

FINAL REPORT

Acute and Chronic Effects of Coal Tar and Asphalt Sealants on Salamanders

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EXECUTIVE SUMMARY

1 The Barton Springs salamander (*Eurycea sosorum*) is a federally endangered species inhabiting
2 pools of the Edwards aquifer in the City of Austin, Texas. The presence of polycyclic aromatic
3 hydrocarbons (PAH) in these pools elicited concern for the conservation of the species because
4 PAHs have acute and chronic effects on aquatic organisms, including amphibians. These PAHs
5 appear to be coming from sealants applied to pavements within the watershed of these ponds.
6 To determine if Barton Springs salamanders could be a risk to these PAHs three laboratory
7 experiments were funded by the Barton Springs Conservation Foundation and conducted by at
8 Southern Illinois University. These experiments used adult eastern newts (*Notophthalmus*
9 *viridescens*), larval spotted salamanders (*Ambystoma maculatum*) and adults of the closely
10 related San Marcos salamander (*Eurycea nana*) as surrogates. The respective primary
11 objectives of the experiments were to assess: 1) a dose/response relationship between
12 concentrations of PAHs and acute or chronic effects using asphalt and coal tar sealants; 2) if
13 exposure to realistic levels of ultraviolet radiation affect the toxicity of PAHs from coal tar
14 sealant; and 3) the comparative toxicity of *E. nana* to the other surrogates species.

15 In the first experiment mortality was very light and there was no evidence that it was
16 associated with sealant or PAH concentration. After a 28 day exposure, body measurements of
17 eastern newts including mass, snout vent length or total length did not differ among
18 concentrations of PAH in water or sediment. Righting ability, as determined by the amount of
19 time newts needed to reorient after being turned on their backs, was significantly affected by
20 PAH concentration. Animals that were exposed to coal tar sealants and total PAH
21 concentrations of PAH in water $> 177 \mu\text{g/L}$ took longer to turn themselves than those exposed to
22 lower concentrations. Righting ability may be related to survival through changes in the ability
23 to capture prey or escape predation. Liver enzymes including lactate dehydrogenase, aspartate
24 aminotransferase and alanine aminotransferase, decreased in livers with exposure to higher
25 concentrations of aqueous PAH. Because the liver is a major site for the production of these

26 enzymes, reduced levels suggest hepatic changes consistent with observed liver damage in other
27 studies. Newts exposed to the highest coal tar sealant concentrations (1500 mg/kg sediment) had
28 measurable concentrations of benzo[a]pyrene and two other PAH analytes.

29 As with experiment 1, there was no evidence of PAH-related mortality in spotted
30 salamander larvae. There were effects on growth and body size over the 28 days, however.
31 Growth was reduced under high PAH concentration and non-UV light compared to lower
32 concentrations or UV light exposure. Reduced growth under non-UV light seems
33 counterintuitive and we are unable to explain it at this time. Most types of white blood cells
34 significantly in frequency with exposure to ultraviolet radiation indicating alterations in immune
35 functions. Neutrophils were the only white blood cell to respond to PAH concentrations.

36 *Eurycea nana* began dying independently of PAH concentration within several days after
37 the beginning of exposure to contaminated sediments. We do not believe that the cause of death
38 can be attributed to PAHs but we cannot explain why animals died. Although body
39 measurements and liver enzymes were measured from these animals, the short exposure period
40 and possible confounding influences from whatever was causing their mortality prevented any
41 meaningful interpretations. *E. nana* did bioconcentrate several PAHs and had higher body
42 concentrations than those displayed by eastern newts. Other studies have shown that
43 salamanders can assimilate relatively high concentrations of PAHs early in exposure only to
44 achieve a lower level homeostasis after several days. Thus we should over accentuate the
45 significance of these elevated body burdens.

46 Although *Eurycea*, *Notophthalmus* and *Ambystoma* are not closely related amphibian
47 genera, they have some common features such as similar diets and permeable skin. Larval
48 *Ambystoma*, like *Eurycea sosorum* and *E. nana*, have external gills that can serve as portals for
49 organic contaminants and *Notophthalmus* and the eurycids are totally aquatic as adults. While
50 it makes sense to use *E. nana* as a surrogate for *E. sosorum*, the limited data on comparative
51 amphibian toxicity does not show any consistent trends among taxonomic groups to support or

52 refute the use of *Ambystoma* or *Notophthalmus* as surrogates. One factor of potential
53 consequence is the difference in exposure between the laboratory studies and the natural
54 environment of the Barton Springs salamander. *E. sosorum* inhabits an environment that is
55 essentially flow-through. At least in the open water column and surface waters, PAHs entering
56 pools inhabited by *E. sosorum* could be expected to have short durations. Most likely, PAH
57 contamination of these pools would be in discrete spurts following rain events. The continual
58 recharge of the pools through underground springs and above ground currents would tend to
59 flush contaminants through the water column and reduce exposure. Circulation may be reduced
60 at the sediment/water interface or in pore water, however, so it would be informative to study
61 PAH dynamics *in situ*.

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127

128 **1. INTRODUCTION**

129 In 1995, biologists with the city of Austin Texas detected elevated concentrations of polycyclic
130 aromatic hydrocarbons (PAHs) in sediments entering and settling upstream of and in the Barton
131 Springs pools, an area inhabited by the federally endangered Barton Springs salamander
132 (*Eurycea sosorum*). The Barton Springs salamander is an entirely aquatic species that is confined
133 to Barton Springs of the Edwards Aquifer in downtown Austin, Texas, USA (Chippindale et al.
134 1993; Hauwert et al. 2002). The city of Austin is a rapidly growing community and urban
135 development within the Barton Springs watershed may threaten the continued existence of *E.*
136 *sosorum* (Chippindale et al. 1993; USFWS 1997). Most notably, coal-tar and asphalt pavement
137 sealants from nearby roads and parking lots may run off into waters occupied by this species.

138 Coal-tar, a byproduct of the production of coke from coal, is 50% or more PAHs by
139 weight (U.S. Department of Health and Human Services 2002), and coal-tar-emulsion sealers are
140 20 to 35% coal-tar by weight (Mahler et al. 2005). Asphalt is a byproduct of refining crude
141 petroleum and has PAH concentrations 5 to 600 times less than coal-tar (Takada et al. 1990;
142 Mahler et al. 2003, City of Austin 2004). Coal-tar sealants are used to extend the life of asphalt
143 parking lots. Runoff from these lots is a major source of urban PAHs (ATSDR 2002), resulting
144 in localized hot spots (Mahler et al. 2005) that may be toxic to aquatic organisms.

145 Unaltered parent compounds of PAHs can directly affect aquatic organisms, but
146 oxidation and ultraviolet radiation (UV) can create degradation products that are many times
147 more toxic than parent compounds (Albers 2003). A wide variety of harmful effects on
148 amphibians and other aquatic organisms are caused by PAHs such as cancerous growths and
149 cellular abnormalities (Eisler 2000), genotoxicity and micronucleated erythrocytes (Jaylet 1971;
150 Fernandez and l'Haridan 1992; Gauthier et al. 1993), inhibition of growth and metamorphosis
151 (Fernandez and l'Haridan 1994), edema and impaired gas exchange (McGrath and Alexander
152 1979), activation of mixed function oxidases or other cellular metabolic defense mechanisms

153 (Schwen and Mannering 1982; Noshiro and Omura 1984), liver damage (Myers et al. 2003;
154 Shallaja and D'Silva 2003) and death (Hedtke and Puglisi 1982; Lefcort et al. 1997).
155 Amphibians are vulnerable to bioaccumulation of PAHs during metamorphosis, and may
156 bioconcentrate these chemicals (Grinfield et al. 1986; Vojinovic-Mildoradov et al. 1996).
157 However a low-level equilibrium can be reached after several days (Garrigues et al. 2004).
158 Exposure of PAHs to ultraviolet (280-400 nm) sunlight induced micronucleated erythrocytes in
159 bullfrogs (*Rana catesbeiana*) and Spanish ribbed newt (*Pleurodeles waltl*) at concentrations
160 1/100 of those when ultraviolet light was not present (Fernandez and l'Haridan 1992).

161 Evidence that *E. solorum* is exposed to sealants and their PAHs comes from three
162 sources. First, some concentrations of PAHs in Barton Creek and Barton Springs Pool sediment
163 exceeded the U.S. Environmental Protection Agency's (EPA) Probable Effects Concentrations
164 for aquatic species (MacDonald et al. 2000, Hayward et al. 2002). Second, Hayward et al. (2002)
165 found that mortality was significant in *Hyaella azteca*, a prey species for *E. solorum*, when
166 exposed to high concentrations of PAHs and ultraviolet radiation. Third, an on-site investigation
167 identified deteriorated coal-tar sealant from adjacent parking lots as the probable source of PAHs
168 in Barton Springs (Geomatrix Consultations Inc. 2003; Little, pers. comm.). Preliminary studies
169 found that wetland sediments near sealed parking lots had higher concentrations of PAHs than
170 those near unsealed lots (Van Metre et al. 2000).

171 The overall objective of this study was to determine if coal-tar and asphalt-based sealants
172 can pose toxic risks to Barton Springs salamanders. Physiological damage, including sublethal
173 and lethal effects to individuals may affect the remaining population of *E. solorum*. Because of
174 their endangered status, experiments using *E. solorum* could not be conducted; instead the
175 eastern newt (*Notophthalmus viridescens*), spotted salamander (*Ambystoma maculatum*) and San
176 Marcos salamander (*Eurycea nana*) were used as surrogate species.

177 **2. EXPERIMENT 1: EFFECTS OF ASPHALT AND COAL TAR**
178 **SEALANTS ON EASTERN NEWTS**

179 **2.1. Objectives**

180 The objectives of this experiment were to determine:

181 1) If sediments contaminated with sealants negatively affect eastern newts through lethal
182 or sublethal means.

183 2) If there is a difference between coal-tar and asphalt sealants in their risk of producing
184 lethal and sublethal effects in eastern newts and to assess that risk in regards to *Eurycea*
185 *sosorum*.

186 **2.2. Materials and Methods**

187 **2.2.1. Sediment and Water Preparation**

188 We collected and homogenized 128 kg of sediment from Little Crab Orchard Creek, Carbondale,
189 IL (UTM Zone 16, 0301377E, 4175002N), a source of uncontaminated sediment used in
190 previous toxicology studies conducted at Southern Illinois University. Coal-tar and asphalt
191 sealants were provided by chemists at Austin, Texas. The sealants were prepared by applying
192 sealant to sheets of glass, allowing them to dry and the most volatile PAHs to dissipate, and
193 scraping the dried substance into containers. Coal-tar and asphalt sealants were mixed with 800
194 g of sediment to create final concentrations of 15, 31, 62, 125, 250, 500 and 1500 mg sealant/kg
195 sediment. Because asphalt sealant has a lower total PAH concentration than coal-tar, the
196 sediments were expected to have different PAH concentrations for given concentrations of
197 sealant.

198 The mixtures were placed in 8 L aquaria and covered with 7 L of water for 10 days to
199 allow suspended sealant to sink. Water from each tank was completely drained and the sealant
200 and sediment were mixed again. The mixture was again covered with 7 L of water and allowed
201 to stabilize for two weeks. Sediments were disturbed as little as possible during water exchange.

202 Tap water was filtered through gel ion-exchange columns and activated charcoal and
203 reconstituted (ASTM 1988) to approximate the hardness and conductivity of natural waters
204 around Austin, Texas. Water was aerated for at least 24 hours prior to adding it to aquaria and
205 gently aerated throughout the experiment.

206 **2.2.2. Lighting Conditions**

207 Eastern newts were exposed to coal-tar or asphalt based sealants under a combination of UV
208 (290-400 nm) and visible lighting (400-700 nm). To provide shelter from direct light, each
209 aquarium contained a 10 cm long piece of 5.1 cm wide PVC pipe that was cut lengthwise. A
210 combination of cool white (Ecologic, Osram Sylvania, Danvers, MA, USA) and UV (UVA-340,
211 Q-Panel Company, Cleveland, OH, USA) fluorescent lamps provided visible and UV conditions
212 to emit the approximate lighting found in south central Texas during summer months
213 (Chamberlain, pers. comm.). The lamps were calibrated using a hand held radiometer (Macam
214 UV 203; Macam Photometrics, Livingston, Scotland) sensor at 12 equally distributed locations
215 for each light setup. Light intensity for both treatments was set to follow a natural summer
216 photoperiod of 16L:8D; where each of the 4 sets of lights were programmed to turn on and off at
217 predetermined times to control the amount of UV and visible light (Table 1).

218 **2.2.3. Newt Experimental Conditions**

219 Newts were acquired commercially (Connecticut Valley Biological Supply Company,
220 Southampton, Massachusetts, USA) and were given 10 days to acclimate to laboratory
221 conditions. Air temperature within the room was controlled to maintain the water between 19-
222 23°C. Seventy percent of the water was replaced twice a week without disturbing sediments to
223 maintain quality and clarity. Before the water in each tank was replaced, its pH, ammonia
224 content, and hardness were recorded using a pH/mV/Ion meter (YSI 550DO, YSI
225 Environmental, Yellow Springs, Ohio, USA).

226 Each aquarium contained three adult eastern newts and was randomly assigned to one of
 227 the 16 sealant treatments and five replicates per treatment. Each newt was fed approximately 0.5
 228 mL of blood worms (*Chironomus tentans*) every other day. Newt health and status was recorded
 229 daily for the 28 day exposure period through observing their activity, food consumption, and
 230 condition of their skin.

231
 232

233 Table 1. Light sets that provided the required amount of light that eastern newts were exposed to, the
 234 length of time each light set was on, and the mean (SE) levels of UVB, UVA, and visible light.

	Time	Light Sets	UVB ($\mu\text{W}/\text{cm}^2$)	UVA ($\mu\text{W}/\text{cm}^2$)	Visible ($\mu\text{W}/\text{cm}^2$)
Level A	0700-2300 (16 hours)	Set A	0.02 (0.04)	0.03 (0.01)	5.40 (± 1.50)
Level B	0800-2200 (14hours)	Sets A & B	0.50 (0.47)	4.73 (4.73)	25.2 (1.4)
Level C	12:15-17:45 (5.5 hours)	Sets A, B, & C	1.17 (0.29)	8.7 (1.5)	50.3 (5.4)
Level D	14:45-15:15 (30 minutes)	Sets A, B, C, & D	1.75 (0.24)	15.3 (2.55)	70.6 (4.0)

235

236 Prior to and at the end of the experiment, newts' physical measurements were recorded.
 237 Weights were recorded using a Fisher XT scale (Fisher Scientific, Hampton, New Hampshire,
 238 USA) to the nearest 0.001 g and snout-vent-length (SVL) and total-length (TL) were measured
 239 using Fisher Digital Calipers (Fisher Scientific, Hampton, New Hampshire, USA) to the nearest
 240 0.1 mm. At the end of the study the newts were humanely euthanized with MS-222 and stored at
 241 -75°C until analyzed.

242 **2.2.4. Chemical Analyses**

243 Polycyclic aromatic hydrocarbon (PAH) analyses were conducted on sediment, water, and newt
244 bodies at the Southern Illinois University, Carbondale, IL. Sample extraction, cleanup, and
245 quantification followed EPA methods 3510C (USEPA 1994), 3541 (USEPA 1994), 3630C
246 (USEPA 1996), and 8100 (USEPA 1986), respectively, using an Agilent 5975C Series Gas
247 Chromatograph/Mass Spectrometer, Inert XL EI/CI MSD with Agilent 6850 network GC system
248 and 6850 series autosampler (Agilent Technologies, Wilmington, Delaware, USA). Control
249 sediment samples were spiked with 2-fluorobiphenyl (2-FBP) and PAH Mix (Ultra Scientific,
250 Kingstown, RI, USA), and were extracted and analyzed to examine method extraction efficiency,
251 with a recovery rate > 80%. Concentrations of PAHs in sediments were measured at the
252 beginning and end of the experiment. Liquid-liquid extraction and quantification of PAHs in
253 water followed EPA method 3510C. One liter samples were collected from each treatment and
254 spiked with 1.0 mL of 2-fluorobiphenyl.

255 **2.2.5. Righting Ability**

256 Midway through the experiment, righting ability was conducted by placing each newt in a
257 shallow dish lined with plastic netting and with just enough reconstituted water to cover the
258 animal. Each animal was inverted onto its back three separate times and the time needed to right
259 itself was measured with a stopwatch (Fischer Scientific Traceable Stop Watch, Fisher Scientific,
260 Hampton, New Hampshire, USA). Delays between trials were 10 seconds, and the means of the
261 three trials were tested statistically to determine if differences existed among sealants or
262 treatments.

263 **2.2.6. Liver Enzymes**

264 Activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate
265 dehydrogenase (LDH) were tested on one animal per aquarium according to the methods of
266 Searcy (1969). Livers were dissected from newts and homogenized in a 50:1 dilution (v/m) of
267 Tris pH 7.4 buffer. Samples were centrifuged at 6,500 rpm for 15 minutes at 2°C, and the
268 supernatant was stored at -75°C until analyzed. LDH reagent (200 µL; Thermo Electron,

269 Louisville, Colorado, USA) was mixed with 25 μL of liver supernatant. AST and ALT were
270 analyzed using 30 μL of liver supernatant with 200 μL of ALT or AST reagent. Plates were
271 scanned at a wavelength of 340 nm in a spectrofluorimeter (Biotek Synergy, Biotek Instruments,
272 Winooski, Vermont, USA) at 30°C. Results were deemed acceptable when 4 readings were
273 within 10 percentage points of each other. Results are expressed as U/L (= mg/L).

274 **2.2.7. Data Analysis**

275 Statistical analyses were performed using SAS 9.1 (SAS Institute Inc., Cary, North Carolina,
276 USA) with $\alpha=0.05$. All dependent variables were examined for meeting the assumptions of
277 parametric statistics; normality was checked with the Shapiro-Wilks statistic and
278 homoscedasticity by examining the relationship between means and standard deviations. End
279 point data were initially analyzed using regressions based on measured water concentrations of
280 PAHs to determine if dose-response relationships could be identified. Analyses of variance
281 (ANOVA) were used to test for specific differences in end points among treatments and sealants.
282 When interaction terms were statistically significant but one or both main effects were not, we
283 ran additional ANOVAs on each sealant type. Since newts were tested individually in the
284 behavioral experiments, a conventional two-way ANOVA without repeated measures was used
285 to test if there were differences in behavior among PAH concentrations, sealant types or their
286 interaction.

287 **2.3. Results**

288 **2.3.1. Sediment and Water Chemistry**

289 Water conditions were within acceptable levels (ASTM 1988) throughout the study. Mean values
290 were: water temperature=20.0 °C, pH=7.4, hardness=53.7 $\mu\text{S}/\text{cm}$, dissolved oxygen =5.8 mg/L
291 and ammonia =10 ng/L. No differences were found between sealants for these measurements.
292 The sediment was composed of 60% sand, 35% silt, 5% clay and had 2% organic matter.

293 Initial analysis of the dried sealants revealed that they contained 17 different PAHs with
 294 phenanthrene, fluoranthene and pyrene having the highest concentrations. Coal-tar was 84.3%
 295 total PAH (TPAH) and asphalt was 6.7% TPAH (Table 2). There was no statistical difference
 296 and no net loss of sediment TPAH between treatments at the beginning and end of the study
 297 (Appendix 1), so the mean of the two samples for each treatment was used in subsequent
 298 analyses (Table 3).

299
 300

301 Table 2. Analysis of coal-tar and asphalt sealant (from City of Austin)

PAHs	Solubility mg/L ^a	Log k_{ow} ^a	Coal-Tar mg/kg	Asphalt mg/kg
Acenaphthene	4.08	3.92	382	15.7
Acenaphthylene	N/A	3.89	16.7	1.01
Anthracene	0.058	4.61	4800	381
Benzo(a)anthracene	0.011	5.91	4790	430
Benzo(a)pyrene	0.001	6.13	4360	319
Benzo(b)fluoranthene	0.001	6.45	4520	365
Benzo(e)pyrene	0.006	6.05	2840	224
Benzo(g,h,i)perylene	0.0001	6.22	1980	165
Benzo(k)fluoranthene	0.0001	6.11	3610	320
Chrysene	0.002	5.81	5020	468
Dibenzo(a,h)anthracene	0.002	6.86	1030	82.6
Fluoranthene	0.220	5.20	16700	1300
Fluorene	1.84	4.18	1470	59.8
Indeno(1,2,3-cd) perylene	N/A	7.09	1960	157
Naphthalene	30.7	3.35	35.7	8.67
Phenanthrene	1.11	4.55	17300	1400
Pyrene	0.129	5.14	13500	1040

Total

84314.4

6736.78

302 ^aLog k_{ow} values taken from Crunkilton and DeVita (1997) and van Noort (2009), solubility
 303 data taken from van Noort (2009).

304 The percentage of TPAH differed between coal-tar and asphalt. For asphalt TPAH
 305 averaged (SE) 2.8% (0.8) and for coal-tar TPAH averaged 70.7% (8.8) of sealant concentrations
 306 across treatments. TPAH in water averaged 0.2% (0.1) of sealant concentrations for asphalt and
 307 0.4% (0.1) for coal-tar (Table 4); this converted to 17.2% (7.7) and 0.7% (0.3) of sediment
 308 TPAH for asphalt and coal-tar, respectively. The concentration of TPAHs in coal-tar and
 309 asphalt-spiked sediments corresponded very well to the nominal values of sealant ($r^2 = 0.993$, $p <$
 310 0.001 ; $r^2 = 0.803$, $p = 0.006$, respectively). Aqueous concentrations of TPAHs were positively
 311 related to nominal sediment concentrations in both coal-tar ($r^2 = 0.973$; $p < 0.001$) and asphalt (r^2
 312 $= 0.919$; $p = 0.002$).

313

314

315

316 Table 3. Measured concentrations of total PAHs in sediment and water of asphalt and coal-tar treatments.

Sealant Treatment (mg/kg)	Asphalt Sealant		Coal-tar Sealant	
	Sediment (mg/kg)	Water ($\mu\text{g/L}$)	Sediment (mg/kg)	Water ($\mu\text{g/L}$)
Control	0.07	30	1.51	30
15	0.44	112	7.66	182
31	1.27	71	34.98	160
62	1.12	102	54.36	219
125	8.63	105	58.99	177
250	7.94	105	149.4	302

500	14.39	114	297.7	388
1500	20.58	281	1149	1464

317

318 **2.3.2. PAH Concentration in Newts**

319 We analyzed whole body residues of three eastern newts exposed to 1500 mg/kg coal-tar sealant
 320 (=1464 μg TPAH /L). Only four analytes were detected. All three of the animals had
 321 measurable concentrations of benzo(a)pyrene ranging from 1.060 $\mu\text{g/g}$ to 4.334 $\mu\text{g/g}$ wet body
 322 weight. One animal had two other analytes (pyrene and chrysene; 1.176 and 0.591 $\mu\text{g/g}$,
 323 respectively) and another had 2.642 $\mu\text{g/g}$ benzo(b)fluoranthene in its tissues. Measurable
 324 TPAHs ranged from 1.060 to 6.976 $\mu\text{g/g}$ wet weight.

325 **2.3.3. Survival and Growth**

326 There was no statistical difference in mortality between newts exposed to coal-tar (4/120) or to
 327 asphalt (2/120) sealant following 28 days of exposure. At the end of the study there was no
 328 significant relationship between water and sediment PAH concentrations and either body mass,
 329 total length or snout vent length (Appendix 2). Nor were there any significant differences in
 330 these variables between type of sealant, treatment, or their interaction for these measurements.

331 Table 4. Detectable levels of PAHs ($\mu\text{g/L}$) found within the water column of coal-tar and asphalt
 332 treatments

Coal-tar	Control	15 mg/kg	31 mg/kg	62 mg/kg	125 mg/kg	250 mg/kg	500 mg/kg	1500 mg/kg
Napthalene	5	15	11	10	13	17	18	10
Acenathphylene	ND	ND	ND	10	ND	12	10	19
Acenaphthene	5	10	ND	ND	ND	17	16	16
Fluorene	5	10	10	10	10	10	10	10
Phenanthrene	ND	12	10	10	10	11	12	19
Anthracene	ND	10	10	10	10	13	12	35
Fluoranthene	ND	13	10	10	10	15	16	79

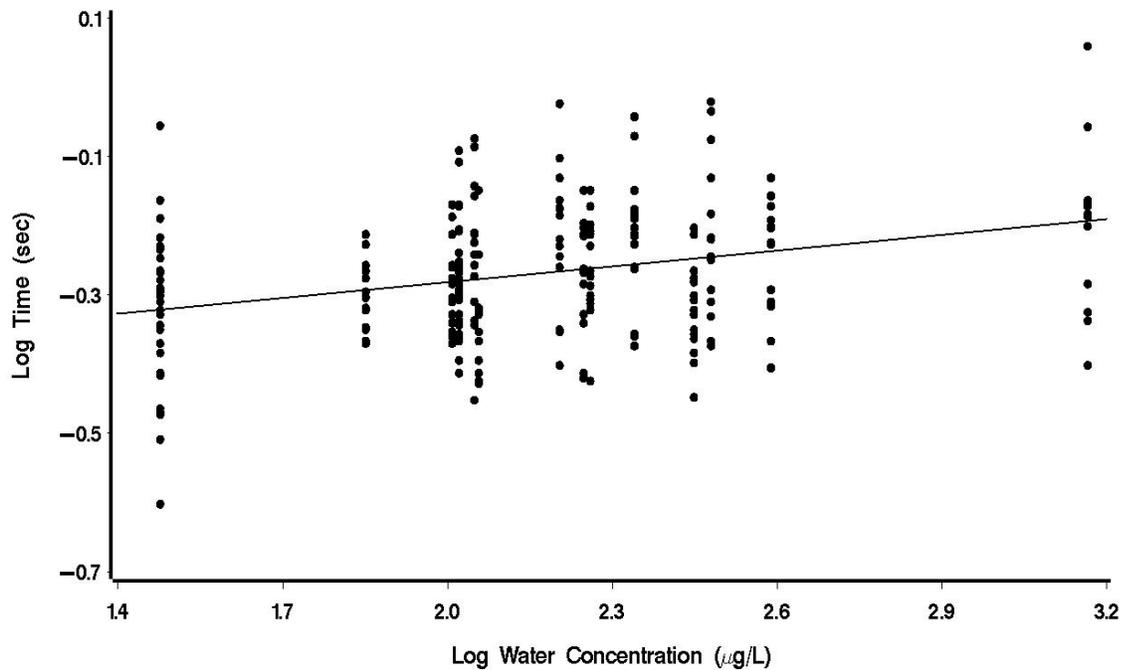
Pyrene	ND	11	10	10	10	11	15	85
Benzen(a)anthracene	ND	20	20	20	20	35	44	220
Chrysene	ND	13	11	13	19	39	55	130
Benzo(b)fluoranthene	5	39	39	38	44	51	72	190
Benzo(k)fluoranthene	5	29	29	28	31	35	48	120
Benzo(a)pyrene	5	ND	ND	ND	ND	36	60	190
Indeno(1,2,3-cd)perylene	ND	ND	ND	ND	ND	ND	ND	120
Dibenzo(a,h)anthracene	ND	ND	ND	ND	ND	ND	ND	120
Benzo(g,h,i)perylene	ND	ND	ND	50	ND	ND	ND	101
Total	30	182	160	219	177	302	388	1464
Asphalt^b	Control	15 mg/kg	31 mg/kg	62 mg/kg	125 mg/kg	250 mg/kg	500 mg/kg	1500 mg/kg
Napthalene	5	17	11	11	13	12	12	33
Acenaphthylene	ND	10	10	10	10	10	10	28
Acenaphthene	5	10	10	11	ND	ND	ND	65
Fluorene	5	10	10	10	10	10	10	20
Phenanthrene	ND	13	10	10	10	10	11	10
Anthracene	ND	10	ND	ND	10	10	10	21
Fluoranthene	ND	12	10	10	10	10	11	24
Pyrene	ND	10	10	10	10	10	10	10
Benzen(a)anthracene	ND	20	ND	20	20	20	20	20
Chrysene	ND	ND	ND	10	12	13	20	23
Benzo(a)pyrene	5	ND	ND	ND	ND	ND	ND	27
Total	20	112	71	102	105	105	114	281

333 ^a ND indicates no instrument response was detected for this parameter.

334 ^b Benzo(b)fluoranthene, benzo(k)fluoranthene, indeno(1,2,3-cd)perylene,
335 dibenzo(a,h)anthracene, and benzo (g,h,i)perylene were not detected in any of the asphalt
336 treatments.

337 2.3.4. Righting Ability

338 There was a significant dose-response relationship between log righting time and log water
339 concentrations ($r^2 = 0.094$, $p < 0.001$, Figure 1). Righting times tended to increase with PAH
340 concentrations. The ANOVA indicated there was a significant difference in the righting time of
341 newts by sealant ($F_{1,64} = 4.31$, $p = 0.042$) and the interaction of sealant type and treatment ($F_{7,64} =$
342 3.45 , $p = 0.003$), but not by treatment. Within coal-tar treatments, control newts had the quickest
343 righting times and were significantly different ($p = 0.05$) from all other treatments. Those
344 exposed to asphalt sealant righted themselves more quickly than those in coal-tar sealant.

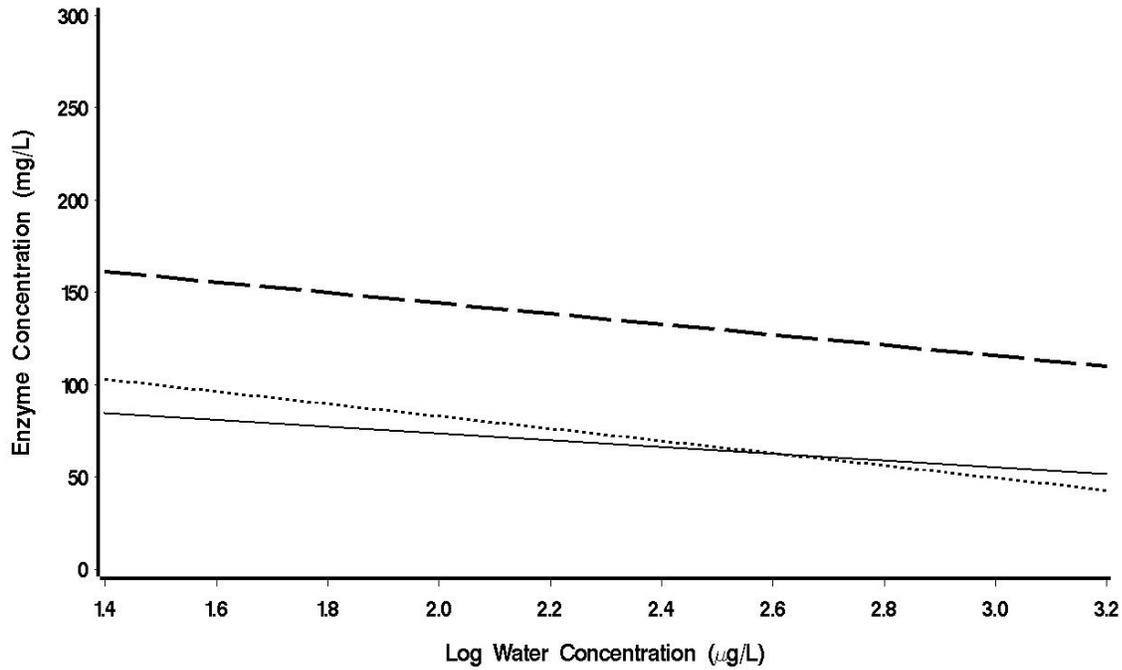


345
346 Figure 1. Righting times for eastern newts exposed to PAH concentrations coming from asphalt
347 and coal-tar sealants.

348 2.3.5. Liver Enzymes

349 Regression analysis on ALT showed a significant declines with increasing log of water ($r^2 =$
350 0.069 , $p = 0.022$, Figure 2) and sediment ($r^2 = 0.125$, $p = 0.001$) TPAH concentrations. The
351 ANOVA indicated that ALT differed in the interaction of sealant type and concentration ($F_{7,79} =$
352 2.74 , $p = 0.015$) but was not significant for the main effects of sealant type or treatment. No

353 differences existed among asphalt treatments when sealant types were analyzed separately , but
354 there was a significant difference among coal-tar treatments ($F_{7,33} = 4.71, p = 0.001$). Enzyme
355 activity was lower at water PAH concentrations greater than $177 \mu\text{g/L}$ than below that level.



356
357 Figure 2. Relationship between enzyme concentrations and log water PAH concentration for
358 eastern newts exposed to asphalt and coal-tar sealants. Solid line = LDH, dashed line=AST,
359 dotted line=ALT.

360
361 Aspartate aminotransferase significantly declined as the log of water ($r^2 = 0.069, p =$
362 0.022) and sediment ($r^2 = 0.067, p = 0.023$) TPAH concentrations increased. In an ANOVA,
363 AST did not vary between sealant types, among sealant concentrations or in the interaction
364 between sealant type and concentration.

365 Lactate dehydrogenase declined as the log TPAH concentrations in water ($r^2 = 0.066, p =$
366 0.023) and sediments ($r^2 = 0.1016, p = 0.004$) increased. In the ANOVA of sealant type and

367 treatment, concentrations of this enzyme differed significantly between sealant types ($F_{1,78} = 8.18$,
368 $p = 0.005$), and the interaction of sealant and treatment ($F_{7,78} = 3.70$, $p = 0.002$) but not across
369 treatments. Within coal-tar treatments, LDH was significantly lower at water concentrations
370 greater than 302 $\mu\text{g TPAH /L}$ than at lower concentrations ($F_{7,38} = 2.85$, $p = 0.020$).

371 **2.4 Discussion**

372 **2.4.1. Sediment, Water and Newt Concentrations of PAHs**

373 Across sediment concentrations coal-tar had a mean of 27 (9) times the TPAH concentration of
374 their asphalt counterparts. However, water concentrations with coal-tar were only 2.7 times those
375 with asphalt. Sediment-bound PAH has little or no bioavailability (Garrigues et al. 2004) and it
376 is the aqueous compartment, whether in the water column itself or in pore water, that provides
377 most of the exposure. Because the volatile, low molecular weight PAHs dissipated during the
378 formation of flakes used in this experiment, most of the remaining analytes were relatively high
379 molecular weight, low solubility hydrocarbons. These analytes also had moderate to high $\log k_{ow}$
380 values which suggest high affinity for binding to organic matter (Van Noort 2009). The analyte
381 with the highest solubility was naphthalene which was found in the water column of all
382 treatments except controls. The next most soluble analytes were acenaphthene, fluorene and
383 phenanthrene with solubilities that were 3 to 13% of naphthalene. These analytes were
384 detectable in the water from the highest treatments of both coal-tar and asphalt. Many of the
385 other analytes had $\log k_{ow}$ values of 5 to 7 which suggest low bioavailability. However,
386 benzo(a)pyrene, with a k_{ow} of 6.13, was the only analyte found in the three newts examined.

387 Toxicity varies roughly with molecular weight of PAHs (Eisler 2000). Those with lower
388 molecular weights often have higher acute toxicity than heavier PAHs but heavy PAHs may be
389 more carcinogenic and produce other chronic effects (Eisler 2000). Most of the PAHs that newts
390 were exposed to could be expected to have lower acute toxicity but higher potential for chronic
391 effects. Therefore, longer term studies could reveal complications not seen during the 28 days of
392 this study.

393 Residue concentrations in newts could have been influenced by self-regulation.
394 Garrigues et al. (2004) found that when the salamander *Pleurodeles waltl* was constantly
395 exposed to sediments contaminated with phenanthrene, pyrene and benzo(a)pyrene, the
396 salamanders bioconcentrated TPAHs to approximately 2.5 times that found in water within 24
397 hours of exposure. However, after a few days, tissue concentrations of phenanthrene dropped by
398 80% and by 15 days tissue concentrations were near pre- exposure levels. Pyrene took longer to
399 deplete but it dropped by 80-85% after 20 days. Benzo(a)pyrene was the slowest to deplete,
400 after dropping to approximately 0.5 of its peak at the end of 10 days, it remained nearly stable for
401 an additional 20 days. The biological stability of benzo(a)pyrene and pyrene is consistent with
402 our finding them in the animals we tested. Garrigues et al. (2004) also reported that water
403 concentrations of the PAHs ranged from 0.08 to 0.58% of that in sediment, similar to what we
404 found.

405 **2.4.2. Survival and Growth**

406 At the end of 28 days of exposure to coal-tar and asphalt sealant, the newts experienced only
407 minimal, non-significant, mortality. At least under the test conditions, this demonstrates that
408 neither coal-tar nor asphalt sealants cause acute mortality. Bryer et al. (2006) exposed *Xenopus*
409 *laevis* embryos to coal-tar sealant ranging from 3 to 300 mg TPAH /kg sediment. They found
410 that the highest concentration of sealant caused 100% mortality within 6 days of exposure and
411 that 30 mg/kg caused retarded development compared to controls and those exposed to 3 mg/kg.
412 *Xenopus laevis* is not a native amphibian species and its use to estimate toxicity concentrations
413 of contaminants has been criticized (Birge et al. 2000). Also, toxicity may differ across life
414 stages (Allran and Karasov 2001, Ortiz-Santaliestra et al. 2006). Our sediment concentrations
415 exceeded Bryer et al. (2006) highest concentration by more than four times and we did not see
416 any PAH-related mortality. Because our study used adult newts, it is not possible to determine if
417 the difference in sensitivity was due to developmental stage or to species.

418 Growth is indeterminate in amphibians but its rate is markedly slower in adults than in
419 larvae or juveniles (Jørgensen 1992); thus it would be unlikely to find significant increases
420 within 28 days. However, it was possible for the newts to lose mass due to decline in appetite or
421 malaise and this could have resulted in loss of tail length due to autolysis (Fraser 1980), but we
422 did not observe this. In experiment 2 larval spotted salamanders did show reduced growth with
423 elevated PAH concentrations and UVB light.

424 **2.4.3. Righting Ability**

425 Righting time appeared to be a sensitive indicator to PAH exposure. Elevated PAH
426 concentrations in the coal-tar exposures led to increased righting times. At the lower
427 concentrations of PAH in asphalt there were no differences among treatments. However,
428 righting times tended to increase with coal-tar sealant concentrations. This suggests that PAHs at
429 the higher concentrations found in coal-tar (ca. 177 $\mu\text{g/L}$ total PAH in water) may cause stress,
430 resulting in newts that are weaker or are exhausted more quickly than those in controls and lower
431 concentrations. Righting responses integrate several factors involving cognition (an animal
432 recognizing that it is upside down), muscular strength and coordination. Poorer reflexes could be
433 related to reduced survival if they hamper food capture or escape from predators.

434 **2.4.4. Liver Enzymes**

435 Differences in hepatic enzyme activity were observed among coal-tar treatments. As the
436 concentration of coal-tar sealant increased, activity for all three enzymes evaluated declined.
437 Plasma is often the matrix of choice for enzyme determinations but was not obtainable from
438 euthanized animals. Therefore, we used liver tissue instead. The liver is an active site for the
439 production of AST, ALT and LDH (Ozmen et al. 2006). Ozmen et al. (2006) examined liver
440 samples in the European carp (*Cyprinus carpio*) for signs of hepatic enzyme activity and they
441 reported a decline in the concentration of LDH, AST, and other enzymes in the liver when
442 exposed to increasing concentrations of metals. Liver damage including neoplasms, lesions,
443 cancer, and cell necrosis are common results of PAH toxicity in fish (Walker et al. 1998, Myers

444 et al. 2003, Shallaja and D’Silva 2003, Vogelbein and Unger 2006). The reduced activities of
445 LDH, ALT and AST reflect changes in the liver and suggest hepatic damage. The livers of
446 newts in the current study were not examined histologically to determine if tissue damage had
447 occurred.

448 **2.5. Conclusions**

449 This study did not demonstrate lethal toxicity to newts, even when sediment concentrations were
450 three times higher than the maximum concentration found in Barton Springs. Certainly,
451 interspecies differences in toxicity can occur but our study suggests that *Eurycea sosorum* might
452 not be acutely at risk from current levels of sealant influx. However, we did find that PAH
453 affected righting ability and liver enzymes of newts. To the degree that *E. sosorum* and *N.*
454 *viridescens* are similar in their sensitivity, these findings suggest that there may be some health
455 and behavioral risks to *E. sosorum* from coal-tar sealant. We have no information on the
456 availability of prey organisms to *E. sosorum* or on potential predators. If there are avian or
457 mammalian predators, they would not likely be exposed to PAH in run off. However, aquatic
458 prey and predators would be subject to the same exposures encountered by *E. sosorum* and their
459 sensitivities would have to be factored into any risk formulation. If potential predators were
460 debilitated at lower PAH concentrations than *E. sosorum*, PAHs could actually be of some
461 benefit to the salamander (Boone and Semlitsch 2001). Alternatively, if *E. sosorum* is more
462 sensitive, PAHs could have a synergistically negative effect on their populations (Relyea 2004).

463 Decreased liver enzyme activity, in itself, may not be harmful. Additional studies
464 involving histopathology and longer term exposures to look for neoplasms or cancer would have
465 to be conducted to determine if hepatic changes are deleterious.

466 This study demonstrated that that at the same concentration of sealant, asphalt is less
467 harmful than coal-tar. The results of this study support the decision of the city of Austin, Texas

468 to use asphalt sealant in place of coal-tar sealant to lessen the risk of harm to the Barton Springs
469 salamander.

470 **3. EXPERIMENT 2: RESPONSE OF SPOTTED SALAMANDERS TO**
471 **COAL TAR AND UV LIGHT.**

472 **3.1. Objectives**

473 Because ultraviolet radiation can significantly affect the toxicity of PAHs, this study was designed to
474 determine if exposure to ultraviolet radiation altered toxicity due to coal tar sealants in the surrogate
475 species, *Ambystoma maculatum*. Specific objectives were to determine:

476 1) If ultraviolet light at a level commiserate with that of the middle of the summer in Austin,
477 Texas interacted with PAH in producing lethal or sublethal effects

478 2) If PAHs from coal tar sealants produced similar effects in a spotted salamanders and eastern
479 newts

480 **3.2 Specific Methods for Experiment 2**

481 **3.2.1. Husbandry, Sediment and Lighting Conditions**

482 During June and July 2007 120 wild caught, larval spotted salamanders were exposed to 0, 60, 280, and
483 1500 mg/kg of coal-tar sealant in sediment under visible light (400-700 nm) for 28 days. A second group
484 of 120 salamanders was exposed simultaneously to the same concentrations of coal-tar sealant but under
485 a combination of visible lighting and ultraviolet radiation (290-400 nm). Six salamanders were placed in
486 each tank with five replicates for each treatment. Other husbandry conditions were the same as in
487 experiment 1. At the end of the exposure period all salamanders were weighed and measured for snout
488 vent length (SVL) and total length (TL) as in experiment 1. They were euthanized with MS-222.

489 The light setup for the UV treatment varied slightly from that in Experiment 1 (Table 5). For the
490 non-UV treatment each fixture contained only 1 cool white bulb. The lights were calibrated to emit the
491 light consistent with mid summer in Austin, Texas.

492 Table 5. Light sets in experiment 2 that provided the required amount of light that larval spotted
493 salamanders were exposed to in the UV phase, the length of time each light set was on, and the mean (SE)
494 levels of UVB, UVA, and visible light.

	Time	Light Sets	UVB ($\mu\text{W}/\text{cm}^2$)	UVA ($\mu\text{W}/\text{cm}^2$)	Visible ($\mu\text{W}/\text{cm}^2$)
Level A	0700-2300 (16 hours)	Set A	0.04 (0.01)	0.04 (0.02)	8.5 (2.7)
Level B	0800-2200 (14hours)	Sets A & B	0.5 (0.03)	4.14 (1.0)	29.6 (1.3)
Level C	12:15-17:45 (5.5 hours)	Sets A, B, & C	1.3 (0.3)	7.9 (0.5)	52.5 (4.0)
Level D	14:45-15:15 (30 minutes)	Sets A, B, C, & D	1.8 (0.2)	14.4 (2.9)	71.6 (3.8)

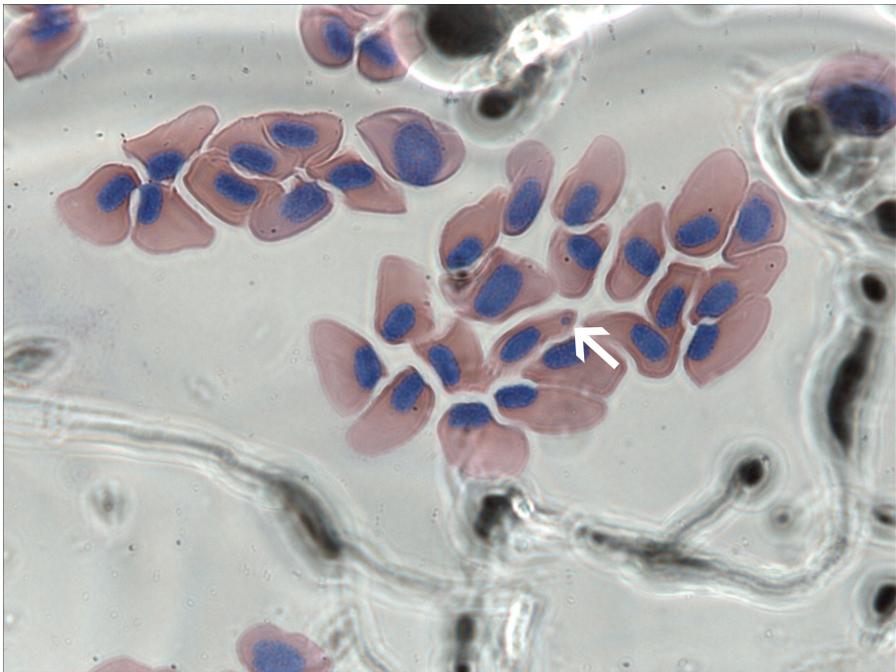
495 **3.2.3. Behavioral Response**

496 Larval spotted salamanders were tested for speed and swimming distance. They were too small to be
497 safely inverted as in Experiment 1. Instead, each individual was placed in a 122 cm section of 7.6 cm
498 diameter PVC pipe that was cut lengthwise and capped on the ends. A tape measure was affixed to
499 measure the distance swam and time was measured with a stopwatch. Each individual was gently
500 prodded with a stainless steel probe to evoke swimming behavior. If no swimming occurred the duration
501 and distance were recorded as zero. The distance (± 0.5 cm) and duration (± 1 sec) of the initial
502 movement trial for each trial were measured and rate was calculated as cm swam/sec. Each bout
503 consisted of 10 trials with a 10 sec delay between trials; means for the bouts were tested statistically to
504 determine differences in swimming duration, distance and rate of travel.

505 **3.2.4 Micronucleus & White Blood Cell Test**

506 At euthanasia, salamanders were decapitated and blood was collected in a heparinized capillary tube.
507 Blood smears were applied to slides that were sent to the histology department at Southern Illinois
508 University where they were fixed and stained with Wright-Giemsa. The frequency of micronucleated
509 erythrocytes (MNC) was determined by randomly observing 1000 erythrocytes on each slide using
510 1000X magnification under oil immersion. A micronucleus was identified as a small dark staining body
511 that was adjacent but not connected to the principle nucleus (Meintieres et al. 2001; Figure 3).

512 Lymphocytes, erythroblasts, monocytes, basophils, eosinophils, and neutrophils were also counted at
513 this time.



514
515 Figure 3. Blood smear from a spotted salamander, arrow indicates a micro-nucleated red blood cell

516 3.3. Results

517 3.3.1 Sediment and Water Chemistry

518 Sealant and sediment were highly related across treatments and light phases $r^2=0.980$, $p<0.0001$ (Table
519 6). Neither the non-UV nor UV phases showed significant differences in sediment concentrations
520 between the start and end of the study (Appendix 3), so we took the mean of those two measurements to
521 represent sediment concentrations of TPAH. Overall, mean sediment TPAH concentrations were 92%
522 (18) of the sealant concentrations. In the UV phase sediment TPAH concentrations averaged 115% (21)
523 of nominal sealant concentrations and in the non-UV phase they were 70% (25). There was no
524 systematic differences in PAH concentration among sealant concentrations for the two light treatments.
525 (paired-t test, $t=-0.89$, $p=0.437$). Water TPAH concentrations (Table 7) related strongly to both nominal
526 sealant concentrations ($r^2=0.995$, $p < 0.0001$) and TPAH concentrations in sediment ($r^2=0.979$,
527 $p<0.0001$). Over both light exposures water TPAHs were 0.05 (0.02)% of nominal sealant

528 concentrations and 1.6 (1.0)% of TPAH in sediments. Basic water quality parameters were within
 529 acceptable limits as listed by ASTM (1988; Table 8).

530 **3.3.2. Survival and Growth**

531 Percent mortality was less than 1% (1/120) under non-UV and 2.5 % (3/120) under UV following 28
 532 days of exposure. This low mortality negated any need for detailed statistical analysis.

533 Body mass ($r^2=0.091$, $p=0.006$), SVL ($r^2=0.071$, $p=0.017$) and TL ($r^2=0.063$, $p=0.025$) were
 534 significantly related to water TPAH concentrations. In each case, increasing PAH concentrations
 535 resulted in decreased body size. In ANOVAs there were no significant differences in body mass(Fig.
 536 4A) or SVL (Fig. 4B) by treatment, phase or their interaction. There were differences in TL due to light
 537 phase and marginally to treatment but not in their interaction (Fig. 4C). Salamanders exposed to non-UV
 538 light were shorter than those exposed to UV light and those at 1500 mg/kg sealant were shorter than
 539 those at 280 mg/kg sealant.

Table 6. Total PAHs in sediment (mg/kg) and water ($\mu\text{g/L}$) for sediments exposed to laboratory light (non-UV) and ultraviolet radiation (UV) in experiment 2.

Treatment	Non-UV		UV	
	Sediment	Water	Sediment	Water
0 mg/kg	1.15	72.3	1.03	62
60 mg/kg	22.9	72.0	46.5	72.4
280 mg/kg	147.8	92.9	429.8	101
1500 mg/kg	1771	303	1728	273

540

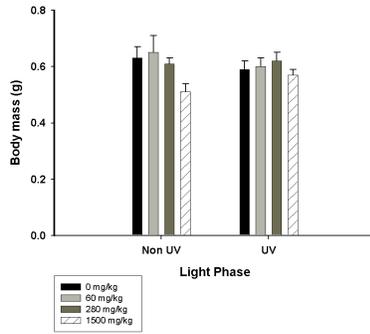
541 **3.3.3 Speed Trials**

542 There was a significant difference in the swimming rate (distance/time) among coal-tar concentrations
 543 treatment and between phases but not in the interaction of the two terms (Table 9). Salamanders in the

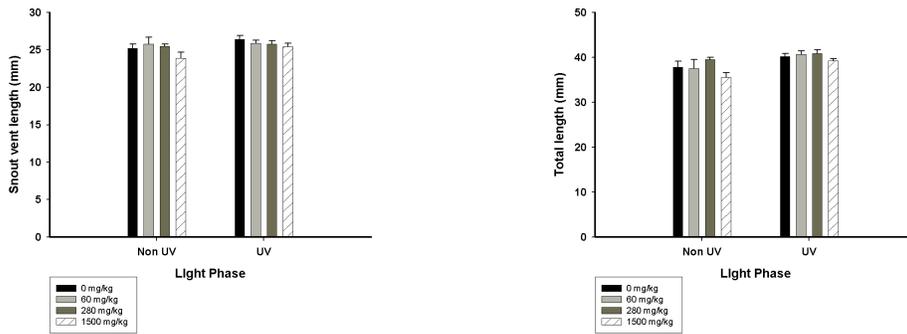
544 UV phase had a slower swimming rate than those in the non-UV phase (Table 10). Animals exposed to
 545 1500 or 60 mg/kg sealants were slower than controls. Specifically, in the non-UV phase the swimming
 546 rate of salamanders exposed to 280 and 1500 mg/kg treatments declined compared to controls with the
 547 slowest occurring in 1500 mg/kg. In the UV phase the swimming rates of salamanders in all of the coal-
 548 tar treatments were significantly less than controls. Both components of rate (duration and distance)
 549 were affected by exposure to coal-tar and UV light. Swimming duration significantly differed across
 550 phases and the interaction of treatment and phase but not by treatment. Salamanders in the non-UV
 551 exposure had longer swimming times than those in the UV phase. The distance swam differed by phase,
 552 treatment and their interaction. Again, those in the non-UV phase swam longer distances than those in
 553 the UV phase and those at 1500 mg/kg sealants swam shorter distances than controls.

Table 7. Water concentration ($\mu\text{g/L}$) of specific PAH analytes during experiment 2.

Analyte	Non-UV				UV			
	0 mg/kg	60 mg/kg	280 mg/kg	1500 mg/kg	0 mg/kg	60 mg/kg	280 mg/kg	1500 mg/kg
Napthalene	20	22	22	23	24	20	21	24
Acenathphylene	ND	ND	3	10	ND	ND	ND	14
Acenaphthene	6	9	9	14	8	9	11	12
Fluorene	7	9	9	14	7	7	9	14
Phenanthrene	7	6	11	93	4	7	12	30
Anthracene	22	23	29	65	21	21	19	42
Fluoranthene	< DL	< DL	< DL	13	< DL	< DL	< DL	11
Pyrene	< DL	< DL	< DL	11	< DL	< DL	< DL	11
Benzene(a)anthracene	< DL	ND	1	ND	< DL	< DL	1	ND
Chrysene	< DL	< DL	8	ND	< DL	< DL	ND	44
Benzo(a)pyrene	ND	ND	ND	23	ND	ND	ND	12
Ideno(1,2,3-cd)pyrene	ND	< DL	< DL	7	< DL	< DL	< DL	< DL
Dibenzo(a,h)anthracene	ND	ND	ND	10	ND	8	8	10
Benzo(g,h,i)perylene	ND	3.4	ND	10.3	5.2	ND	3.9	9



570



571

572 Figure 4. Body mass (top), snout vent length (left) and total length (right) of spotted salamanders exposed to coal
 573 tar sealants and either laboratory lighting or ultraviolet radiation.

574

Table 9. Results of ANOVAs on swimming rate, distance and duration in spotted salamanders exposed to coal tar sealants (treatment) and either UV or non-UV light (phase)

Factor	df	F	p
Swimming Rate			
Phase	1,224	8.08	0.005
Treatment	3,224	3.69	0.013
Interaction	3,224	2.08	0.104
Swimming Duration			
Phase	1,224	79.27	< 0.0001
Treatment	3,224	0.75	0.526
Interaction	3,224	2.98	0.032
Swimming Distance			
Phase	1,224	39.54	< 0.0001
Treatment	3,224	2.74	0.044
Interaction	3,224	2.69	0.047

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Table 10. Mean (SE) of swimming times, distances and rates for spotted salamanders exposed to coal tar sealants under standard laboratory lighting (Non-UV) and ultraviolet radiation (UV) in experiment 2.

Treatment	Time (sec)	Distance (cm)	Rate (cm/sec)
Non-UV			
0 mg/kg	1.1 (0.1)	10.46 (0.77)	10.8 (0.6)
60 mg/kg	1.5 (0.3)	11.62 (0.93)	10.8 (0.8)
280 mg/kg	1.0 (0.1)	10.04 (1.03)	9.8 (0.6)
1500 mg/kg	1.2 (0.1)	10.69 (1.07)	9.4 (0.7)
UV			
0 mg/kg	0.7 (0.04)	8.80 (1.09)	10.9 (0.8)
60 mg/kg	0.6 (0.03)	5.71 (0.76)	7.2 (0.8)
280 mg/kg	0.7 (0.04)	7.30 (0.73)	9.0 (0.8)
1500 mg/kg	0.6 (0.02)	5.03 (0.57)	7.7 (0.7)

582

Table 11. Results of ANOVA on the frequencies of multi-nucleated erythrocytes (MNC) and leucocytes in spotted salamanders exposed to UV and non-UV light (phase) and sediment PAH (treatment).

Factor	df	F	P
MNC			
Phase	1,72	5.97	< 0.0001
Treatment	3,72	1.27	0.196
Interaction	3,72	1.39	0.128
Erythroblasts			
Phase	1,72	7.18	0.009
Treatment	3,72	0.35	0.786
Interaction	3,72	0.83	0.479
Monocytes			
Phase	1,72	23.70	< 0.0001
Treatment	3,72	0.86	0.466
Interaction	3,72	1.38	0.256
Basophils			
Phase	1,72	8.93	0.003
Treatment	3,72	0.20	0.898

Interaction	3,72	0.74	0.533
Eosinophils			
Phase	1,72	5.08	0.027
Treatment	3,72	1.16	0.332
Interaction	3,72	0.72	0.542
Neutrophils			
Phase	1,72	4.60	0.035
Treatment	3,72	3.10	0.031
Interaction	3,72	2.46	0.069
Lymphocytes			
Phase	1,72	0.01	0.916
Treatment	3,72	1.95	0.129
Interaction	3,72	2.44	0.071

583

584 **3.4. Discussion**

585 **3.4.1 Sediment and Water Chemistry**

Although the difference among treatment concentrations were not statistically significant, concentrations of TPAH in sediment differed between light phases and in pre and post exposure measurements. In the pre exposure, 1500 mg/kg sealant non-UV treatment and UV treatments TPAH concentrations (1634 mg TPAH/kg sediment and 2644 mg TPAH/kg sediment, respectively) exceeded the nominal sealant concentration (Appendix 3). Similarly, in the post exposure, non-UV treatment the TPAH concentration in the 1500 mg sealant/kg sediment treatment was 1907 mg TPAH/kg sediment and that in the UV, 60 mg sealant/kg sediment

Table 12. Mean (SE) of micronucleated erythrocytes and white blood cells of salamanders exposed to coal-tar sealant in the UV and non-UV phases of experiment 2. Values are expressed as number of cells per 1000 erythrocytes.

Treatment	Micronucleated	Erythroblast	Monocyte	Eosinophil	Lymphocyte	Neutrophil
Non-UV						

0	0	6.3 (1.4)	0.1 (0.1)	0.8 (0.4)	23.4 (6.1)	1.1 (0.3)
60 mg/kg	0.1 (0.1)	4.8 (1.4)	1.0 (0.4)	2.6 (0.6)	18.4 (5.3)	1.7 (0.8)
280 mg/kg	0	9.8 (2.2)	1.9 (0.7)	3.9 (1.1)	24.9 (2.1)	1.8 (0.6)
1500 mg/kg	0.1 (0.1)	5.5 (2.1)	1.2 (0.5)	4.0 (1.8)	24.2 (7.6)	5.2 (1.6)
UV						
0	0.1 (0.1)	9.2 (2.1)	3.7 (0.7)	4.8 (1.3)	17.4 (6.6)	1.1 (0.6)
60	0.1 (0.1)	14.8 (5.3)	2.8 (0.6)	5.8 (1.9)	23.0 (6.7)	1.4 (0.5)
280	0.4 (0.2)	12.0 (3.5)	3.5 (0.9)	3.6 (0.8)	12.1 (3.3)	1.1 (0.6)
1500	0.3 (0.1)	15.2 (5.3)	2.6 (0.6)	7.6 (3.3)	40.3 (9.7)	1.4 (0.7)

586

587 Treatment was 70.6 mg TPAH/kg sediment. The most likely explanation for these elevated
588 TPAH concentrations relates to the difficulty of maintaining a homogeneous mixture of sealant
589 flakes and sediment. Even if the initial mixture was homogeneous, because the flakes were
590 mobile in the sediments they could be redistributed by salamander activity; a random selection of
591 sediment from each tank for a given treatment could include errant flakes. The possibility of
592 non-homogeneous sampling is supported in that concentrations before and after the experiment
593 across sealant treatments showed some variation whereas water concentrations were more
594 consistent between sealant concentrations. Given these caveats, spotted salamanders were likely
595 to have been exposed to concentrations more consistent with nominal values than indicated by
596 the available data.

597 **3.4.2 Growth and Survival**

598 As with Experiment 1, spotted salamanders did not experience any treatment-related mortality and the
599 experimental conditions cannot be considered to be acutely or subchronically lethal. However, there
600 were important sublethal effects produced by PAH and lighting conditions. Overall, salamanders
601 experienced a 68% increase in body mass and a 23% increase in total length over the 28 d period. The
602 rate of growth was related to PAH concentration in that, regardless of light phase, there was a negative
603 relationship between PAH concentrations and body size at the end of the experiment. Because the

604 spotted salamanders were larvae, growth during the 28 days of the experiment could be expected. The
605 negative relationship between body size and PAH concentration indicated that elevated PAH
606 concentrations reduced the rate of growth. In the ANOVAs no PAH or light phase effects were detected
607 in body mass or SVL at the end of the study but there was a difference between phases in TL. Larvae in
608 the 1500 mg/kg treatment were shorter than in other PAH concentrations and those exposed to UV light
609 were actually longer than those in non-UV. It is difficult to explain the increased growth under UV
610 lights.

611 Larvae of the Spanish ribbed newt (*Pleurodeles waltl*) died when exposed to benzo(a)pyrene
612 (BaP) in water. Exposure to 500 ppb of BaP and only visible light resulted in mortalities, but larvae died
613 at an accelerated rate when exposed to 25 $\mu\text{g/L}$ of BaP and UVA (Fernandez and l'Haridan 1994). BaP
614 is one of the most chronically toxic PAHs (Eisler 2000). Tadpoles of northern leopard frogs (*Rana*
615 *pipiens*) experienced similar results when they were exposed to 30.6 $\mu\text{g/L}$ of fluoranthene and 25 $\mu\text{g/L}$
616 of anthracene. When tadpoles were exposed to UV light, mortalities increased (Kagan et al. 1984;
617 Monson et al. 1999). In our study measured concentrations of BaP, fluoranthene and anthracene under
618 ultraviolet exposure were 12 $\mu\text{g/L}$, 11 $\mu\text{g/L}$, and 42 $\mu\text{g/L}$, respectively. While the concentration of
619 anthracene exceeded lethal levels found for *R. pipiens*, the dose of ultraviolet radiation would have an
620 important influence. Our simulation of Austin City conditions resulted in less total UV exposure than
621 that used by previous studies.

622 Other studies have reported that developmental differences in tadpoles of the African clawed
623 frog (*Xenopus laevis*) occurred when exposed to 3 mg/L of coal-tar sealant in water (Bryer et al. 2006).
624 Exposure to 625 $\mu\text{g/L}$ of fluoranthene without ultraviolet radiation, a concentration that was > 50 times
625 our levels, affected the growth of *R. pipiens* (Hatch and Burton 1998). However, exposure of larval
626 spotted salamanders (*Ambystoma maculatum*) to fluoranthene and UV light did not affect their growth.

627 This raises an important factor in evaluating PAH toxicity. The majority of previous studies
628 used single PAHs because the dose/effect relationships are more straightforward. Our exposures
629 involved a more realistic 'cocktail' of PAHs found in sealants, but each PAH has its own unique

630 characteristics, water solubility and toxicity. Unfortunately, there are no toxic equivalencies (TEQs or
631 TEFs) for PAHs as there are for polychlorinated biphenyls, dioxins and furans (Van den Berg et al.
632 1998). Therefore, direct comparisons of expected toxicity for different combinations of PAHs is not
633 possible.

634

635 **3.4.3 Speed Trials**

636 Salamanders exposed to coal-tar sealant and UV light experienced noticeable effects on their speed,
637 time, and distance they swam. Swimming measures were affected by both UV phase and PAH
638 concentrations and the significant interaction of the two factors indicates that they are synergistic. As in
639 experiment 1, behavior proved to be a sensitive end point to PAH. Also as in Experiment 1, sluggish
640 responses and reduced swimming could interfere with capture of prey or escape from predators.

641 **3.4.4 Micronucleus & White Blood Cell Test**

642 In this study the frequency of micronucleated erythrocytes (MNC) significantly increased as a result of
643 exposure to UV light, but apparently not from exposure to PAHs. Fernandez and l'Haridan (1994)
644 added PAHs directly into water and reported a significant increase in MNC when larvae of the Spanish
645 ribbed newt were simultaneously exposed to oil refinery effluent and UVA light compared to oil or
646 UVA light alone. The lack of an interaction in our study is consistent with low aqueous concentrations
647 of PAH.

648 Similarly, the number of erythroblasts, monocytes, basophils, eosinophils and total leukocytes
649 were higher in the UV phase than in the non-UV. The total white blood cell count was significantly
650 higher in the UV phase than in the non-UV phase. Ultraviolet radiation causes cell damage (Licht and
651 Grant 1997) which could stimulate the production of these cells. Erythroblasts are the immediate
652 precursors of normal red blood cells (Alberts et al. 2002) and an increase in their numbers suggests
653 increased production. Monocytes ingest dying or damaged cells and their increased frequency suggests
654 the occurrence of cell damage or necrosis. Basophils stimulate inflammation as part of the body's
655 defense, while eosinophils have an anti-inflammatory function (Alberts et al. 2002). Neutrophils

656 phagocytize microorganisms; they were the only leukocyte that showed a response to PAH. Elevation
657 of total white blood cells, therefore, was essentially indicative of cellular damage and elevated immune
658 response under UVB. Very similar differential white blood cell response to ultraviolet radiation occurs
659 in fish (Salo et al. 2000).

660 UV light can be lethal to anuran larvae (Tietge et al. 2001). Tadpoles of the red legged frog
661 (*Rana aurora*) and the Pacific tree frog (*Pseudacris regilla*) experienced prominent lens opacities and
662 substantial skin burns when exposed to UVB (Flamarique et al. 2000), but no studies are known on the
663 effects of UV light on the immune system in amphibians.

664 **4. EXPERIMENT 3 – EXPOSURE OF SAN MARCOS SALAMANDERS** 665 **TO PAHS**

666 **4.1 Objectives**

667 This experiment was part of the original study plan and was intended to use a closely related species of
668 salamander as a surrogate for the endangered Barton Springs salamander. The objective was to compare
669 the sensitivity of San Marcos salamanders to sealants with that of eastern newts by first determining the
670 dose/response relationships for various end points in the eastern newt and comparing these relationships
671 with a smaller number of salamanders. Unfortunately, San Marcos salamanders began dying
672 indiscriminate of sealant concentration and no dose/response relationship could be developed.

673 **4.2. Methods**

674 In March 2007 one of us (TB) drove from Austin, TX to Carbondale, IL with a shipment of 80 *Eurycea*
675 *nana*. After a three week quarantine period the animals were placed on study, two per aquarium. Water
676 chemistry was monitored during the first seven days of the study but was not continued when animals
677 began dying to avoid possible contamination of other tanks if a disease was involved. Within a week
678 animals began to die regardless of PAH concentration (Appendix 4). This mortality continued until
679 most of the animals had perished. Live and recently dead animals were sent to the USGS National
680 Wildlife Health Research Center for necropsy and to determine the cause of death. The cause for the
681 mortality was idiopathic, although a fungal infection was detected by pathologists at the research center.

682 Other dead animals were frozen at -75°C until they were analyzed for PAH. Their livers were
 683 subsequently analyzed for enzyme activity as in Experiment 1. Whole bodies were tested for PAH
 684 concentrations following the methods in Experiment 1.

685 **4.3. Results**

686 **4.3.1. Water and Sediment Chemistry**

687 Water chemistry before (Table 13) and after the experiment (Table 14) seemed to be within guidelines
 688 established by ASTM (1988; Table 13) but water quality criteria are not known for San Marcos
 689 salamanders.

690 Total PAH concentrations in *E. nana* whole bodies corresponded with sealant concentrations in
 691 that as sealant concentrations increased so did total PAH, but the relationship was not significant due to
 692 the small number of treatments (Table 15). The PAHs with the highest concentrations included
 693 benzo(a)pyrene, chrysene, fluoranthene, phenanthrene and pyrene.

694 **4.3.2 Enzyme activities**

695 We found no differences among light phases, sealant concentrations or their interactions for either LDH
 696 (phase $F_{1,39}=0.47$, $p=0.499$, sealant $F_{3,39}=1.58$, $p=0.213$, interaction $F_{3,39}=0.30$, $p=0.825$) or AST (phase
 697 $F_{1,39}=2.63$, $p=0.115$, sealant $F_{3,39}=0.23$, $p=0.874$, interaction $F_{3,39}=0.39$, $p=0.759$; Appendix 5). Because
 698 there were no significant differences for these two enzymes, ALT was not analyzed.

Table 13. Mean (SE) water quality conditions during the exposure experiment with *Eurycea nana*. Sample size is three tanks per day

Day	pH	Temperature (°C)	Hardness (mg/L Ca)	Ammonia (mg/L)	Oxygen (mg/L)
1	7.30 (0.03)	20.6 (0.1)	53.7	0.03 (0.03)	5.36 (0.02)
2	7.31 (0.03)	20.8 (0.1)	53.7	0	5.34 (0.02)
3	7.29 (0.02)	20.4 (0.0)	53.7	0.06 (0.06)	5.39 (0.06)
7	7.33 (0.04)	20.3 (0.1)	53.7	0.09 (0.03)	5.38 (0.02)
11	7.35 (0.04)	20.7 (0.1)	53.7	0.19 (0.11)	

15	7.30 (0.06)	20.8 (0.1)	53.7	0.03 (0.03)
19	7.31 (0.05)	20.5 (0.1)	53.7	0.06 (0.04)

699

Table 14. Mean (SE) water quality conditions measured after the termination of the experiment with *Eurycea nana*, N=10 tanks per concentration

Sealant concentration (mg/kg)	Dissolved oxygen (mg/L)	Temperature (°C)	Conductivity (mS/m)
0	5.74 (0.03)	17.9 (0.1)	200.9 (3.5)
60	5.63 (0.05)	17.9 (0.1)	210.8 (4.8)
280	5.71 (0.02)	18.0 (0.0)	183.8 (8.8)
1500	5.60 (0.03)	17.9 (0.0)	207.1 (1.7)

700

Table 15. Mean (SE) of PAH concentrations in *Eurycea nana* exposed to sediments dosed with coal tar sealant. N= 3 animals per sealant concentration. Units are $\mu\text{g/g}$ wet weight.

Analyte	0 mg/kg Sealant	60 mg/kg Sealant	280 mg/kg Sealant	1500 mg/kg Sealant
Acenaphthylene	0	0	0.17 (0.17)	0 (0.17)
Acenaphthene	0.33 (0.17)	0	0	0.17 (0)
Phenanthrene	2.06 (0.99)	0.59 (0.38)	1.45 (0.54)	8.82 (0.54)
Anthracene	0	0	0.37 (0.37)	0.74 (0.37)
Fluoranthene	0	0	1.88 (1.88)	6.12 (1.88)
Pyrene	0.83 (0.60)	2.33 (1.12)	4.58 (3.40)	7.67 (3.40)
Benzo(a)anthracene	0.50 (0)	0.50 (0)	0.50 (0)	0.17 (0.0)
Chrysene	0	0.17 (0.17)	0.58 (0.58)	7.22 (0.58)
Benzo(b)fluoranthene	3.29 (0.48)	1.77 (1.77)	5.68 (0.66)	2.48 (0.66)
Benzo(k)fluoranthene	0.17 (0.17)	0	0.92 (0.92)	1.58 (0.92)
Benzo(a)pyrene	2.41 (0.98)	5.67 (2.16)	18.73 (14.92)	14.02 (14.92)

Total PAH	8.08 (0.91)	10.03 (4.55)	34.03 (22.91)	48.14 (22.91)
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701

702 **4.4 Discussion**

703 Most of the data collected during the San Marcos experiment were inconclusive. However, we can say
704 that the animals appeared to quickly assimilate PAH concentrations in their tissues in a dose dependent
705 fashion. Because PAHs are naturally occurring compounds found in all living organisms, a TPAH of
706 8.08 $\mu\text{g/g}$ in controls is not unexpected. We did not analyze water concentrations in this experiment but
707 if data from the other two experiments are considered, it appears that San Marcos salamanders may
708 bioconcentrate PAHs, at least initially. The concentrations of individual and TPAH in these animals
709 exceeded those found in eastern newts. As reported above, Garrigues et al. (2004) found that
710 salamanders quickly bioconcentrated PAHs but reached a lower equilibrium after a few days. Each of
711 the individual PAHs they examined behaved differently, so the diversity of PAHs found in the bodies of
712 our salamanders after a few days of exposure may not represent what would be present after longer
713 exposures. The carcinogenic benzo(a)pyrene is often readily assimilated by aquatic organisms and
714 accounted for 29 to 56% of the TPAH found in San Marcos salamanders.

715 There was considerable and appropriate concern after the *Eurycea nana* experiment failed due to
716 high mortality in all groups, including controls. This concern focused on why the *E. nana* experiment
717 failed and what to do in its place. We are still uncertain why the *E. nana* died. The possibility of
718 hypoxic conditions was raised by the review team in Austin. For most species of aquatic amphibians
719 this would not be of great concern. Aquatic amphibians in general have high tolerance to hypoxic
720 conditions with many physiological and behavioral methods to counteract hypoxia (Boutilier et al.
721 1992). The dynamics of respiratory gas exchange in amphibians is complex with many back up
722 methods to sustain gaseous exchange, as is evidenced by the detail presented within Feder and Burggren
723 (1992).

724 During the first week of the experiment oxygen concentrations were above 5 mg/L ($\text{PO}_2 > 300$
725 torr) and measurements taken after the study was terminated were consistent with those concentrations.

726 Air was bubbled into every tank continuously to avoid hypoxia. However, while these conditions were
727 suitable for the spotted salamander and eastern newts in that mortality of these species was less than 3%
728 and for many other species of amphibians we have worked with, it is possible that they may not have
729 been optimal for San Marcos salamanders. Feder and Buggren (1992) provide several chapters on the
730 physiology of oxygen uptake, consumption, and needs as related to other environmental factors such as
731 temperature. Interestingly, the book seems to omit a defined criterion for hypoxia but perusal of their
732 charts and text shows no evidence of adverse oxygen concentrations above a PO₂ of around 40 torr, well
733 below our measured levels.

734 **4.5 Conclusions**

735 The limited data from this experiment shows that *Eurycea nana* can bioconcentrate PAHs over a short
736 period of time. Whether they would depurate these PAHs after a longer exposure and reach an
737 equilibrium such as that found by Garrigues et al. (2004) in another species of salamander is not known.
738 No other effects associated with sealant concentrations were found but the exposures may have been too
739 short and confounded by factors related to premature mortality to reveal chronic effects.

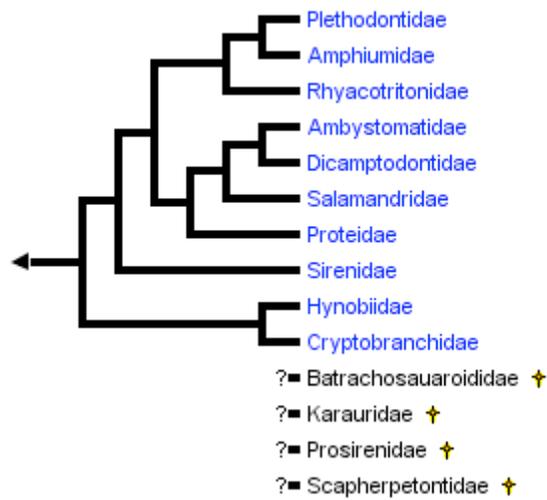
740 **5. GENERAL DISCUSSION**

741 **5.1 Choice of Surrogate Species**

742 The choice of surrogate species in toxicological studies is always of concern. Other species that
743 were considered as a substitute for *E. sosorum* and *E. nana* include the salamanders *A. gracile*,
744 and *Pleurodeles waltl* because there is at least some information on their responses to PAH.
745 *Ambystoma gracile* would not have offered any advantages over *A. maculatum* and we had a
746 large population of *A. maculatum* available. *P. waltl* is a nonendemic, large salamander and was
747 omitted from consideration almost immediately. Another salamander with less information
748 about PAH responses under consideration was the tiger salamander (*Ambystoma tigrinum*) which
749 is wide spread but occurs regionally as many subspecies, each having some unique
750 characteristics and which could differ in sensitivity. Also, some populations of tiger salamanders

751 are declining precipitously. Among anurans we considered the Texas toad (*Bufo speciosus*)
752 which has a distribution that partially overlaps that of *Eurycea nana* and *E. sosorum*. However,
753 this species is rare and is a protected species. Narrow-mouthed toads (*Gastrophryne*
754 *carolinensis* or *G. olivacea*) were also considered. They seem to be very sensitive to
755 contaminants even they have a broad distribution that overlaps that of the *Eurycea*. For varying
756 reasons, each of these species was rejected by the Austin advisory committee.

757 In theory, the more closely related a surrogate is to the target species, the more similar
758 their responses should be. While this makes intuitive sense, there has been no critical
759 examination of this in amphibians or other taxa of which we are aware. Salamanders belong in
760 the order Caudata (alternatively Urodela) of the Class Amphibia. The order consists of 10 living
761 families and 4 extinct families with unresolved lineage (Figure 5). The genus *Eurycea* is in the
762 subfamily Spelerpinae which falls along the main branch of the family Plethodontidae or
763 lungless salamanders (Figure 6). Of the two surrogate species used in this study, eastern newts
764 are in the family Salamandridae and spotted salamanders are in the family Ambystomatidae.
765 It is clear that there is some taxonomic distance among the families and, subsequently, between
766 the surrogates and the species of concern, *Eurycea sosorum*. What is less clear is what, if any,
767 significance this may have on toxicological responses.



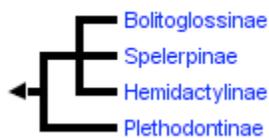
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769

770 Figure 4. Relationships among Caudata families (after Wiens 2005; cladogram from Larson et al.
 771 2006). The families not connected to the cladogram are extinct and their relationships have not
 772 been resolved.

773

774



775

776 Figure 5. Subfamily relationships within the family Plethodontidae. The genus *Eurycea* is in the
 777 subfamily Spelerpinae, part of the main root of the family (after Chippendale 2004, cladogram
 778 from Larson et al. 2006).

779

780 Other factors that need to be considered include the natural history of each species and available
 781 information on interspecific sensitivities to contaminants among other amphibians. All three families
 782 are native to North America and all three species are native to the United States. *Eurycea nana* and *E.*

783 *sosorum*, like other Plethodontids, are lungless. Unlike most other Plethodontids, however, these two
784 species are totally aquatic and neotenic, retaining their gills into sexual maturity. They have highly
785 restricted distributions and are limited to freshwater springs in central Texas. They are closely related
786 (Chippindale et al. 2004). Both species feed on small aquatic invertebrates and live amongst rocks or
787 heavy cover within their respective springs. There is no disagreement that the federally threatened *E.*
788 *nana* would have been the best surrogate for the endangered *E. sosorum*.

789 *Ambystoma maculatum* has a wide distribution in the eastern United States, ranging from
790 northern Maine to Southern Louisiana with some extension into eastern Texas (Conant and Collins
791 1998). Larvae have external gills as do the *Eurycea* species and both feed on similar foods. Spotted
792 salamanders breed in vernal wetlands and pools. Adults are primarily terrestrial, living beneath stones
793 or boards in moist environments. Unlike *Eurycea*, Ambystomids have lungs as larvae and as adults.

794 *Notophthalmus viridescens* has a life history that is considerably different from either of the
795 other two species or from many other salamanders. Adults lay their eggs in water and, after a period of
796 development, immature efts become terrestrial. Newts breed in a variety of habitats including ponds,
797 vernal wetlands, and quiet portions of streams. They appear to avoid swift moving waters. Some
798 populations lack this terrestrial stage. Upon reaching sexual maturity the efts return to water and
799 become newts. The adult newts lack gills but have lungs. Like the other species, newts are carnivorous,
800 feeding on aquatic invertebrates. Eastern newts have four recognized subspecies and collectively have a
801 range similar to that of spotted salamanders (Conant and Collins 1998). In summary, while natural
802 history differences exist among the species, there are similarities including external gills in *Eurycea* and
803 larval *Ambystoma*, adult aquatic stages in *Eurycea* and *Notophthalmus* and common qualities of dermal
804 respiration, permeable skin, and similar diets among all the species.

805 Cross-species comparisons in toxicological responses are hampered by the relatively few studies
806 conducted on amphibians and even fewer on salamanders. Sparling et al. (2000 and in press)
807 demonstrated that there have been fewer ecotoxicological papers published on amphibians than any
808 other vertebrate class except reptiles. Moreover, among amphibians, more ecotoxicological papers have

809 been published on members of the family Ranidae than on all salamander species combined and the
 810 family Plethodontidae is one of the least represented groups among amphibians.

811 Interspecific comparisons in toxicological responses, therefore, must include other amphibians in
 812 addition to salamanders. Available literature shows that dose/response relationships can vary among
 813 amphibian species but not in entirely predictable ways. For example, Sparling and Fellers (2009) found
 814 that *Pseudacris regilla* (Family Hylidae) were approximately 10 times less sensitive to the pesticide
 815 endosulfan and half as sensitive to chlorpyrifos than was *Rana boylei* (Family Ranidae). However, *Bufo*
 816 *boreas* (Family Bufonidae) was almost exactly as sensitive to endosulfan as was *P. regilla* (Sparling,
 817 unpublished). Sparling et al (2000) contains many chapters that have comparative data for a variety of
 818 amphibian species. Table 16 summarizes LC50 data from this reference for a variety of chemicals to
 819 show how variable inter-taxon, inter-study and inter-chemical comparisons can be. Most studies have
 820 been conducted on anuran larvae so caudate representation is scant. Data from the same sources were
 821 collected under similar conditions for each species. Note in particular that there are no apparent
 822 consistencies in inter-familial sensitivities. Thus, there is no way to predict *a priori* if *E. nana* and *E.*
 823 *sosorum* are more or less sensitive to PAHs than the two surrogate species. We can say, however, that
 824 in terms of mortality and sublethal responses there was considerable similarity between the two
 825 surrogates, which we believe underscores that aspects of this study can be generalized to *Eurycea* spp.

Table 16. Summary of LC50 data for amphibians and a variety of chemicals from Sparling (2000) and Sparling et al. in press.

Chemical	Species	Family	Life Stage	Duration	Effect	Source
Guthion	<i>Xenopus laevis</i>	Pipidae	Larva	96 hr	LC50: 2.94 mg/L	1
Guthion	<i>Hyla regilla</i>	Hylidae	Larva	96 hr	LC50:– 4.14 mg/L	1
Guthion-2S	<i>X. laevis</i>	Pipidae	Larva	96 hr	LC50: 0.59 mg/L	1
Guthion-2S	<i>H. regilla</i>	Hylidae	Larva	96 hr	LC50: 0.84 mg/L	1
Guthion-2S	<i>H. regilla</i>	Hylidae	Larva	96 hr	LC50:1.47 mg/L	2

						mg/L	
Guthion-2S	Ambystoma gracile	Ambystomatidae	Larva	96 hr	LC50:1.90	2	
					mg/L		
Guthion-2S	A. maculatum	Ambystomatidae	Larva	96 hr	LC50:1.90	2	
					mg/L		
Endosulfan	Rana boylei	Ranidae	Larva	2 week	50% mortality: 0.3 µg/L	3	
Endosulfan	Bufo boreas	Bufo	Larva	2 week	50% mortality: 3.5 µg/L	3	
Endosulfan	Pseudacris regilla	Hylidae	Larva	2 week	50% mortality: 3.8 µg/L	3	
Chlorpyrifos	Rana pipiens	Ranidae	Larva	96 hr	LC50: 3	4	
					mg/L		
Chlorpyrifos	Bufo americanus	Bufo	Larva	96 hr	LC50: 1	4	
					µg/L		
Chlorpyrifos	Rana boylei	Ranidae	Larva	24 hr	LC50: 3	5	
					mg/L		
Phenol	R. pipiens	Ranidae	Larva	96 hr	LC50: 0.04 mg/L	6	
Phenol	Bufo americanus	Bufo	Larva	96 hr	LC50: 0.10 mg/L	6	
Phenol	R. catesbeiana	Ranidae	Larva	96 hr	LC50: 0.23 mg/L	6	
Phenol	Bufo fowleri	Bufo	Larva	96 hr	LC50: 2.45 mg/L	6	
Phenol	R. palustris	Ranidae	Larva	96 hr	LC50: 13.0 mg/L	6	

826 Sources: 1) Schuytema et al. 1995; 2) Nebeker et al. 1998; 3) Sparling and Fellers 2009; 4) Barron and
827 Woodburn 1995; 5) Sparling and Fellers 2007; 6) Birge et al. 2000.

828

829 A factor that must be considered is how the habitats of the four species may influence exposure.
830 Eastern newts and spotted salamanders prefer quiescent bodies of water in which to breed. These sites
831 are similar to our test conditions in that water flow and recycling are restricted. We may surmise that in
832 these situations PAHs in solution are in near continuous contact with the salamanders. In our visits to
833 Austin it appeared that Barton Springs salamander habitats were flow-through systems. Rapid

834 percolation and flow of water could diminish actual exposure and risk in that effluent from sealant-
835 coated surfaces and any PAHs that become uncoupled from sediments may be flushed through the
836 waterways. Because Barton Springs salamanders are often found beneath stones where circulation rates
837 may differ from open water columns, it would be useful to estimate exposure conditions and PAH
838 concentrations in the preferred microhabitats.

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1012 **APPENDICES**

1013 **Appendix 1. PAH concentrations in sediment before and after the 28 day exposure**
 1014 **period of experiment 1.**

1015
 1016 Detectable levels of PAHs found within the sediment of control and coal-tar treatments prior to exposure
 1017 to UV and visible light.

Coal-tar	Control	15 mg/kg	31 mg/kg	62 mg/kg	125 mg/kg	250 mg/kg	500 mg/kg	1500 mg/kg
Napthalene	ND	0.05	<RL	<RL	<RL	<RL	<RL	<RL
Acenathphylene	0.00	ND	0.17	0.41	0.78	0.80	1.64	2.02
Acenaphthene	ND	<RL	<RL	<RL	<RL	<RL	<RL	8.70
Fluorene	<RL	0.08	0.21	0.54	<RL	0.86	2.66	18.12
Phenanthrene	0.03	0.30	2.91	6.33	1.73	11.09	35.04	174.36
Anthracene	ND	0.25	1.72	3.10	1.24	5.92	14.20	67.27
Fluoranthene	0.10	0.97	8.24	14.55	4.91	29.91	57.98	244.93
Pyrene	0.07	0.73	6.19	10.61	3.15	23.28	46.58	192.58
Benzene(a) anthracene	0.13	0.58	5.11	6.75	3.86	13.88	21.97	88.80
Chrysene	0.12	0.48	3.72	5.88	3.45	12.26	19.21	69.11
Benzo(b) fluoroanthene	ND	0.50	3.90	6.19	3.46	12.00	17.68	81.29
Benzo(k) fluoranthene	ND	0.39	2.43	3.74	3.08	6.37	11.61	24.63
Benzo(a)pyrene	ND	0.53	4.51	7.70	3.45	13.86	21.11	82.62
Indeno(1,2,3-cd)perylene	0.13	0.45	6.14	7.43	3.74	17.13	22.79	77.68
Dibenz(a,h) anthracene	0.19	ND	0.59	1.42	2.38	2.84	5.10	7.92
Benzo(g,h,i) perylene	0.15	0.31	2.47	4.21	2.82	7.25	11.17	30.40
Total	0.92	5.62	48.30	78.92	38.05	157.44	288.74	1170.45

1018 ^a ND indicates no instrument response was detected for this parameter.

1019 ^b <RL indicates the concentration of a PAH was less than the reporting limit of 50 µg/g.

1020 ^c Recovered concentrations are reported as µg/g.

1021 Detectable levels of PAHs found within the sediment of control and coal-tar treatments at the
 1022 conclusion of exposure to UV and visible light.

Coal-tar	Control	15 mg/kg	31 mg/kg	62 mg/kg	125 mg/kg	250 mg/kg	500 mg/kg	1500 mg/kg
Napthalene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	1.17
Acenathphylene	0.08	0.08	0.16	0.39	0.78	0.78	1.57	1.98
Acenaphthene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	6.51
Fluorene	<RL	<RL	0.11	<RL	<RL	0.55	1.71	13.79
Phenanthrene	0.05	0.40	1.30	1.16	2.95	7.39	22.14	140.56
Anthracene	0.09	0.31	0.78	0.98	2.43	4.99	10.96	58.88
Fluoranthene	0.14	1.33	3.22	4.16	11.27	23.87	50.34	247.70
Pyrene	0.11	1.01	2.40	3.26	9.45	19.93	41.01	190.34
Benzene(a) anthracene	0.25	0.95	2.06	3.09	8.14	13.33	27.28	100.05
Chrysene	0.16	0.74	1.66	2.55	6.82	10.70	22.88	78.23
Benzo(b) fluroanthene	0.22	1.18	2.38	3.00	8.68	14.03	29.23	70.76
Benzo(k) fluoranthene	0.22	0.41	1.06	1.88	4.18	5.50	13.86	52.23
Benzo(a)pyrene	0.16	1.15	2.11	2.93	8.75	14.69	30.34	79.97
Indeno(1,2,3-cd)perylene	0.21	1.33	2.70	3.34	8.93	15.52	33.56	50.96
Dibenz(a,h) anthracene	0.22	0.25	0.52	1.22	2.65	2.95	6.11	ND
Benzo(g,h,i) perylene	0.19	0.56	1.21	1.86	4.91	7.18	15.67	34.42
Total	2.10	9.71	21.67	29.81	79.94	141.40	306.70	1127.55

1023 ^a ND indicates no instrument response was detected for this parameter.

1024 ^b <RL indicates the concentration of a PAH was less than the reporting limit of 50 µg/g.

1025 ^c Recovered concentrations are reported as µg/g.

1026 **Appendix 2. Body measurements of eastern newts after a 28 day exposure to coal**
 1027 **tar and asphalt sealants.**

Table 8. Mean (SE) of body mass, snout vent length (SVL) and total length (TL) of eastern newts exposed to asphalt and coal tar sealants

Sealant Concentration (mg/kg)	Asphalt Sealant				Coal Tar Sealant			
	PAH (mg/kg)	Body mass (g)	SVL (mm)	TL (mm)	PAH (mg/kg)	Body mass (g)	SVL (mm)	TL (mm)
0	0.07	1.72 (0.09)	42.9 (0.2)	82.5 (1.1)	1.51	1.90 (0.10)	44.0 (0.3)	84.7 (1.2)
15	0.44	2.12 (0.36)	43.8 (0.7)	84.5 (1.3)	7.67	1.89 (0.13)	43.1 (0.6)	82.7 (1.9)
31	1.12	1.53 (0.14)	37.8 (3.7)	71.2 (6.9)	35.0	1.86 (0.09)	43.2 (0.47)	82.7 (0.5)
62	1.27	2.04 (0.12)	44.2 (1.1)	85.8 (1.5)	54.4	1.83 (0.06)	43.1 (0.4)	82.7 (1.0)
125	7.94	1.85 (0.09)	43.2 (0.6)	82.9 (1.3)	59.0	1.55 (0.12)	37.2 (3.2)	71.4 (6.1)
250	8.63	2.44 (0.39)	44.9 (0.6)	87.4 (0.8)	149	1.75 (0.11)	40.4 (3.1)	77.1 (5.5)
500	14.4	1.90 (0.11)	43.4 (0.3)	84.4 (0.7)	297	1.85 (0.09)	40.4 (2.4)	78.6 (4.6)
1500	20.58	1.97 (0.11)	43.5 (0.5)	84.5 (1.3)	1148	1.66 (0.09)	38.2 (3.2)	71.4 (6.3)

1028

1029 **Appendix 3. PAH Concentrations in sediments before and after the 28 day exposure**
 1030 **period for experiment 2.**

1031 Detectable levels of PAHs found within the sediment of control and asphalt treatments prior to exposure
 1032 to UV and visible light.

Asphalt	15 31 62 125 250 500 1500							
	Control	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Napthalene	ND	0.012	0.013	0.015	ND	<RL	<RL	<RL
Acenathphylene	ND	0.027	0.027	0.027	0.667	0.268	0.67	0.67
Acenaphthene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Fluorene	<RL	ND	<RL	ND	0.396	<RL	<RL	<RL
Phenanthrene	0.006	0.037	0.051	0.043	0.387	0.198	0.40	<RL
Anthracene	0.017	0.025	0.033	0.032	0.565	0.157	0.38	0.28
Fluoranthene	0.002	0.063	0.380	0.145	0.773	0.383	1.36	0.50
Pyrene	ND	0.141	0.245	0.137	0.675	0.319	1.26	0.37
Benzene(a) anthracene	ND	0.038	0.205	0.190	1.230	0.469	1.71	0.95
Chrysene	ND	0.035	0.192	0.171	0.746	0.408	1.27	0.49
Benzo(b) fluoroanthene	0.003	0.041	0.209	0.175	1.328	1.030	2.02	1.29
Benzo(k) fluoranthene	ND	0.004	0.083	0.064	0.911	0.390	0.94	0.65
Benzo(a)pyrene	ND	0.035	0.198	0.189	1.224	1.085	1.97	1.12
Indeno(1,2,3-cd)perylene	0.009	0.043	0.210	0.185	1.493	1.231	2.33	1.70
Dibenz(a,h) anthracene	0.002	0.003	0.039	0.024	1.068	0.490	1.18	1.11
Benzo(g,h,i) perylene	0.003	0.024	0.129	ND	0.936	0.768	1.41	1.02
Total	0.042	0.527	2.014	1.397	12.398	7.197	16.90	10.14

1033 ^a ND indicates no instrument response was detected for this parameter.

1034 ^b <RL indicates the concentration of a PAH was less than the reporting limit of 50 µg/g.

1035 ^c Recovered concentrations are reported as µg/g.

1036

1037 Detectable levels of PAHs found within the sediment of control and asphalt treatments at the conclusion
 1038 of exposure to UV and visible light.

Asphalt	Control	15 mg/kg	31 mg/kg	62 mg/kg	125 mg/kg	250 mg/kg	500 mg/kg	1500 mg/kg
Napthalene	<RL	0.011	0.012	0.012	<RL	<RL	<RL	<RL
Acenathphylene	0.028	0.028	0.027	0.027	0.267	0.268	0.669	0.670
Acenaphthene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Fluorene	ND	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Phenanthrene	0.026	0.012	0.009	0.016	0.096	0.192	0.289	1.105
Anthracene	0.022	0.018	0.018	0.019	0.128	0.159	0.326	0.578
Fluoranthene	0.009	0.012	0.017	0.026	0.217	0.619	0.654	3.575
Pyrene	0.005	0.006	0.026	0.019	0.164	0.561	0.528	2.746
Benzene(a) anthracene	ND	0.038	0.049	0.068	0.377	0.958	1.000	2.124
Chrysene	ND	0.038	0.048	0.081	0.241	0.793	0.607	1.989
Benzo(b) fluroanthene	0.001	0.048	0.070	0.139	0.680	1.118	1.642	3.614
Benzo(k) fluoranthene	ND	0.014	0.024	0.048	0.295	0.445	ND	1.382
Benzo(a)pyrene	ND	0.049	0.069	0.127	0.643	1.062	1.576	4.317
Indeno(1,2,3-cd)perylene	0.006	0.051	0.087	0.152	0.832	1.249	2.141	4.597
Dibenz(a,h) anthracene	ND	0.004	0.017	0.026	0.443	0.505	1.168	1.439
Benzo(g,h,i) perylene	ND	0.028	0.055	0.093	0.490	0.753	1.271	2.892
Total	0.099	0.353	0.529	0.854	4.873	8.683	11.872	31.029

1039 ^a ND indicates no instrument response was detected for this parameter.

1040 ^b <RL indicates the concentration of a PAH was less than the reporting limit of 50 µg/g.

1041 ^c Recovered concentrations are reported as µg/g.

1042 **Appendix 4 – History of *Eurycea nana***
1043

1044 **3/13/07** – Acquired and transported San Marcos salamanders from San Marcos TX to Carbondale IL.

1045 **3/16/07** – A few of them are acting strange where their body is kinked most of the
1046 time in the shape of a U. Even when they swim they have trouble and often swim
1047 in the direction their body is kinked toward, like clockwise or counterclockwise.

1048 **3/21/07** – Small hemorrhages observed on belly and tail of quarantine salamander

1049 **3/22/07** (Day 0) – All salamanders weighed and measured

1050 **3/23/07** (Day 1) – All salamanders fed and doing fine. Most were found within their shelters.

1051 Not much movement was observed

1052 All tanks aerating well

1053 Room temp staying pretty constant around 68-69°F

1054 Much worse hemorrhages observed on a salamander in quarantine.

1055 **3/24/07** (Day 2) – All looking fine and most found within their shelters

1056 All tanks aerating well

1057 **3/25/07** (Day 3) –

1058 Tank #1/0ppm – 2 dead

1059 Tank #20/500ppm – 1 dead

1060 Tank #16/0ppm – 2 dead

1061 Tank #13/500ppm – 2 dead

1062 Tank #8/280ppm – 1 dead

1063 Tank #38/500ppm – 1 dead

1064 Tank #25/0ppm – 2 dead

1065 Tank #35/60ppm – 1 dead 12 total

1066 Tank #29/60ppm – 1 individual occasionally being found on its back

1067 Tank #2/500ppm – 1 individual also showing found on its back

1068 Room temp staying pretty constant around 68-70°F

1069 Water quality tests done

1070 All tanks aerating well

1071 Not much movement was observed

1072 All salamanders fed

1073 **3/26/07** (Day 4) –

1074 Tank #2/500ppm – 1 dead

1075 Tank #4/280ppm – 1 dead

1076 Tank #6/280ppm – 1 dead

1077 Tank #14/280ppm – 1 dead

1078 Tank #7/280ppm – 2 dead

1079 Tank #2/500ppm – 1 dead

1080 Tank #8/280ppm – 1 dead

1081 Tank #11/0ppm – 1 dead 9 dead - - 21 total

1082 Tank #3/500ppm – Both individuals have a fungal growth on their body. One has it on its front left limb,
1083 and the other has it on its tail. Both individuals were removed from their experimental tank and placed in
1084 a shallow container. I used tweezers to gently pry the fungus off their body and found that the front limb
1085 on one individual was almost completely gone. The epidermis on the other individual was completely
1086 gone under the fungal growth. The growth was brown in color because sediment was mixed in with it.
1087 Both were placed back into their experimental tank.

1088 Water quality tests done

1089 All tanks aerating well

1090 More individuals found dead that had no noticeable indications of sickness such as fungus or lying on
1091 their back.

1092 Room temp staying pretty constant around 68-69°F

1093 Two salamanders in the quarantine (Non-experimental tank) were found dead.

1094 **3/27/07** (Day 5) –

1095 Tank #3/500ppm – 1 dead

1096 Tank #15/60ppm – 1 dead

1097 Tank #29/60ppm – 1 dead

1098 Tank #34/280ppm – 1 live salamander shipped away for necropsy

1099 Quarantine – 2 dead salamanders shipped away for necropsy

1100

1101 3 dead – experimental

1102 1 shipped away – experimental

1103 2 dead - quarantine

1104 Not much movement observed

1105 Room temp staying pretty constant around 68-69°F

1106 Changed water in all tanks & water analysis

1107 All salamanders fed

1108 All tanks aerating well

1109 **3/28/07** (Day 6) –

1110 Tank #27/60ppm – 1 dead

1111 Tank #7/280 ppm – 1 dead

1112 Tank #17/500ppm – 1 dead

1113 Tank #24/0ppm – 1 dead 4 dead - - 30 total

1114 Tank #18/280ppm – 1 individual with fungal growth by its head

1115 Tank #38/1500ppm, #13/1500ppm, #40/0ppm, #28/60ppm - Numerous individuals showing a reduction

1116 in the size of their gills

1117 **3/29/07** (Day 7) –

1118 Tank #4/280ppm – 1 dead

1119 Tank #20/500ppm – 1 dead

1120 Tank #19/280ppm – 1 dead

1121 Tank #18/280ppm – 2 dead

1122 Tank #17/50ppm – 1 dead

1123 Tank #29/60ppm – 1 dead

1124 Tank #24/0ppm – 1 dead

1125 Tank #40/0ppm – 1 dead

1126 Tank #37/500ppm – 1 dead

1127 Tank #22/500ppm – 1 dead

1128 Tank #9/60ppm – 1 dead

1129 Tank #14/280ppm – 1 dead

1130 Tank #12/0ppm – 2 dead

1131 Tank #21/0ppm – 1 dead 16 dead, 46 total

1132 Tank #33/500ppm - 1 individual lying on its back

1133 Nothing obvious that could be causing mortality other than the fungus that is occasionally spotted on
1134 some individuals. Water quality tests are normal with most tanks having very low ammonia levels.

1135 Not much movement observed

1136 More individuals of all concentrations showing a reduction in gill size

1137 All salamanders fed

1138 **3/30/07** (Day 8) –

1139 Tank #28/60ppm – 1 dead

1140 Tank #21/0ppm – 1 dead

1141 Tank #33/500ppm – 2 dead 4 dead - - 50 total

1142 Tank #19/280ppm – 1 individual with open sore on back

1143 Individuals still found under their shelters and in the open.

1144 **3/31/07** (Day 9) –

1145 Tank #11/0ppm – 1 dead 1 dead – 51 total

1146 Nothing new observed that would indicate the cause of mortality.

1147

1148 Changed water in all tanks & water analysis

1149 All salamanders fed

1150 **4/1/07** (Day 10) –

1151 Tank #38/500ppm – 1 dead

1152 Tank #36/0ppm – 1 dead 2 dead – 53 total

1153 **4/2/07** (Day 11) –

1154 Tank #31/60ppm – 1 dead 1 dead – 54 total

1155 All salamanders fed

1156 **4/3/07** (Day 12) –

1157 Tank #15/60ppm – 1 dead 1 dead - - 55 total

1158 **4/4/07** (Day 13) –

1159 Tank #40/0ppm – 1 dead

1160 Tank #27/60ppm – 1 dead

1161 Tank #23/500ppm – 1 dead

1162 Tank #26/280ppm – 2 dead

1163 Tank #32/60ppm – 1 dead

1164 Tank #31/60ppm – 1 dead

1165 Tank #30/280ppm – 1 dead 8 dead – 63 total

1166 Nothing new observed other than a reduction in gills on some individuals.

1167 Changed water in all tanks & water analysis

1168 All salamanders fed

1169 **4/5/07** (Day 14) –

1170 0 mortalities 0 dead - - 63 total

1171 **4/6/07** (Day 15) –

1172 Tank #32/60ppm – 1 dead 1 dead – 64 total

1173 All salamanders fed

1174 **4/7/07** (Day 16) –

1175 Tank #39/60ppm – 1 dead

1176 Tank #30/280ppm – 1 dead 2 dead - - 66 total

1177 **4/8/07** (Day 17) –

1178 Tank #10/0ppm – 1 dead

1179 Tank #34/280ppm – 1 dead

1180 Tank #23/500ppm – 1 dead

1181 Tank #39/60ppm – 1 dead 4 dead – 70 dead

1182 Changed water in all tanks & water analysis

1183 All salamanders fed

1184 **4/9/07** (Day 18) –

1185 Tank #27/60ppm – 1 dead

1186 Tank #9/60ppm – 1 dead 2 dead - - 72 total

1187 **4/10/07** (Day 19) –

1188 0 mortalities 0 dead

1189 All salamanders fed

1190 **4/11/07** (Day 20) –

1191 Tank #10/0ppm – 1 dead 1 dead - - 73 total

1192 **4/12/07** (Day 21) –

1193 0 mortalities 0 dead

1194 Changed water in all tanks & water analysis

1195 All salamanders fed

1196 **4/13/07** (Day 22) –

1197 0 mortalities 0 dead - - 73 total

1198 **4/14/07** (Day 23) –

1199 0 mortalities 0 dead

1200 All salamanders fed

1201 **4/15/07** (Day 24) –

1202 Tank #16/0ppm – 1 dead 1 dead - - 74 total

1203 Changed water in all tanks & water analysis

1204 All salamanders fed

1205 **4/16/07** (Day 25) –

1206 0 mortalities

1207 Remaining 5 salamanders were euthanized

1208

1209 Summary:

1210 1 salamander sent to Madison Wildlife Health Laboratory while still alive

1211 79 salamanders died on study or in quarantine.

1212

1213

1214 **Treatments by Tank**

1215 500ppm – 2, 3, 13, 17, 20, 22, 23, 33, 37, 38

1216 280ppm – 4, 6, 7, 8, 14, 18, 19, 26, 30, 34

1217 60ppm – 5, 9, 15, 27, 28, 29, 31, 32, 35, 39

1218 0ppm – 1, 10, 11, 12, 16, 21, 24, 25, 36, 40

1219

1220

1221

1222

1223 **Appendix 5. Mean (SE) liver enzymes from individual *Eurycea nana*. Treatment**
1224 **values are in mg sealant/kg sediment and enzyme values are in mg/L= (U/L). N=5**
1225 **animals per treatment.**

Treatment	LDH	AST
Non-UV		
0 mg/kg	85.84 (3.59)	39.89 (1.42)
60 mg/kg	70.02 (6.71)	38.03 (3.45)
280 mg/kg	76.32 (5.98)	35.65 (2.06)
1500 mg/kg	71.81 (2.89)	38.56 (3.16)
UV		
0 mg/kg	76.75 (6.35)	35.81 (2.40)
60 mg/kg	71.15 (4.44)	33.89 (2.27)
280 mg/kg	76.75 (10.32)	36.11 (2.15)
1500 mg/kg	67.52 (5.64)	33.49 (4.35)

1226