



Effects of mercury on behavior and performance of northern two-lined salamanders (*Eurycea bislineata*)

John N. Burke, Christine M. Bergeron, Brian D. Todd¹, William A. Hopkins*

Wildlife Ecotoxicology and Physiological Ecology Program, Department of Fisheries and Wildlife Sciences, Virginia Polytechnic Institute and State University, 100 Cheatham Hall, Blacksburg, VA 24061, USA

Mercury contamination may alter behavior and performance in the northern two-lined salamander (*Eurycea bislineata*).

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ABSTRACT

Mercury (Hg) causes a range of deleterious effects in wildlife, but little is known about its effects on amphibians. Our objective was to determine whether Hg affects performance and behavior in two-lined salamanders (*Eurycea bislineata*). We collected salamanders from Hg-contaminated and reference sites and assessed speed, responsiveness, and prey capture ability. Mercury concentrations were $>17\times$ higher in salamanders from the contaminated sites and were among the highest documented in amphibians. In the first, but not in the second, locomotion trial, we found a significant effect of Hg on speed and responsiveness. In the prey capture experiment, reference salamanders ate approximately twice as many prey items as the contaminated salamanders. Together, our results suggest that sublethal Hg concentrations may negatively affect salamanders by reducing their ability to successfully execute tasks critical to survival. Future work is warranted to determine whether Hg has other sublethal effects on salamanders and whether other amphibians are similarly affected.

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1. Introduction

Mercury (Hg) is a widespread contaminant of considerable ecological concern due to its toxicity to fish and wildlife (e.g., Scheuhammer et al., 2007; Wolfe et al., 1998) and its tendency to biomagnify in ecosystems (e.g., Hall et al., 1998). Although Hg occurs naturally, environmental occurrence and concentrations have increased due to redistribution associated with industrial processes (Fitzgerald et al., 1998). Mercury occurs in both inorganic and organic forms, but it is the highly toxic methylmercury (MeHg) that efficiently bioaccumulates in organisms (Hill et al., 1996; Watras and Bloom, 1992) and biomagnifies in food webs (Hall et al., 1998). Methylmercury is primarily synthesized by anaerobic sulfate-reducing bacteria in marine and freshwater sediments (Gilmour et al., 1992; Benoit et al., 1998), which make fauna associated with these environments particularly susceptible to contamination. The adverse effects of Hg exposure are often associated with its neurotoxicity; however, Hg has also been linked to immune suppression, endocrine disruption, physical malformations, and mortality in organisms (Eisler, 2006; Scheuhammer et al.,

2007; Tan et al., 2009; Wada et al., 2009; Weiner and Spry, 1996; Wolfe et al., 1998).

Behavior is the action exhibited by an organism in response to stimuli, and it is the direct manner by which an organism interacts with its environment. Behavioral changes result from interactions of environmental, chemical, and neurological variables between the organism and its surroundings and can be advantageous, deleterious, or have no effect on the individual's well-being. Exposure to certain environmental contaminants, especially neurotoxicants such as Hg, can promote the expression or suppression of behaviors and affect performance (Henry and Atchison, 1991), the proficiency with which an organism executes a behavior. The neuron degeneration caused by Hg (Sakamoto et al., 1998) suggests that the metal could potentially disrupt the brain's ability to effectively control motor functions. The resulting behavioral effects may inhibit an organism's ability to capture prey, avoid predators, or successfully compete with others (Little et al., 1990; Walker et al., 2005). For example, a study of mosquitofish (*Gambusia affinis*) aqueously exposed to mercurial chloride demonstrated altered swimming activity and decreased swimming speed (Jakka et al., 2007). Similarly, Alvarez et al. (2006) noted concentration-dependent effects of maternally-transferred MeHg in larval Atlantic croaker (*Micropogonias undulatus*), including decreased response speed and increased response time to a vibratory stimulus. Thus, functional impairments, such as altered prey capture ability and

* Corresponding author.

E-mail address: hopkinsw@vt.edu (W.A. Hopkins).

¹ Present address: University of California, Davis, Department of Wildlife, Fish, and Conservation Biology, One Shields Ave, Davis, CA 95616, USA.

reduced performance in individuals, could theoretically reduce fitness, lower recruitment, and even cause population declines.

Many studies regarding the effects of Hg have focused on fish, birds, and mammals (Eisler, 2006; Scheuhammer et al., 2007; Wolfe et al., 1998), but amphibians have received little attention apart from measuring and reporting Hg-tissue concentrations (e.g., Bank et al., 2007; Bergeron et al., 2010a; Eisler, 2006; Linder and Grillitsch, 2000). However, the life history traits and ecological strategies of many amphibian species, such as a complex lifecycle with an aquatic larval stage, omnivorous and carnivorous diets, and use of a wide range of habitats, make them particularly appropriate for studying the effects of Hg contamination on wildlife (Hopkins, 2007). Amphibians can also have important roles in many ecosystems. In certain areas, amphibians represent a critical energy source to predators due to their high biomass, especially in eastern forests of the United States (Burton and Likens, 1975). Due to their abundance and life history, amphibians with complex lifecycles often link trophic levels and facilitate the transfer of nutrients and energy between aquatic and terrestrial habitats (Beard et al., 2002; Regester et al., 2006; Wyman, 1998). Additionally, amphibians are capable of using more of their energy intake for tissue production than endotherms at similar trophic levels (Burton and Likens, 1975; Grayson et al., 2005), increasing their potential to accumulate high Hg concentrations in their tissue (Unrine et al., 2007). Finally, in the last 30 years, biologists have observed marked declines in amphibian populations, putting many of them at greater risk of extinction than birds or mammals (Stuart et al., 2004). While past research indicates that habitat loss and disease are causing many declines, anthropogenic contaminants, like Hg, may also contribute (Beebee and Griffiths, 2005).

Our objective in this study was to determine the effects of Hg contamination along the South River, VA, USA on the performance and behavior of northern two-lined salamanders (*Eurycea bislineata*). We chose northern two-lined salamanders for this study because they have been shown to have the highest total Hg (THg) concentrations of the three amphibian species studied on the South River, and also have some of the highest THg concentrations (up to 5,785 ng/g, dry wt prior to this study) reported in the literature for amphibians (Bergeron et al., 2010a). To accomplish our objective, we used two experiments to determine whether northern two-lined salamanders from the Hg-contaminated portion of the South River would exhibit reduced locomotor performance and reduced prey capture ability when compared with conspecifics from an upstream, reference portion of the river.

2. Materials and methods

2.1. Site description

The South River, VA, USA is a tributary of the Shenandoah River and has a history of anthropogenic Hg contamination from an acetate fiber manufacturing plant in Waynesboro, VA that used Hg in its manufacturing processes from 1929 to 1950 (Carter, 1977). Mercury concentrations in the river currently remain elevated (Southworth et al., 2004) and continue to contaminate wildlife species (Bergeron et al., 2007; Bergeron et al., 2010a; Brasso and Cristol, 2008; Cristol et al., 2008; Wada et al., 2009). Salamanders used in the experiments were collected from reference sections of the river upstream (river mile [RM] -1.7 and -5) and from impacted sections downstream (RM 16 and 20) from the historic source of Hg contamination (RM 0; see Fig. 1 in Bergeron et al., 2010a).

2.2. Animal collection and husbandry

We collected adult northern two-lined salamanders by turning cover objects within 1 m of the streamside from both the reference ($n = 19$) and contaminated ($n = 15$) portions of the South River, VA from 23–25 May 2009. We then transferred the salamanders from the field sites to a temperature-controlled environmental chamber in Blacksburg, VA. We housed all salamanders separately in ~400 mL plastic containers with a substrate of paper towels moistened with dechlorinated tap water. The containers were kept under controlled environmental conditions

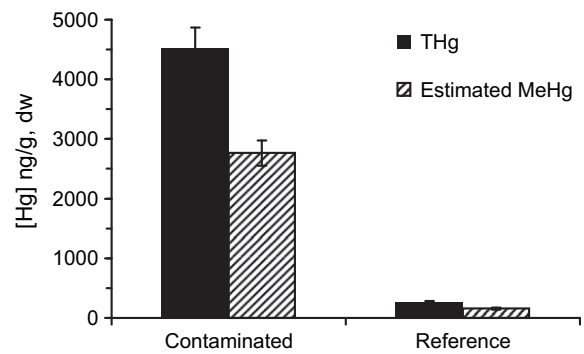


Fig. 1. Total mercury (THg) and estimated methylmercury (MeHg) tissue concentrations (ng/g, dry weight) for *Eurycea bislineata* from reference ($n = 18$) and contaminated ($n = 15$) sites of the South River (VA, USA).

with an ambient air temperature of 18 °C and 12L:12D photoperiod. Salamanders were not fed prior to experiments. We assigned each northern two-lined salamander a random number and all observers involved in data analysis and experimentation were blind to animal origin. We subjected the salamanders to experiments quantifying locomotor performance and prey capture ability beginning on 27 May and ending on 15 June 2009. Upon completion of the experiments described below, all salamanders were humanely euthanized on 15 June 2009 in a buffered solution of 1% tricaine methanesulfonate (MS-222) and analyzed for THg. We measured and recorded mass, total length, and snout-vent length of all salamanders at the time of euthanasia.

2.3. Locomotor performance

We conducted a terrestrial locomotion test to determine whether northern two-lined salamanders from contaminated sites exhibited reduced responsiveness to a stimulus and crawling speed compared to those from upstream reference sites. We conducted a preliminary acclimation trial and two recorded locomotion trials over three days from 27–29 May 2009 using a 3-m long racetrack constructed of 3.6 cm wide polyurethane with 1 cm demarcations down its length. Each salamander was prompted to flee down the raceway once per day over three consecutive days. The first race was used as a training and acclimation period for salamanders to become conditioned to the performance environment (DuRant et al., 2007). Each experimental trial was recorded with a digital video camera (Sony HD Handycam SR85 Cybershot) at 9 Mb/sec (30 frames/sec). We viewed and analyzed all trials to quantify an individual's distance and rate of travel using Adobe® Premiere Pro CS3. To begin the experiment, one test subject was lowered into the racetrack corridor and allowed to take shelter under a moistened paper towel saturated with 5 mL of dechlorinated tap water. After 5 min of acclimation, we lifted the paper towel and gently prodded the individual by tapping the base of its tail. The individual was only tapped when it ceased forward motion and was completely stationary. Each subject was gently prodded at 1-s intervals when stopped. The stimulus was repeated continuously until the salamander reached 1 m or until the animal rested and would not resume progress after 30 prods (Alvarez et al., 2006; Hopkins et al., 2000). One person recorded each trial while another person worked with the test subject. When reviewing videos, we determined the time it took each salamander to travel 1 m and the number of prods it took each salamander to reach 1 m (i.e., responsiveness) (Hopkins et al., 2000). Time to 1 m was calculated as net movement and included only portions of the trial during which the salamander was in motion down the track. In other words, stoppage time during which individuals were prodded to resume forward movement was excluded from the final times.

2.4. Prey capture analysis

We conducted a prey capture experiment to determine whether salamanders from contaminated sites had altered predatory behavior. Prey capture trials were recorded and analyzed using the same equipment and software described in the locomotor performance experiment. At the start of each trial, we placed a single individual into a 14-cm plastic Petri dish lined with a sheet of coarse filter paper saturated with 5 mL of dechlorinated tap water to prevent evaporative water loss. We allowed each individual to acclimate for 5 min after introduction to the dish. Following the acclimation period, we introduced 8 flightless fruit flies (*Drosophila melanogaster*) into the Petri dish from behind a blind. We recorded the entire trial using a video camera mounted overhead. Each Petri dish was visually isolated by a ring of paper and the researcher remained behind a blind during the entirety of each trial (Placyk and Graves, 2002). For this analysis, similar to the locomotor experiment, each of the 34 salamanders underwent an initial conditioning trial on 3 June 2009 to acclimate the salamanders to the testing environment and to allow

them to develop a search image for the test prey. A week later, on 10 June 2009, salamanders underwent the actual recorded experimental trial. We reviewed each videotape to determine the total number of strikes each salamander made and the total number of flies they consumed during the 5 min period (Arnold, 1976; Placyk and Graves, 2002; Watson et al., 2003).

2.5. Mercury tissue analysis

Salamander carcasses were individually lyophilized and homogenized. Whole-body percent moisture, calculated from weights before and after lyophilization, was $73.6 \pm 4.2\%$. Subsamples (~ 20 mg) of each homogenized carcass were analyzed for THg content by combustion–amalgamation–cold vapor atomic absorption spectrophotometry (Direct Mercury Analyzer 80, Milestone, Monroe, CT USA) according to U.S. Environmental Protection Agency method 7473. For quality assurance, each group of 10 to 15 samples included a replicate, blank, and standard reference material (SRM; DOLT-4 dogfish liver or DORM-3 fish protein [National Research Council of Canada (NRCC), Ottawa, ON]). We calibrated the instrument using solid SRMs (DOLT-4 and DORM-3). Method detection limits (MDLs; 3 times the standard deviation of procedural blanks) for samples were 0.33 ng, and all samples had THg concentrations that exceeded the limit. Average relative percent differences between replicate sample analyses were $4.47 \pm 1.06\%$ ($n = 14$). Mean percent recoveries of THg for the SRMs, DOLT-4 and DORM-3, were $96.68 \pm 0.30\%$ ($n = 20$) and $99.22 \pm 1.57\%$ ($n = 20$), respectively.

2.6. Statistical analyses

Prior to analyses, we verified whether data met the assumptions for parametric statistical models. Where assumptions could not be met via data transformations, we used non-parametric tests as described below. We used $\alpha = 0.05$ to define significance but we report unaltered P -values. We used an analysis of variance (ANOVA) to determine whether total length of the salamanders differed between the two sites (reference versus contaminated). We used a non-parametric Mann–Whitney test to determine whether THg concentrations differed between salamanders collected from reference and contaminated sites.

For the locomotor performance trials, our response variables were the number of prods to 1 m (responsiveness) and the total moving time for an animal to reach 1 m, calculated in both Trial 1 and Trial 2. Any animals that did not reach 1 m (Trial 1: $n = 2$; Trial 2: $n = 5$) were excluded from locomotor analyses. The resulting data were highly non-normal and had heterogeneous variances so we were restricted to using more conservative non-parametric analyses in all cases. We determined whether total length in salamanders was correlated with any of our response variables using Spearman correlations. In all cases, there was no correlation between an animal's total length and its locomotor performance (P -values > 0.2). Consequently, we omitted total length as a covariate in the final analyses. We used non-parametric Mann–Whitney tests to determine whether locomotor performance in each of the two experimental trials differed between animals from reference or contaminated sites.

As with the locomotor performance trials, data from the feeding trials were non-normal. Thus, we used non-parametric Spearman correlations to determine whether total length of salamanders was correlated with the total number of strikes or with the total number of flies eaten by each individual. Because we found no correlations between total length of salamanders and their feeding performance (P -values > 0.6), body size was omitted as a covariate from subsequent analyses. We used non-parametric Mann–Whitney tests to determine whether the total number of strikes or total number of flies eaten differed between animals from contaminated or reference sites. Lastly, we use a non-parametric Spearman correlation to determine whether the number of flies eaten by a salamander was correlated with the total number of strikes it made.

3. Results

Average THg tissue concentrations (Fig. 1) for northern two-lined salamanders collected from contaminated sites were ~ 17.5 times higher than those from reference sites ($4,519 \pm 348$ and 256 ± 26 ng/g, respectively) ($Z = -4.88$, $P < 0.001$). Using the percent MeHg (61.2%) found by Bergeron et al. (2010b) for northern two-lined salamanders in a previous study at the South River, the average THg concentrations are equivalent to 2,766 and 157 ng/g MeHg (Fig. 1) at the contaminated and reference sites, respectively.

We found no significant difference in total length of salamanders collected from the contaminated (74.44 ± 2.64 mm) and reference (73.84 ± 1.84 mm) sites ($F_{1,31} = 0.04$, $P = 0.85$). We found a significant difference in responsiveness of salamanders collected from the contaminated versus those collected from reference sites in the first locomotor performance trial ($Z = -2.09$, $P = 0.037$). On

average, it took contaminated salamanders ~ 5 times as many prods to reach 1 m than for reference salamanders to traverse the same distance (Fig. 2). In addition, salamanders from contaminated sites took ~ 3 times longer to reach 1 m than those from uncontaminated sites in the first locomotor performance trial (Fig. 3; $Z = -1.9$, $P = 0.057$). However, we did not find significant differences in either responsiveness (Fig. 2; $Z = -0.399$, $P = 0.69$) or time to reach 1 m (Fig. 3; $Z = -0.38$, $P = 0.71$) in the second locomotor performance trial. For both response variables during the second trial, salamanders from the contaminated sites tended to improve their performance whereas reference salamanders declined in performance.

There was a tendency for contaminated salamanders to both consume and strike at fewer flies compared to the reference salamanders. Reference salamanders ate twice as many flies as contaminated salamanders (3.74 ± 0.79 versus 1.67 ± 0.69 , respectively) (Fig. 4A; $Z = -1.754$, $P = 0.079$). Additionally, reference salamanders made, on average, twice as many strikes as did salamanders from contaminated sites (2.0 ± 0.9 versus 5.1 ± 1.1 , respectively) (Fig. 4B; $Z = -1.825$, $P = 0.068$). There was a significant correlation between the number of strikes made by a salamander and the number of flies consumed (Spearman's $\rho = 0.98$, $P < 0.001$), suggesting that animal motivation, rather than strike efficiency, was responsible for the observed feeding trend. In fact, strike efficiency, the number of prey caught per strike, was similar between the groups and averaged 0.73 for reference salamander salamanders and 0.83 for contaminated salamanders.

4. Discussion

Anthropogenic contaminants are suspected contributors to global amphibian declines but the ways in which they may contribute to these declines is poorly understood (Beebe and Griffiths, 2005). The purpose of our study was to examine the potential impacts of Hg contamination on amphibians in light of these declines. While other studies have identified the tendency for Hg to accumulate in amphibian tissues (e.g., Bank et al., 2007; Bergeron et al., 2010a; Eisler, 2006; Linder and Grillitsch, 2000; Unrine et al., 2007), and have demonstrated detrimental health effects in other wildlife (Eisler, 2006; Scheuhammer et al., 2007; Wolfe et al., 1998), there has been little focus on the possible effects of sublethal contaminant exposure on post-metamorphic amphibians (Linder and Grillitsch, 2000). Despite this lack of attention, a previous study found that Hg concentrations (as MeHg) in northern two-lined salamanders from the South River (Bergeron et al., 2010a) exceeded threshold concentrations for sublethal adverse effects (i.e., growth, reproduction, development, and behavior) in juvenile and adult fish (Beckvar et al., 2005). Similarly, our results suggest that salamanders that exceed the estimated

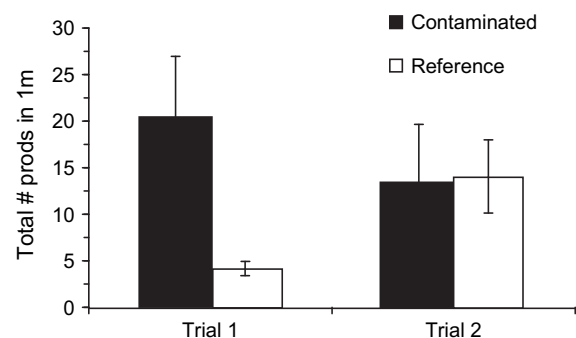


Fig. 2. Responsiveness (number of prods to 1 m) for *Eurycea bislineata* from reference ($n = 19$) and contaminated ($n = 15$) sites of the South River (VA, USA).

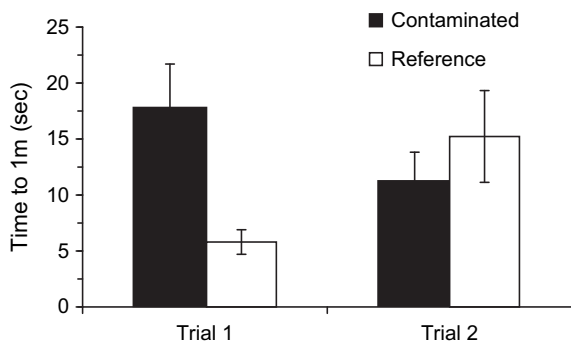


Fig. 3. Total time to 1 m for *Eurycea bislineata* from reference ($n = 19$) and contaminated ($n = 15$) sites of the South River (VA, USA).

threshold effect levels for fish may also exhibit sublethal adverse effects in terms of altered locomotor performance and prey capture behavior.

There was an order of magnitude difference between THg concentrations in northern two-lined salamanders inhabiting the contaminated versus the reference sites on the South River. Total Hg concentrations in northern two-lined salamanders from the reference sites were similar to those reported in Bergeron et al. (2010a) for reference areas along the South River and in studies at Shenandoah and Acadia National Parks (Bank et al., 2005). Conversely, THg concentrations for northern two-lined salamanders downstream from the source of Hg contamination on the South River were extremely high. In fact, two salamanders from contaminated sites had whole-body THg concentrations of 7,507 and 7,377 ng/g, which is among the highest Hg concentrations out of at least 12 previous studies on amphibians (Bank et al., 2005; Bank et al., 2007; Bergeron et al., 2010a; Eisler, 2006; Gerstenberger and Pearson, 2002; Rimmer et al., 2010; Unrine et al., 2005; Unrine et al., 2007; Weir et al., 2010), with the exception of bullfrogs (*Rana catesbeiana*) from a Hg-contaminated watershed in northern CA, USA (Hothem et al., 2010). Mercury uptake in wildlife is affected by numerous variables including

dietary composition and feeding niche (Eisler, 2006). This relationship between THg and feeding niche was made apparent at the South River where both American toads (*Bufo americanus*) and red-backed salamanders (*Plethodon cinereus*), amphibians associated with terrestrial habitats, were sampled for THg levels by Bergeron et al. (2010a) and yielded values lower than those of the more riparian northern two-lined salamanders. The elevated Hg concentrations in northern two-lined salamanders were presumably due to their dependence on prey closely associated with the contaminated river (Bergeron et al., 2010a). Taken together, it is clear that northern two-lined salamanders are likely more susceptible to Hg exposure and accumulation than more terrestrially associated species.

Behavior and performance are important for an animal's survival because they facilitate activities such as foraging, predator avoidance, and reproduction. Reduced ability to successfully execute these tasks may negatively affect an individual's survival. For example, reduced escape speed in guppies (*Poecilia reticulata*) and bullfrogs (*Rana catesbeiana*) has been linked to susceptibility to predators (Raimondo et al., 1998; Walker et al., 2005). Because Hg, as a neurotoxicant, has been shown to affect behavior and performance in many species (e.g., Alvarez et al., 2006; Jakka et al., 2007; Samson et al., 2001), we predicted Hg would reduce speed and responsiveness in northern two-lined salamanders. However, our locomotor performance study yielded mixed results. The results of the first performance trial indicated that speed and responsiveness were significantly inhibited in the salamanders from the contaminated sites, suggesting Hg may affect locomotor performance. However, the second trial did not support this trend, making it difficult to determine whether the result from the first trial actually represents an effect of Hg or may be an artifact of some other variable, such as sample size. Alternatively, because reference salamanders were faster in the first recorded trail, it is possible their decline in performance in the second recorded trial was due to fatigue. Conversely, it may have taken the contaminated salamanders an additional day to acclimate to the performance trial, allowing them to improve in the second recorded trial. Because we observed a seemingly strong effect in the first trial and changes in performance due to Hg contamination is a common response in studies involving other ectotherms, this response in amphibians warrants further investigation. We acknowledge that our animals were drawn from only two contaminated sites and two reference sites along a single river. If limited gene flow occurs among collection sites, it may lead to variations in the quality of individuals from the contaminated and reference sites as a result of the effects of Hg or other unquantified environmental factors. Future studies could sample more thoroughly across the South River contamination gradient (Bergeron et al., 2010a) to minimize potential variation attributed to genetic or environmental factors other than Hg.

As predicted, Hg contamination in northern two-lined salamanders was associated with changes in feeding activity and behavior. In our study, reference salamanders ate twice the number of flies relative to contaminated salamanders. Reference salamanders also made nearly twice as many attempts to capture prey, suggesting that the effects of Hg exposure led to a reduced motivation to feed. These results are consistent with similar work involving other vertebrates. For example, Rodgers and Beamish (1982) observed reduced appetite and motivation to feed in rainbow trout (*Salmo gairdneri*) exposed to MeHg, and Zhou et al. (2001) observed reduced prey capture ability in larval mummichogs (*Fundulus heteroclitus*) after aqueous MeHg exposure. Other anthropogenic contaminants have also been shown to alter feeding behavior. For example, Little et al. (1990) found that static exposure to several agricultural chemicals (carbaryl, chlordane, dimethylamine salt of 2,4-dichlorophenoxyacetic acid (2,4-DMA), tributyl phosphorotrithioate (DEF1), methyl parathion, and

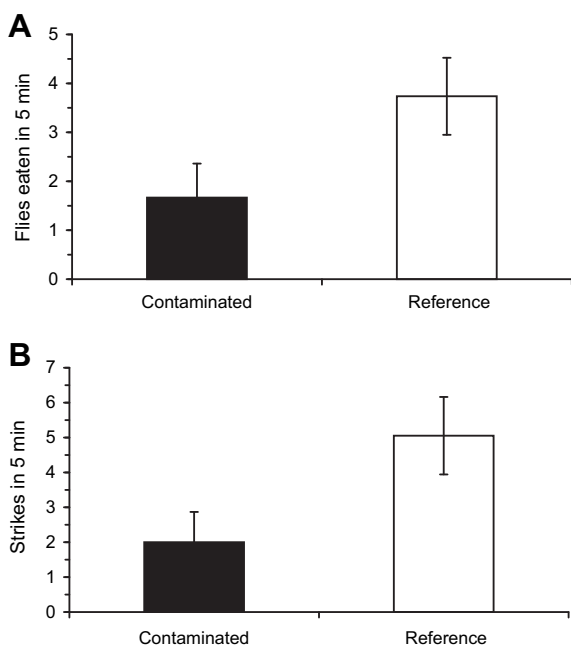


Fig. 4. Feeding Trial: (A) The average number of *Drosophila melanogaster* eaten in 5 min and (B) total strikes by *Eurycea bislineata* from reference ($n = 19$) and contaminated ($n = 15$) sites of the South River (VA, USA).

pentachlorophenol) significantly impaired the ability of rainbow trout to capture prey. Additionally, glyphosate herbicides and cadmium have been linked to reduced prey capture in fish (Morgan and Kiceniuk, 1992; Scherer et al., 1997). Notably, Scherer et al. (1997) found that over a 96 hr period, reference lake trout (*Salvelinus namaycush*) consumed eight times more fingerling rainbow trout than their counterparts exposed to aqueous cadmium. While our prey capture findings were only marginally significant, the magnitude of difference between study groups suggests Hg may have biologically significant effects on feeding efficiency in this species. In order to more closely emulate natural conditions, future research involving prey capture experiments could present prey in a more physically complex environment that would challenge the salamanders to locate and pursue prey in a semi-natural feeding environment.

Behavior and physiological performance are mechanisms through which chemical changes in organisms can produce population level consequences (Weis et al., 2001). However, the subtle sublethal effects of contaminants on behavior and performance can occur at concentrations much lower than would cause morphological abnormalities, thus making it difficult to draw conclusions on the large ecological scale of impacts of contaminants (Scheuhammer et al., 2007; Weis et al., 2001). The present study is important because it is one of the few to consider the effects of Hg exposure in amphibians, one of the first to address Hg-induced changes in behavior and performance in post-metamorphic amphibians, and reports some of the highest amphibian THg concentrations to date. Based on our results, salamanders exposed to sublethal Hg concentrations may suffer from altered prey capture behavior and terrestrial performance. While the goal of this study was to evaluate ecologically relevant tasks, future research should determine how variations in these behaviors influence the survival of northern two-lined salamanders. Despite the inconclusiveness of our locomotor trials, our research adds to the extremely limited data focusing on amphibian behavior and physiology in relation to environmental contaminants, and serves as a useful starting point for future research on this topic.

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