Bull Environ Contam Toxicol (2010) 84:71–75
DOI 10.1007/s00128-009-9909-z

Parental Dietary Effect on Embryological Development Response to Toxicants with the Sea Urchin *Arbacia punctulata*

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Received: 23 February 2009 / Accepted: 6 November 2009 / Published online: 21 November 2009

Abstract The role of echinoid parental nutrition in early-life stage toxicity is not well understood. *Arbacia punctulata* were fed either a fresh diet consisting of organic lettuce and carrots or a dry feed. Embryos from parents fed the dry feed exhibited lower sensitivity to copper, whereas the opposite occurred with 1,3,5-trinitrobenzene and sodium dodecyl sulfate (SDS). EC_{50} values for the dry and fresh feed treatments, respectively, were 41.0 and 29.9 µg/L for copper, 0.5 and 1.8 mg/L for 1,3,5-trinitrobenzene, and 3.5 and 5.6 mg/L for SDS. The data suggests that nutritional standardization for sea urchins in ecotoxicological laboratories needs to be addressed and further investigated.

Keywords Echinoid · Toxicity · Nutrition · Sea urchin

The use of sea urchin embryos in toxicological tests has become accepted internationally for a variety of liquid media including seawater, pore water and elutriates from estuarine and marine sediments, sea surface microlayer, and industrial and domestic effluents (e.g., Nacci et al. 1986; Dinnel et al. 1988; ASTM 1995; USEPA 2002), yet there is lack of standardization in nutritional requirements for laboratory-maintained test organisms.

Differential sensitivity of aquatic organisms to toxicants when fed either different amounts of food or diets of different quality has been documented (e.g., Cripe et al. 1989; Bridges et al. 1997; De Schamphelaere and Janssen 2004). However, potential effects of parental diet on the sensitivity of the offspring to contaminants are poorly understood.

Interestingly, sea urchins in culture tanks are commonly maintained on diets consisting of romaine lettuce and carrots (e.g., USEPA 2002; Carr, RS, USGS, Corpus Christi, TX, personal communication). This diet consisting of two terrestrial plant sources is likely to be suboptimal for meeting parental dietary requirements. Further, the nutrient levels in carrots and lettuce are variable. Carotenoids in the sea urchin, *Lytechinus variegatus*, parental diet have been shown to be essential nutrients for production of viable offspring (George et al. 2001). Feeds specifically formulated for sea urchins have a defined, balanced nutrient content and are expected to represent an adequate nutritional standard to minimize variability in sea urchin research results.

Earlier reports using formulated feeds showed that the parental diet affects gonad development in *L. variegatus* (George et al. 2001), as well as larval development (George et al. 2004). Very recently, several species of sea urchins have been shown to have good gonad development when fed formulated feeds very similar to the one used in this study (Hammer et al. 2006; Schlosser et al. 2006). These reports suggest that a formulated feed would better supplement the nutritional requirements of the sea urchin species selected for use in the current study, *Arbacia punctulata* (Lamarck 1816), compared to a fresh feed comprised of lettuce and carrots. The minimization of variables that might affect the outcome of toxicity tests is

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essential to ensure consistency and confidence in results, and comparability among tests.

The objective of the current research was to determine if the parental nutrition of sea urchins affects the sensitivity of early-life stages of their offspring to a variety of contaminants. This study analyzed if there was a statistically significant difference in the toxicity of three different contaminants (a metal and two organic chemicals, one of which is routinely used as a reference toxicant) to the embryological development of the sea urchin *A. punctulata* when parental adults were fed either a fresh, organic romaine lettuce and carrot diet or a dry, formulated feed.

Materials and Methods

Arbacia punctulata were obtained from Gulf Specimen Company, Inc. (Panacea, FL) and were maintained in the laboratory for 9 months prior to this study. Sea urchins were maintained in recirculating systems with seawater filtered through a 1 μM cartridge filter at 30 ± 1 psu salinity and 16 ± 1°C, and fed romaine lettuce and carrots ad libitum. Three days before feed trials began the sea urchins were completely spawned by gentle electric stimulation from a 12 V transformer to the area adjacent to their gonopores. This was performed to prevent any residual effects from mature gametes produced prior to the initiation of the feeding experiment. Spawned sea urchins (n = 67) were weighed and randomly partitioned into two populations with similar mean wet weights and subdivided by sex: 15 females and 19 males on a fresh diet, and 15 females and 18 males on the formulated dry diet. *A. punctulata* were fed ad libitum three times/week either a fresh, organic feed that consisted of organic romaine lettuce hearts (Newman's Own® or Earthbound Farms®) and organic carrots (Earthbound Farms®), or a dry feed formulated for sea urchins (Table 1). Feces and uneaten food, which was always present, were removed and filters were cleaned at the time of feeding. Fresh seawater was added after siphoning tanks. Organisms were maintained on the fresh and dry feed for 68 days prior to use in embryological development toxicity tests. No differences were observed between the two laboratory populations based on sea urchin survival, appearance, or weight at the end of this period. Temperature was gradually increased to 19 ± 1°C 3 days prior to gamete collection for the tests.

Table 1 Calculated nutrient values of semi-purified formulated feed on an "as fed" basis (Lawrence et al. 2006) and USDA (2008) published nutrient levels of romaine lettuce and carrots

Nutrient	Feed type			Nutrient	Feed type		
	Dry feed	Carrots	Lettuce		Dry feed	Carrots	Lettuce
Crude protein (%)	28	0.93	1.23	Methionine (%)	0.51	0.02	0.02
Carbohydrate (%)	37	9.58	3.29	Tyrosine (%)	1.03	0.04	0.02
Carotenoid (%)	0.72	0.01	0.004	Valine (%)	1.26	0.07	0.06
Sodium (%)	1.30	0.69	0.008	Vitamin E (ppm)	240	6.60	1.30
Iron (ppm)	365	3.00	9.7	Riboflavin (ppm)	48	0.58	0.67
Copper (ppm)	50	0.45	0.48	Niacine (ppm)	99	9.83	3.13
Histidine (%)	0.67	0.04	0.02	Choline (%)	0.10	0.009	0.01
Lysine (%)	1.79	0.10	0.06	Moisture (%)	5.47	88	95
Phenylalanine (%)	1.38	0.06	0.06	Total ash (%)	34	0.97	0.58
Tryptophan (%)	0.28	0.01	0.01	Cholesterol (%)	0.33	0.00	0.00
Vitamin D (IU)	3,000	–	–	Phosphorus (%)	1.96	0.04	0.03
Thiamin (ppm)	36	0.66	0.72	Magnesium (%)	4.60	0.01	0.01
Pantothenic acid (ppm)	36	2.73	0.14	Manganese (ppm)	115	1.43	1.55
Inositol (%)	0.1	–	–	Arginine (%)	2.28	0.09	0.05
Vitamin B12 (ppm)	19	0.00	0.00	Leucine (%)	2.11	0.10	0.08
Lipid (%)	5.98	0.24	0.30	Cystine (%)	0.27	0.08	0.006
Crude fiber (%)	4.18	2.80	2.10	Threonine (%)	1.11	0.19	0.04
Calcium (%)	7.64	0.03	0.03	Vitamin A (IU)	4,800	16,811	5,807
Potassium (%)	1.38	0.32	0.25	Vitamin C (ppm)	349	59	240
Zinc (ppm)	91	2.40	2.30	Pyridoxine (ppm)	96	1.38	0.74
Selenium (ppm)	0.24	0.001	0.004	Biotin (ppm)	1.00	–	–
Isoleucine (%)	1.19	0.08	0.04	Folic Acid (ppm)	24	0.19	1.36

Stock and test solutions for toxicity tests were prepared in dilution water, which consisted of 0.45-μm Millipore® filtered seawater at 30 psu salinity. Reagent grade copper ($CuSO_4$ $5H_2O$) and 1,3,5-trinitrobenzene (TNB) were purchased from Chem Service (660 Tower Lane, West Chester, PA) and 99% purity sodium dodecyl sulfate (SDS) was purchased from Sigma-Aldrich (St. Louis, MO). Cu and TNB stock solutions were stirred on a magnetic stirrer for 24 h, while the SDS stock solution was made just prior to the toxicity test due to its higher solubility and degradability. Stock solutions of Cu and TNB were serially diluted with dilution water, with measured concentrations ranging from 4.3 to 68.9 μg/L and 0.40 to 12.7 mg/L, respectively. The reference toxicant SDS was tested concurrently with the copper and TNB treatments, and served as a positive test control. Based on the control charts of our laboratory, the effective concentration to 50% of the organisms (EC_{50}) values for the sea urchin embryological development test should be between 1.51 and 7.34 mg/L. Therefore, the nominal concentrations of SDS in the present study ranged from 1.25 to 10 mg/L.

Gametes for use in toxicity tests were obtained by mild electric stimulation of sea urchins randomly selected from the tanks given the different feeds. After collection of gametes of apparent good quality from 2 females and 2 males from each diet, a 1:1,250 sperm:dilution water solution was prepared and added to the egg solution. Fertilized eggs were added to the test vials and the embryological development toxicity test was conducted under static conditions. The occurrence of embryo and larvae malformations or arrested development was assessed at the end of the 48-h exposure period. Embryological development was considered successful when echinopluteus larvae with four distinct arms and symmetrical development were observed at the end of the exposure period. Larvae that were asymmetrical, exhibited any sort of malformation, or were at earlier stages of embryological development were considered as departures from normality and, therefore, adversely affected (ASTM 1995).

Copper, TNB and SDS dilution series, as well as a dilution water negative control, were prepared for organisms from each parental diet, with five replicates per treatment. The experiments with the three chemicals using organisms from each diet were done concurrently and placed in the same incubator at 20 ± 1°C for the duration of the exposure. Each replicate consisted of a disposable glass scintillation vial with a Teflon®-lined cap containing 5 mL of sample. Tests were performed at a salinity of 30 ± 1 psu and vials were kept in complete darkness for the 48-h exposure. The test was terminated by the addition of 10% buffered formalin to each vial. The percentage of normally developed embryos to the pluteus larval stage in each test replicate was counted on a compound microscope in a subsample of 100 embryos.

The dilution water control and the stock solution of each contaminant (Cu 69 μg/L; TNB 254 mg/L; SDS 10 mg/L) were analyzed for water quality at the beginning of the embryological development toxicity test by measuring temperature, salinity, DO, total ammonia and pH. The USEPA method 3015 was used to determine accuracy of the Cu stock solution concentration, by measurement with a graphite furnace atomic absorption (GFAA) spectrophotometer after microwave-assisted digestion with nitric acid using a Microwave Accelerated Reaction System (MARS). The stock solution of TNB was analyzed by high-performance liquid chromatography (HPLC), according to USEPA Method 8330. Experimental samples were analyzed against calibration curves using the external standard method. Repetitions and standards were used for quality control and assurance on both analytical instruments.

ToxCalc® 5.0 (Tidepool Scientific Software, McKinleyville, CA) was used for statistical analyses of the toxicity test data. Data were arc sine, square root transformed and the homoscedastic *T*-test was used to compare the results between each treatment with organisms from parents fed dry or fresh feed. When homogeneity of variances was not observed (100% effect in one of the treatments), the heteroscedastic *T*-test was applied instead. EC_{50} values with 95% confidence intervals were also calculated for each contaminant from both diets using the trimmed Spearman–Karber method with Abbott's correction.

Results and Discussion

Differences in sensitivity to toxicants were exhibited by embryos from parents fed fresh and dry feed (Fig. 1). Embryos from sea urchins fed fresh feed were less sensitive to the organic chemicals, TNB and SDS, than those from organisms fed the formulated dry feed in all but the highest concentrations of each toxicant, in which 100% effect was observed with organisms from both feeds. The EC_{50} for these two chemicals was also significantly different between organisms from the two feeds, as shown by the lack of overlap of the 95% confidence intervals (Table 2). Cu-exposed embryos from parents consuming the formulated feed, on the other hand, exhibited a trend towards a larger amount of developed pluteus larvae in the higher copper concentrations, relative to those from parents fed the fresh feed (Fig. 1), with a significantly higher EC_{50} for the former (Table 2), indicating lower sensitivity to copper of the organisms from parents fed the dry feed.

The water quality measurements of the test samples at the beginning of the toxicity test were within the acceptable ranges suggested for the species, with DO between 5.75 and 5.98 mg/L, pH between 8.01 and 8.06, and total ammonia below the detection level (<0.01 mg/L).

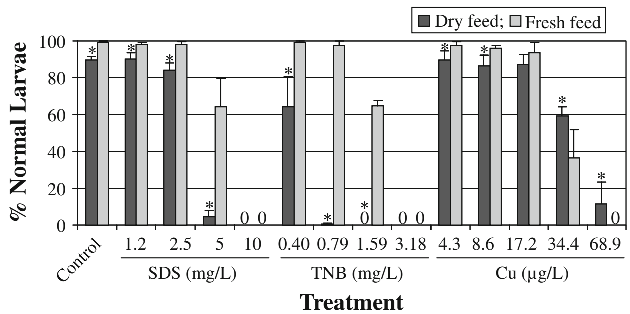

Fig. 1 Percent normal pluteus larvae in each treatment with three different toxicants using gametes from parents fed fresh or dry feed. *Asterisk* indicates significant difference between dry and fresh feed ($\alpha \leq 0.05$)

Table 2 Results of embryological development toxicity tests with *A. punctulata* offspring from parents fed a formulated or fresh diet

Chemical	Feed	
	Dry EC_{50} (95% CI)	Fresh EC_{50} (95% CI)
SDS (mg/L)	3.50 (3.43–3.57)	5.55 (5.39–5.72)
1,3,5-TNB (mg/L)	0.47 (0.45–0.48)	1.75 (1.70–1.81)
Cu (µg/L)	41.05 (39.3–42.9)	29.91 (28.9–31.0)

EC_{50}: Effective concentration to 50% of the organisms; *95% CI*: 95% confidence interval

The different effects of parental nutrition on the response of early-life stages to Cu, TNB and SDS are likely to be related to the levels of nutrients in each feed. The great majority of nutrients exhibit 1 to 2 orders of magnitude higher levels in the dry feed than the fresh feed (Table 1). The lower sensitivity to Cu of embryos from parents fed the dry feed may have been due to higher prior parental exposure to this metal, with 50 ppm Cu in the formulated feed and 0.48 and 0.45 ppm Cu in romaine lettuce and carrots, thus giving the embryos an acclimation advantage. Reproduction rate and/or growth of the cladoceran *Daphnia magna* was enhanced upon exposure to water-borne and dietary cooper, relative to organisms exposed to water-borne copper only (De Schamphelaere and Janssen 2004). EC_{50} values found in the literature for embryological development test with *A. punctulata* exposed to copper range from 14.0 to 44.0 µg/L (Nacci et al. 1986; Carr et al. 1996; USEPA 2006), which encompass the data found in the present study for both feeds (Table 2). None of the cited studies mentioned the parental diets. More relevant than this range, however, is the observed difference in sensitivity of embryos from parents fed different feeds in experiments conducted concurrently under the exact same experimental conditions. The standardization of laboratory sea urchin feed would likely decrease the variability among tests performed by different laboratories.

Nipper et al. (2001) reported *A. punctulata* embryological development effects of TNB, with EC_{50}, NOEC and LOEC values of 1.3, 0.48 and 1.1 mg/L, respectively, using gametes from urchins fed fresh romaine lettuce and carrots. These results are only slightly lower than those of the current study for the same diet, but the formulated feed EC_{50} indicated higher sensitivity of the organisms than in both studies with the fresh diet. The significant difference between the EC_{50} values in the current study is likely to be a result of different parental nutrition levels, since all other factors were identical throughout the adults maintenance period and toxicity tests performance. Effects of nutritional status on the sensitivity to toxicants have been shown for some marine organisms. Survival and growth of the polychaete *Neanthes arenaceodentata* in contaminated sediment were significantly higher in the highest food ration (Bridges et al. 1997). The crustacean *Americamysis bahia* fed sub-optimal amounts of brine shrimp nauplii exhibited higher sensitivity to several organic chemicals in 96-h lethality tests (Cripe et al. 1989).

Kooijman (1991) states that the toxicokinetics of contaminants is dependent on feeding condition, leading to a tight "link between ecotoxicology and energetics." Even though most toxicity values measured in the current study are within the range reported in the scientific literature for the same species and toxicants, the significant differences relative to parental diet are noteworthy and should be taken into consideration when applying sea urchin early-life stage toxicity tests for regulatory purposes. Therefore, it is suggested that it is important to standardize sea urchin feeds in order to allow proper comparisons of temporally separated toxicity data, as well as experiments conducted by different laboratories. Interlaboratory calibrations using early-life stages from urchins fed different diets are recommended for the establishment of a standard diet.

In conclusion, this project has many implications for developmental biology, quality control and marine toxicology. The current results indicate that parental nutrition may affect the sensitivity of echinoid offspring to contaminants. Therefore, the current lack of nutritional standardization for sea urchin diets in ecotoxicological laboratories needs to be addressed to strengthen the validity and reliability of the obtained data for ecological relevance and application, and to allow comparability among studies performed by different laboratories. Ideally, the natural diet and nutritional status of organisms in the field should be analyzed, and a formulated feed with similar nutritional value to the animals' natural diet should be developed, for use of laboratory maintained organisms in toxicity tests.

Acknowledgments Much appreciation and gratitude go to Jeff Barry, Thomas Wahoub, Dr. Mickie Powell, Dr. John Lawrence and Dr. R. Scott Carr. Jim Biedenbach's expertise and guidance in the ecotoxicology laboratory were fundamental to the success of this study.

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