (Cont) sites are indicated.

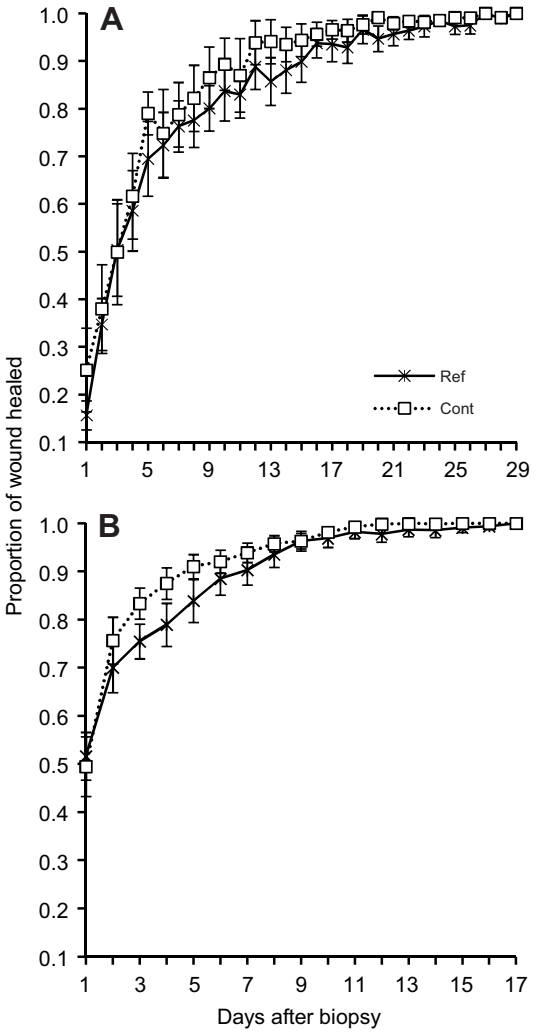

Fig. 2. Mean (±1 s.e.m.) proportion of wound healed over time for female (A) and neonate (B) *Nerodia sipedon* collected from either reference (Ref) or contaminated (Cont) sites in Virginia.

Wound healing progression did not differ between sites for either females (site: $F_{1,17}=0.46$, $P=0.51$; time: $F_{28,406}=66.31$, $P<0.01$; site × time: $F_{28,406}=0.22$, $P=1.00$; Fig. 2A) or neonates (site: $F_{1,17}=0.01$, $P=0.92$, time: $F_{16,266}=71.91$, $P<0.01$; site × time: $F_{16,266}=0.62$, $P=0.86$; Fig. 2B). Similarly, days to full recovery was not significantly related to tissue Hg concentrations within demographic groups (in both cases $P\geq0.47$).

DISCUSSION

To our knowledge, our study is the first to correlate maternal and offspring wound healing ability in a wild vertebrate. We demonstrated that female watersnakes that healed the fastest

produced offspring that also exhibited faster healing rates (Fig. 1). This is consistent with prior studies that demonstrated relationships between maternal and offspring innate, humoral and cell-mediated responsiveness in several bird species and one lizard (Brinkhof et al., 1999; Roulin et al., 2000; Svensson et al., 2001; Råberg et al., 2003) using other immune assessments designed to test the different branches of the immune system independently (Demas et al., 2011). We also demonstrated that healing rate did not differ between females and neonates. This is contrary to previous studies in laboratory mammals (i.e. rodents, rabbits) that report healing time increases with age (reviewed in Davidson, 1998). The relatively constant rates of healing across ontogeny in watersnakes may reflect physiological and life history characteristics of ectothermic vertebrates.

To our knowledge, our experiment is also the first to examine effects of pollutants on wound healing in vertebrates. Despite the fact that individuals collected from the contaminated site had very high tissue concentrations of Hg (female: $5.66\pm0.48\,mg\,kg^{-1}$; neonate: $3.11\pm0.26\,mg\,kg^{-1}$ dry mass) compared with reference individuals (female: $0.36\pm0.08\,mg\,kg^{-1}$; neonate: $0.20\pm0.11\,mg\,kg^{1}$ dry mass), we detected no effects of Hg on healing ability of female *N. sipedon* or their offspring. This is contrary to previous studies that have shown that Hg affects vertebrate immune function (Spalding et al., 2000; Finkelstein et al., 2007; Hawley et al., 2009). However, two recent studies found few adverse sublethal effects of maternally transferred Hg in neonatal *N. sipedon* (Chin et al., 2013a; Chin et al., 2013b), suggesting that our study species may be more tolerant of Hg than many other species. Future studies that examine species known to experience deleterious effects in response to Hg exposure [e.g. *Bufo americanus*, *Thryothorus ludovicianus*, *Tachycineta bicolor* and *Chelydra serpentina* (Bergeron et al., 2011; Hallinger et al., 2011; Jackson et al., 2011; Hopkins et al., 2013)] and that include larger sample sizes are needed to clarify the effects of Hg on wound healing and other aspects of immune function in wildlife.

It remains to be determined whether the effects that we observed have an underlying genetic basis and/or whether non-genomic factors (e.g. maternal effects) are influencing immunity. Previous research demonstrated that both sources can separately contribute to variation in offspring immunity. For example, in captive mice, several studies have shown wound healing to be a highly heritable trait (McBrearty et al., 1998; Li et al., 2001). Alternatively, DuRant et al. (DuRant et al., 2012) demonstrated that the environment can also contribute to immunity independent of genetic contributions: ducklings incubated at sub-optimal temperatures showed a 19–21% decreased swelling response after injection with phytohaemagglutinin. Therefore, we recommend future studies be directed at understanding the relative contribution of parental and environmental factors on offspring immunity.

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AUTHOR CONTRIBUTIONS

B.C.H., J.D.W. and W.A.H. contributed to conception, design and execution of the study, interpretation of the findings, and drafting and revising the article. S.Y.C. contributed to the execution of the experiment and the production of this manuscript.

COMPETING INTERESTS

No competing interests declared.

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