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Cholinesterase-Inhibiting Pesticides

INFLUENCE OF BODY SIZE ON SWIMMING PERFORMANCE OF FOUR SPECIES OF NEONATAL NATRICINE SNAKES ACUTELY EXPOSED TO A CHOLINESTERASE-INHIBITING PESTICIDE

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Abstract—Locomotor performance is an important fitness-related trait in reptiles because of its potential influence on prey capture and predator avoidance. Because cholinesterase-inhibiting pesticides disrupt neuromuscular signaling, reduction in performance seems to be a logical translation of this biochemical disruption to the organism level. In the present study, we compared the swimming performance of four species of natricine snakes acutely exposed to a formulation of carbaryl to determine whether neonatal body size or skin permeability influences responsiveness. Exposure to high concentrations of carbaryl (2.5–5.0 mg/L) resulted in reduced swimming performance in all four species of snakes, and species responded similarly to the pesticide once body size was accounted for allometrically. Using traditional methods in physiological ecology to estimate skin permeability (a parameter that influences the dose of contaminant absorbed), we found that water flux across the integument also scaled allometrically with body surface area and, therefore, was similar among species after controlling for this relationship. We suggest that future studies examining the effects of repeated low-dose exposure to cholinesterase inhibitors on performance parameters will be useful in assessing the ecological significance of our findings.

Keywords—Carbaryl Evaporative water loss Reptiles Snakes Swimming performance

INTRODUCTION

Compared to birds, fish, mammals, and even amphibians, reptiles are the most poorly studied vertebrates in ecotoxicology. Particularly lacking are studies on squamates (lizards and snakes) regarding organism-level traits, such as growth, reproduction, performance, and survival, that can influence an individual's fitness (i.e., lifetime reproductive output) and, perhaps, population-level processes [1–3]. Because contaminants are one of several factors that contribute to the global decline of reptile populations [4], studies examining the effects of contaminants on fitness-related traits are crucial to risk assessment and the conservation of reptiles.

Locomotor performance is an important fitness-related trait that has been well studied in reptiles. In lizards, studies examining locomotor performance are common, because it is a simple metric to quantify, has high repeatability within individuals, and probably is important to prey capture (and, therefore, acquisition of energy) and predator avoidance (and, therefore, survival) [5,6]. Studies directly linking variation in locomotor skills to variation in fitness are rare, primarily because of the inherent difficulties associated with long-term monitoring of individuals in wild populations. However, existing studies strongly support the importance of locomotor performance to survival of reptiles in the wild [5,7,8].

Although the ecology and evolution of locomotor performance have been well studied in squamate reptiles, little is known about the effects of contaminants on locomotion. Because cholinesterase-inhibiting pesticides (organophosphates and carbamates) can disrupt neuromuscular signaling, reductions in performance seem to be a logical translation of this biochemical disruption to the organism level. A recent study confirmed that acute exposure to high concentrations of carbaryl (a commonly used cholinesterase inhibitor) can reduce locomotor performance of snakes [9], but the generality of this response across species has not been adequately addressed. Understanding the relative sensitivity of species to toxicants is important from a conservation perspective, because such knowledge can facilitate the identification of species that require protection from certain activities (e.g., pesticide use in critical habitats). Likewise, understanding the ecological and/or life-history traits of animals that contribute to differences in relative sensitivity can be used to make predictions about unstudied species that may be sensitive and, thus, require protection. In the present study, we compared the swimming performance of four species of natricine snakes (three from the present study and one from a recent study [9]) acutely exposed to a cholinesterase-inhibiting pesticide to determine whether neonatal body size or skin permeability influences responsiveness. We hypothesized that one of the species, *Seminatrix pygaea,* would be more sensitive than the other species because of its relatively small body size and previously described permeable integument [10,11].

MATERIALS AND METHODS

Natural history of the species

We selected four species (*Nerodia taxispilota*, *Nerodia rhombifer*, *Nerodia fasciata*, and *S. pygaea*) of semiaquatic

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Table 1. Descriptive statistics of parameters related to the year 2003 and 2004 study designs, snake body morphology, evaporative water loss (EWL; $n = 9$ per species), and cutaneous water loss (CWL; $n = 3$ per species)[a]

				Neonate body size			Water loss	
Species	Year	*n*	No. of litters used	Mass (g)	SVL (mm)	Surface area (mm^2)	EWL	CWL
Seminatrix pygaea	2003	60	10	1.08 ± 0.02	109 ± 0.67	NA	NA	NA
	2004	35	6	1.28 ± 0.03	110 ± 0.85	1,477 ± 48	0.066 ± 0.01	0.046 ± 0.002
Nerodia fasciata	2004	35	6	4.12 ± 0.10	161 ± 1.55	3,459 ± 70	0.104 ± 0.02	0.052 ± 0.001
N. rhombifer	2003	59	7	9.91 ± 0.18	229 ± 1.22	NA	NA	NA
N. taxispilota	2004	36	6	11.09 ± 0.11	229 ± 1.17	6,603 ± 127	0.095 ± 0.01	0.099 ± 0.006

[a] Sample sizes represent the number of neonates used in statistical comparisons. All values for neonate body size, surface area, and water loss are expressed as the mean ± standard error. NA = not applicable; SVL = snout–vent length.

snakes (family Colubridae, subfamily Natricinae) with different ecological and physiological characteristics. All *Nerodia* sp. used in the present study frequently shuttle between aquatic and terrestrial microhabitats (e.g., adjacent terrestrial habitats and dry perch sites), frequently bask, and forage on aquatic prey. However, *N. taxispilota* generally is restricted to lotic systems, where these snakes primarily use arboreal basking sites (e.g., overhanging trees), and *N. rhombifer* and *N. fasciata* are more general in their habitat preferences. In contrast, *S. pygaea* is much more aquatic than the *Nerodia* sp. and is not known to bask in terrestrial microhabitats [10]. The aquatic lifestyle of *S. pygaea* may be coupled with physiological adaptations distinct from those of *Nerodia* sp. The skin of *S. pygaea* is believed to be more permeable than that of the *Nerodia* sp. (specifically *N. fasciata*), resulting in high rates of evaporative water loss [11]. It is possible that this high skin permeability affords *S. pygaea* the ability to respire cutaneously, as in other highly aquatic snakes [12], or at least allows it a reprieve from the energetic expenses that may be associated with epidermal lipid barriers to water loss [13].

Snake collection and housing

Adult gravid female *N. fasciata* ($n = 6$; snout–vent length [SVL], 601–788 mm), *N. taxispilota* ($n = 6$; SVL, 796–965 mm), and *S. pygaea* ($n = 6$; SVL, 354–383 mm) were collected between May 28, 2004, and July 21, 2004, from uncontaminated wetlands on the Savannah River Site near Aiken (SC, USA) and from the Savannah River. Captive husbandry of gravid females was similar to that described previously [9]. After birth (July 17, 2004, through October 2, 2004), neonates from the same litter (litter size: *N. fasciata*, 25.33 ± 6.19 [mean ± standard error]; *N. taxispilota*, 18.00 ± 1.51; and *S. pygaea*, 16.00 ± 1.55) initially were housed communally, as described previously [9]. Three days before the experiments, neonates selected for the present study were removed from their communal aquaria and housed individually in 737-ml plastic containers (25°C, 14:10-h light:dark photoperiod). No neonate (of any species) was fed before swim trials; therefore, neonates were reliant on yolk reserves. Descriptive statistics of snakes used in trials are presented in Table 1.

Experimental design

For our exposure solutions, we used a commercial formulation containing carbaryl (Sevin®; Garden Tech, Lexington, KY, USA). This formulation is one of the most readily available and commonly used forms of carbaryl, and it recently has become a model formulation for examining effects of cholinesterase inhibitors on amphibian development and life-history traits [14–20]. We chose to work with a common carbaryl formulation, because under natural situations, animals are exposed to formulations, not to commercial-grade compounds [21]. All solutions were made within 1 h of snake exposures. Solutions were made using weighed quantities of Sevin dissolved in aerated, aged tap water (dissolved oxygen, 8.93 ± 0.12 mg/L; pH, 8.04 ± 0.02; conductivity, 138 ± 1.76 μS/cm). Target carbaryl concentrations of 2.5 and 5.0 mg/L were selected based on previous use of similar concentrations [9,17,19]. These concentrations represent the upper limits of what is found in surface waters following field application of carbaryl [22,23]. To verify target concentrations of carbaryl, we sent 5-ml subsamples of water ($n = 3$ per treatment) to the Mississippi State University Chemical Laboratory (Mississippi State, MS, USA) for analysis. Samples were chilled immediately following preparation and were shipped overnight on ice. Samples were analyzed by high-performance liquid chromatography with postcolumn derivatization and fluorescence detection. Actual concentrations in water for the two carbaryl treatments were lower than our nominal concentrations (3.9 and 1.9 mg/L). Control water had no detectable carbaryl (detection limit, 1.0 μg/L).

Thirty-six snakes of each species were assigned to one of three treatments (0, 2.5, or 5.0 mg/L of carbaryl; $n = 12$ per species per treatment) for 48 h. Snakes were drawn from six litters of each species. Allocation of snakes was conducted such that each litter and sex were equally represented among treatments (i.e., stratified random sampling). Each snake was placed in a 1-L flask containing 500 ml of the appropriate test solution. Flasks were randomly arranged on shelves within an environmental chamber (12:12-h light:dark photoperiod, 25°C), where they were left undisturbed for 24 h. Each test solution was replaced with new test solution after 24 h, and snakes were returned to the environmental chamber for the last 24 h of the exposure period. At the end of the exposure (total exposure period, 48 h), each snake was removed from its flask (in the random sequence described above) and then raced (see below). Following the swim trial, each snake was returned to its original housing container holding aged tap water (with no carbaryl) to recover.

Swimming performance

Maximum swim velocity was determined for each snake at 25 ± 0.5°C using a 3-m swim track similar to that described previously [9,24,25]. Briefly, swimming occurred over a background marked at 1.0-cm increments and was recorded using a digital video camera (GL1 Mini DV Camcorder; Canon, Lake Success, NY, USA). Maximum swim velocity for each lap was calculated later using a frame-by-frame advance on a VCR with accuracy to 0.03 s [24–26]. To remove bias from the review process, the identity of snakes was concealed from the videotape reviewer (i.e., tapes were reviewed blindly). The time it took for each individual to swim 30 cm was calculated for each 30-cm segment of the track. The single fastest swim velocity (expressed as cm/s [27]) for each individual was used as an estimate of the maximum swimming performance in statistical comparisons.

In our previous experiment [9], we forced snakes to swim three consecutive laps of the track, resulting in consecutive lap distances of 0 to 3, 3 to 6, and 6 to 9 m. In that study, we determined that snakes achieved their fastest velocity during the first lap 76% of the time and within the first two laps 97% of the time. Therefore, we modified our technique for the present study by racing snakes only two consecutive lengths of the track. Because rapid swimming across short distances probably is more important than long-distance swimming for feeding and predator avoidance in neonate snakes, we believe this modified technique also is more ecologically relevant than our previous long-distance trials.

Evaporative water loss and surface area

The rate of evaporative water loss (EWL) is primarily a function of both respiratory water loss and cutaneous water loss (CWL) in snakes [28–30]. Consequently, for each species, we measured EWL for nine live neonates, and we estimated CWL for three killed neonates. We measured EWL rates following methods similar to those described by Winne et al. [11]. Individuals retrieved from three to six litters per species (equivalent sex allocation) were tested from 9 to 19 d after birth. All snakes were allowed to acclimate individually in plastic containers fitted with a wet paper towel and shallow (depth, ~1.5 cm) water at 25°C for 48 h before the initiation of EWL trials. No snakes were undergoing ecdysis (i.e., shedding) during the trials. All trials were conducted between 9:30 AM and 6:30 PM. Each snake was blotted dry and placed in a flexible fiberglass mesh cylinder (mesh openings, 1 × 1 mm) fit to the contours of each individual's body to restrict movement during the trials (movement is known to increase EWL [13,29]). The combined mass of each cylinder and snake was determined to 0.1 mg. Each cylinder/snake was then immediately placed in an environmental chamber (model 1-37LLVL; Percival Scientific, Perry, IA, USA) at 25°C, 50% relative humidity, and constant darkness. Mass was recorded hourly for 6 h; hourly mass loss was attributed to EWL. Immediately after the trials, snakes were returned to their individual containers to rehydrate. Cutaneous water loss was estimated by measuring EWL rates on freshly killed (lethal dose of tricaine methanesulfonate) neonates, which could lose water only through nonrespiratory pathways (e.g., skin). Samples sizes for CWL trials were intentionally small to limit the number of snakes killed.

Skin surface area is a feature important to EWL and absorption of contaminants. We measured the skin surface area of snakes following the methods described by Tu et al. [31]. Each snake was lightly anesthetized using isoflurane. Once the snake had been anesthetized, we carefully wrapped a thin layer of Parafilm® (American Can, Greenwich, CT, USA) around the snake's head and body contours. The outline of the snake was then cut so that the resulting piece of Parafilm represented the surface area of the snake. Parafilm contours were weighed, and this mass was converted to area using a regression equation derived from Parafilm of known surface areas. For each species, we measured the skin surface area of the same nine live neonates that underwent EWL trials. All surface area measurements were made the day after EWL trials, once the snakes had fully rehydrated.

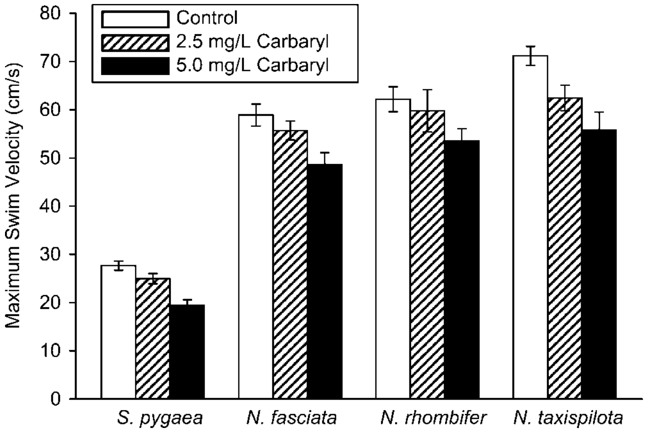

Fig. 1. Comparison of swimming performance of four snake species (*Seminatrix pygaea*, *Nerodia fasciata*, *Nerodia rhombifer*, and *Nerodia taxispilota*) exposed to three concentrations of carbaryl (0, 2.5, and 5.0 mg/L) for 48 h. Snakes are arranged by mean body size in ascending order. Data are presented as the mean ± standard error.

Statistical analyses

Before statistical analysis, data were tested for normality and homoscedasticity using the Ryan-Joiner and Bartlett tests, respectively. Although data were distributed normally, variance was not equivalent among treatments or species, and transformation of data failed to improve this relationship. Therefore, in all comparisons, we used a mixed-model approach (PROC MIXED) to analysis of variance with an unspecified variance matrix structure (SAS, Ver 8.1; SAS Institute, Cary, NC, USA). We included species and pesticide treatment as main effects in the model, swim velocity as the dependent variable, and snake SVL as a covariate in all analyses. Two snakes died during the 48-h dosing period (*N. fasciata*, 2.5 mg/L; *S. pygaea*, 0.0 mg/L), decreasing the sample sizes in those treatments to 11 individuals. To compare our findings directly with those of our previous work [9], we reanalyzed the data from Hopkins et al. [9] for consistency with the present study; only the single maximum velocity achieved over the first two of three laps from that study was used for comparisons.

Evaporative water loss during the 6-h experiment was compared among the three species using a mixed-model approach to analysis of variance (described above), with snake surface area as the covariate. We examined the relationship between body size and surface area and between surface area and EWL using linear regression of log-transformed data.

RESULTS

Acute exposure to carbaryl resulted in reduced swimming performance in all four species of snakes ($F = 20.01$, $p < 0.001$) (Fig. 1). As expected, maximum swim velocity was influenced by snake SVL ($F = 7.67$, $p = 0.006$) (Fig. 2). Velocity also varied among species ($F = 32.34$, $p < 0.001$) independent of snake size; *N. taxispilota* were faster swimmers than *N. rhombifer*, despite having similar body sizes. The interaction between species and treatment was not statistically significant ($F = 1.49$, $p = 0.186$), suggesting that species responded similarly to the pesticide.

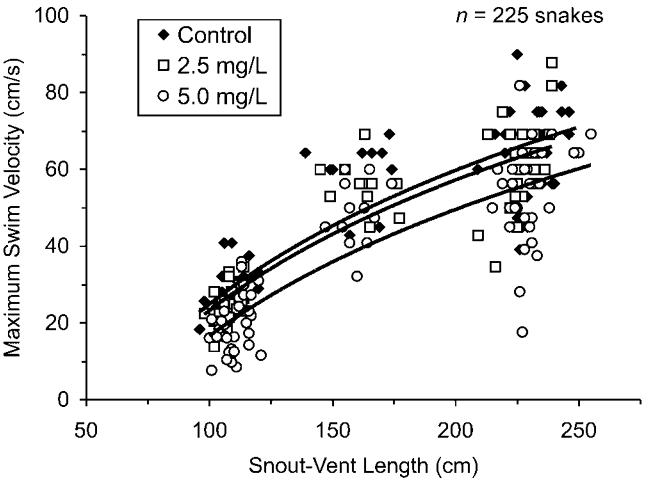

Fig. 2. Relationship between body size and swimming performance of four snake species (*Seminatrix pygaea*, *Nerodia fasciata*, *Nerodia rhombifer*, and *Nerodia taxispilota*) exposed to three concentrations of carbaryl for 48 h. Solid trend lines represent the relationship for snakes exposed to no carbaryl (top line), to 2.5 mg/L of carbaryl (middle line), and 5.0 mg/L of carbaryl (bottom line).

Snake SVL and snake surface area were strongly correlated among the snakes examined in the present study ($r^2 = 0.98$, $p < 0.001$) (Fig. 3A). On average, CWL was less than EWL for two of the three species, but all CWL values fell within the range of EWL values observed for each species (Fig. 3B). Small sample sizes for CWL prohibited statistical comparisons. Evaporative water loss was influenced by SVL ($r^2 = 0.17$, $p = 0.035$) as well as by snake surface area ($r^2 = 0.19$, $p = 0.024$) (Fig. 3B). However, EWL was similar among all three species regardless of whether SVL (species: $p = 0.474$) or snake surface area (species: $p = 0.609$) were included as the covariate in the statistical model. Descriptive statistics for body size, surface area, and water loss are summarized in Table 1.

DISCUSSION

The present study, in combination with data derived from our recent work [9], demonstrated that four species of water snakes responded similarly to exposure to carbaryl and that snake body size explained a significant amount of the variance among snakes in swimming performance. In general, swimming velocity increased with body size. The notable exception to this trend was *N. taxispilota*, which was a faster swimmer than the similarly sized *N. rhombifer* (Fig. 1). Such differences between these two species, independent of body size, are consistent with their ecology and natural history. Whereas *N. rhombifer* primarily inhabits lentic habitats in the geographic region we sampled, *N. taxispilota* is primarily a riverine species, and stronger performance would facilitate swimming against currents. In response to carbaryl exposure, *N. rhombifer* and *N. fasciata* were the least responsive of the four species (e.g., neither exhibited a pronounced reduction in swim velocity when exposed to 2.5 mg/L of carbaryl) but were still within the range described by the allometric scaling relationship with body size. Thus, it is possible that subtle differences in sensitivity among species could emerge under a different experimental approach. For example, if progressively lower concentrations of carbaryl were tested, then the lowest-observable-adverse-effect level might differ among species. However, the fact that our smallest and largest species responded similarly to carbaryl suggests that any subtle differences in sensitivity would be independent of body size.

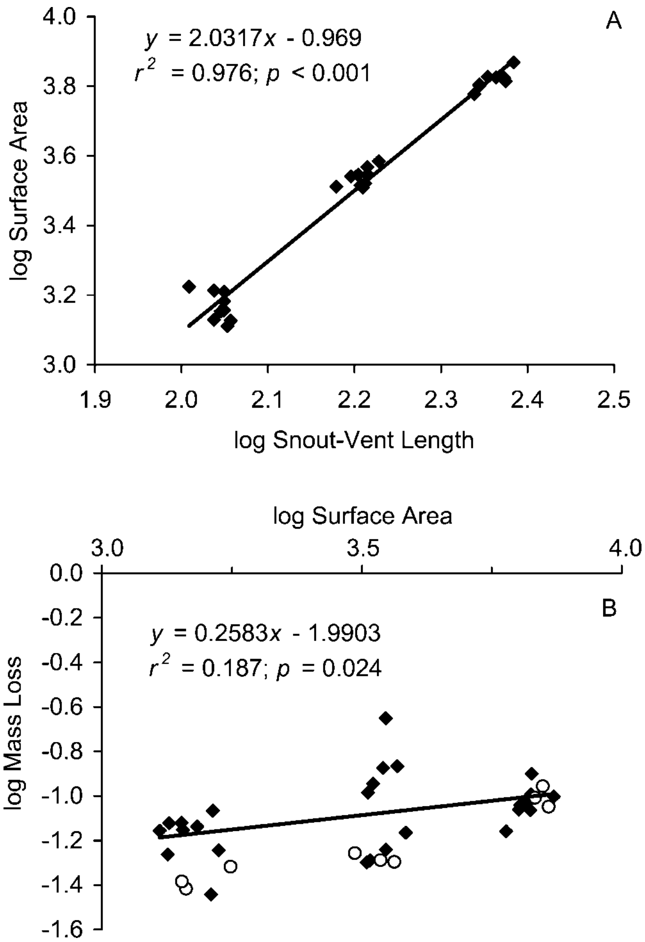

Fig. 3. Relationship between (**A**) snake snout–vent length and surface area and (**B**) snake surface area and mass loss from evaporative water loss. Dark symbols represent individual snakes of three species (*Seminatrix pygaea*, *Nerodia fasciata*, and *Nerodia taxispilota*) in each figure. Open circles (**B**) represent cutaneous water loss of three individuals of each species that were killed; these nine individuals were not used to generate the regression depicted in the figure.

We hypothesized that *S. pygaea* would be more sensitive to carbaryl compared to other species because of its small body size (and, therefore, higher surface area to volume ratio) and previously reported permeable integument, but our results did not support this hypothesis. Instead, the reductions in performance observed in *S. pygaea* following exposure to carbaryl were consistent with those predicted from allometric scaling relationships with body size (Fig. 2). Both EWL and CWL, the latter of which allows inference about water flux (and, possibly, contaminant flux) across the integument, also scaled allometrically with body size and surface area (Fig. 3), contrary to the results of a previous study that demonstrated higher rates of water loss in adult *S. pygaea* compared to similarly sized *N. fasciata*. The discrepancy in EWL between our work and that of Winne et al. [11] may be the result of ontogenetic shifts in the skin permeability of *S. pygaea*. Although adult *S. pygaea* are capable of moving across land to other bodies of water in some situations [32,33], they rarely make terrestrial movements [34]. In fact, recent research suggests that many adult *S. pygaea* aestivate within dried wetlands to survive prolonged droughts rather than migrating over land

(C.T. Winne, J.D. Wilson, and J.W. Gibbons, Savannah River Ecology Laboratory, Aiken, SC, USA, unpublished data). In contrast, *S. pygaea* neonates are routinely captured on land shortly after birth [32,34]. High rates of CWL or EWL likely would be maladaptive for neonates under such terrestrial conditions. Consequently, such ontogenetic changes in terrestrial behavior may be accompanied by shifts in skin permeability [31], explaining the differences in EWL observed between our studies. Ontogenetic shifts in skin permeability within a species could have interesting implications for age-specific sensitivity to contaminants and are worthy of future study.

Our findings generally support those of our previous work, in which two species of snakes exhibited reductions in swimming performance [9], but they also draw attention to the problems associated with broad conclusions from two species comparisons. In ecotoxicology, two species comparisons commonly are used in an effort to detect differences in sensitivity between species. However, inferring differences in inherent sensitivity from two species or two populations can be perilous, because a priori knowledge dictates that the two species are different genetically, physiologically, and ecologically. Thus, the more appropriate null hypothesis is that any two species will differ in sensitivity [35]. Thus, inferences about relative sensitivity to contaminants are much more robust when comparisons of three or more species are used. In our previous study [9], we found that *S. pygaea* was more sensitive than *N. rhombifer*. However, when reanalyzed with greater statistical power and within the context of four species across a broad range of body sizes, *S. pygaea* and *N. rhombifer* fell within the range expected from allometric scaling. Thus, the key finding of the present study was just how remarkably similar the four species were in responsiveness after accounting for body size.

Our findings also raise questions about the usefulness and sensitivity of locomotor performance as an organism-level metric of exposure to cholinesterase inhibitors. In all four species, some individuals were visibly impaired by carbaryl exposure yet had maximum swim velocities that were within the range of controls. Symptoms of carbaryl intoxication included inability to maintain continual upright posture while swimming, suggesting that fine motor skills may be disrupted. Thus, we suggest that in aquatic snakes, processes relying on fine motor skills, such as climbing or prey capture, may be more sensitive organism-level indices of cholinesterase inhibition than maximum swim velocity.

Taken together, our work (present study and [9]) demonstrates that natricine snakes exhibit reductions in maximum swimming performance following acute exposure to high environmental concentrations of carbaryl and that much of the variance among species is accounted for by body size. Our indirect assessment of water flux across the integument, which likely influences the dose of carbaryl received by snakes, suggests that water flux also is similar among species. This observation supports the similar responsiveness of the species examined, but the relationship between water flux and dose received could be strengthened with biochemical measures of cholinesterase inhibition. Although our visual observations suggest that maximum swim velocity may not be as sensitive as other organism-level responses that rely on fine motor skills, we have examined only a single-pulse exposure to high environmental concentrations of carbaryl. Because the effects of multiple, low-dose exposures can be cumulative and probably are more common in nature, future studies evaluating such exposure scenarios may provide additional insight regarding the ecological importance of our findings.

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