

Effects of Culture Duration on Toxicity of Ethanol to Developing Embryos of the Grass Shrimp, *Palaemonetes pugio*

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Embryos of the grass shrimp, *Palaemonetes pugio*, have been utilized by several investigators for determining the toxicity and/or infectivity of chemical and microbial pest control agents. Fisher and Foss (1993) determined the toxicity of #2 fuel oil and two oil dispersants using methodology adapted from Wilson (1985) and Middaugh et al. (1988). Similar tests were used to examine the toxicity of metabolic products of fossil fuel biodegradation (Chapman et al. 1995). Genthner et al. (1994) identified the effects of a fungal microbial pest control agent, *Beauveria bassiana*, on embryos of *P. pugio*. To refine and characterize the grass shrimp test, Rayburn et al. (1996) examined the effects of reduced durations of exposure, sample numbers (N), and test solution volumes on LC50 values for water soluble fractions (WSF) of #2 fuel oil. Rayburn and Fisher (In press) defined the 12 d grass shrimp developmental toxicity test as the Shrimp Embryo Teratogenesis Assay-Palaemonid (SETAP). Sensitivity to WSF of number 2 fuel oil, fractions of oil biodegradation metabolites and the ability to infect embryos of *P. pugio* indicate the potential usefulness of this bioassay in determining developmental toxicity of single chemicals and chemical mixtures to an estuarine crustacean.

Requirements of any toxicity test include the need for a continuous supply of healthy, unstressed test organisms (Perkins 1972) and for consistent experimental results (e.g., LC50 values). Tatem et al. (1976) found that adult *P. pugio* increased in sensitivity (~35%) to dodecyl sodium sulfate after a two-week holding time in the laboratory. If a sensitivity change is also reflected during embryogenesis, then embryos from cultures may show altered sensitivity to toxicants. This research determined if embryos from cultured adult grass shrimp exhibited consistent sensitivity to a single chemical, ethanol (EtOH).

MATERIALS AND METHODS

Adult male and female grass shrimp were collected using push nets from a single site in Escambia Bay, Pensacola, FL. Collections were made during

the months of Sept. 1995, March 1996, and May 1996. For the Sept 1995 collection, ~ 70 gravid females and ~ 60 males were collected during high tide. Water temperature was 28.3°C and salinity was 20 ppt. For the March 1995 collection, ~ 180 non-gravid females and ~ 150 males were collected during low tide. Water temperature was 16.8°C and salinity was 8 ppt. For the final collection in May 1996, ~ 200 gravid females and ~ 200 males were collected during high tide. Water temperature was 28.1°C and salinity was 3 ppt. Shrimp were placed into coolers filled with site water, transported to the Gulf Ecology Division Laboratory, Gulf Breeze, FL, and identified as *Palaemonetes pugio* (Williams 1984). Approximately 60 female and 40 male shrimp from each of three collections were transferred into flow-through aquaria (80 L) and maintained on a flow rate of ~ 24L per hour, salinity of 19-22 ppt, and temperature of 19-25°C for approximately a six-month period. Protective habitats were not furnished in the aquaria. Under these laboratory conditions, the shrimp were able to reproduce and supply adequate numbers of embryos for experiments described here and elsewhere (Little 1968). Shrimp were fed ~ 2.5 grams of flake food (Tetramin[®]) daily and twice a week with 25 ml of concentrated brine shrimp (*Artemia salina*) nauplii.

Experiments were conducted with EtOH during a 9 month period using embryos from shrimp that had been maintained in aquaria for different periods of time. Experiments performed during this time period are referred to as "culture duration" with 0-d being day of collection. A single gravid female shrimp with a clutch of embryos 3-d (after oviposition) was selected for testing. Embryos were removed from the female, placed in disposable 24-well flat bottom plastic culture plates and individually exposed to 2 ml of ethanol at five different dilutions (0.05, 0.10, 0.50, 1.0 and 2.0% v/v%) based on 12-d LC50 values from Rayburn and Fisher (1996). Dilutions were made with histological grade 100% EtOH and 20 ppt 0.22 μ m filtered natural sea water. Filtered sea water was also used as control. Embryos were placed on rotators (Model G2, New Brunswick Scientific Co.) in an incubator maintained at 27°C \pm 1 for 12 d. Rotators were set at 60 rpm to provide a gentle agitation of the embryos in the test wells. After a 12-d exposure, embryos were examined for mortality. 12-d LC50 values and 95% confidence intervals (CI) were calculated using the trimmed Spearman-Kärber method (Hamilton et al. 1977). Average of mean 12-d LC50 values and coefficients of variation (CV) were calculated according to Steel and Torrie (1980).

RESULTS AND DISCUSSION

Adults that were maintained in the laboratory at densities of approximately 60 female and 40 males per aquaria exhibited a 40-50% mortality rate over a six-month period. This mortality rate may have been due to cannibalism (Perkins 1972) and/or aging (Wood 1967). The placement of protective

habitat within aquaria would most likely reduce this observed mortality.

Of 19 females examined, clutch size ranged from 97 to 325 embryos with an average of 207 embryos per female. With the exception of one experiment (Table 1), females with clutches of 150 embryos or more were used. These results indicate that embryos can be obtained year-round from adults maintained in the laboratory.

Table 1. 12-d LC50 values for grass shrimp embryos exposed for 12 days to histological grade ethanol.

Date of Field Collection	Culture Duration	LC 50 (% v/v)	95% CI
September 1995	30 d	0.91	0.75 - 1.10
	60 d	0.59	0.45 - 0.78
	110 d ^{A B D}	0.20	0.10 - 0.37
	130 d	0.52	0.42 - 0.65
	160 d ^B	0.56	0.45 - 0.70
March 1996	2 d ^{B C}	< 0.05	-----
	15 d	0.57	0.48 - 0.69
	30 d	0.50	0.40 - 0.62
	60 d	0.64	0.56 - 0.73
May 1996	1 d ^D	0.26	0.19 - 0.37
	30 d	0.55	0.42 - 0.72
Average LC50 Value		0.53	
CV		37%	

A = Female with < 150 embryos, B = High control mortality, C = Could not calculate 12-d LC50 value and was excluded. D = Significantly lower 12-d LC50 values based on 95% CI.

Average control mortality was 11.7% (31/264) with a standard error of 3.2%. Of eleven experiments performed (Table 1), only three showed control mortalities above 16.7% (4/24), two in the September collection and one in March. Of these three, two showed greater sensitivity to EtOH.

Both the 1 d and 2 d time in laboratory (i.e., embryos oviposited in the field) experiments indicated that the embryos had 2-10x greater sensitivity to EtOH than did embryos oviposited in the laboratory. The 2 d time in laboratory

experiment was excluded from the average mean 12-d LC50 calculation because Spearman-Kärber was unable to generate a 12-d LC50 value. The average 12-d LC50 value for all experiments was 0.53% with a CV of 37%. The 2 d time in laboratory test was performed because no appropriately aged embryos were available on day 1.

These experiments demonstrated that there was low variability in EtOH toxicity for all three collections as indicated by the overlapping 95% CI and low CV values. With one exception, all experiments conducted after 15 d time in laboratory showed similar 12-d LC50 values as indicated by 95% CI values. Therefore, a two-week acclimation time for maintaining adult grass shrimp in the laboratory is recommended before utilizing embryos. This holding time assures that shrimp will have gone through one complete reproductive cycle molt, fertilization and oviposition in the laboratory. These results indicate that, after a two-week acclimation time, adult grass shrimp can be maintained in the laboratory for at least six months without altering the sensitivity of their embryos.

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REFERENCES

- Chapman PJ, Shelton ME, Grifoll M, Selifonov S (1995) Fossil fuel biodegradation: laboratory studies. *Environ Health Perspect* 103: Supplement 5, 79-83
- Fisher WS, Foss SS (1993) A simple experiment for toxicity of number 2 fuel oil and oil dispersants to embryos of grass shrimp, *Palaemonetes pugio*. *Mar Pollut Bull* 26:385-391
- Genthner FJ, Foss SS, Fisher WS (1994) Testing of the insect pest control fungus *Beauveria bassiana* in grass shrimp *Palaemonetes pugio*. *Dis Aquat Org* 20:49-57
- Hamilton MA, Russo RC, Thurston RV (1977) Trimmed Spearman-Kärber method for estimating median lethal concentrations in toxicity bioassays. *Environ Sci Technol* 11:714-719; correction 12:417 (1978)
- Middaugh DP, Hemmer MJ, Loes EM (1988) Teratological effects of 2,4-dinitrophenol, 'produced water' and naphthalene on embryos of the inland silverside *Menidia beryllina*. *Dis Aquat Org* 4, 53-65
- Little G (1968) Induced winter breeding and larval development in the shrimp, *Palaemonetes pugio* Holthuis (Caridea, Palaemonidae). *Studies on decapod larval development. Crustaceana Suppl* 2, 19-26
- Perkins EJ (1972) Some problems of marine toxicity studies. *Mar Poll Bull* 3:13

- Rayburn JR, Glas PS, Foss SS, Fisher WS (1996) Characterization of grass shrimp (*Palaemonetes pugio*) embryo toxicity tests using the water soluble fraction of number 2 fuel oil. *Mar Pollut Bull* 32:860-866
- Rayburn JR, Fisher WS (In press) The developmental toxicity of three carrier solvents using embryos of the grass shrimp, *Palaemonetes pugio*. *Arch Environ Contam Toxicol*
- Steel GR, Torrie JH (1980) Principles and procedures of statistics: A biometrical approach. McGraw-Hill Book Company, New York.
- Tatem HE, Anderson JW, Neff JM (1976) Seasonal and laboratory variations in the health of grass shrimp *Palaemonetes pugio*: Dodecyl sodium sulfate bioassay. *Bull Environ Contam Toxicol* 16:368-375
- Williams AB (1984) Shrimps, lobsters, and crabs of the Atlantic Coast of the Eastern United States, Maine to Florida. Smithsonian Institution Press, Washington, D.C.
- Wilson JEH (1985) Sublethal effects of diflubenzuron (Dimilin) on the reproduction and photobehavior of the grass shrimp *Palaemonetes pugio* Holthuis (Caridea, Palaemonidae). Ph.D. dissertation, Duke University
- Wood CE (1967) Physioecology of the grass shrimp, *Palaemonetes pugio*, in the Galveston Bay estuarine system. *Contrib Mar Sci (Univ Texas)* 12:54-79