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EFFECTS OF BISPHENOL A ON FECUNDITY, EGG HATCHABILITY, AND JUVENILE GROWTH OF *MARISA CORNUARIETIS*

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Abstract—Recent work on the snail *Marisa cornuarietis* has claimed to show endocrine disruption in response to bisphenol A (BPA). The present experimental design was optimized to detect effects of BPA on fecundity, egg hatchability, and juvenile growth, with an emphasis on reproduction, since previous studies suggested this to be a sensitive endpoint. No differences in eggs/female/month between unexposed snails and snails exposed to nominal concentrations of 0.1, 1, 25, and 640 μg BPA/L during six months of exposure were found. No effect of BPA on the percentage of eggs hatching successfully was found, as was no difference in time to hatching between the control and any BPA treatment. We observed a significant decrease in female growth and a marginal effect on female wet weight in the 640-μg/L treatment compared to the control and a significant increase in male growth rate and a marginal increase in male wet weight in the 1-μg/L treatment compared to the control. However, a much greater proportion of the variability in juvenile growth was explained by variation between pairs and between siblings from the same pair than by BPA treatment. We conclude that effects of BPA in the nominal exposure range 0.1 to 640 μg/L (measured range 0.062–696 μg/L) are unlikely to be of significance for field populations of this species. An additional adult fecundity trial at 22°C (in contrast to all other experiments that were conducted at 25°C) found no evidence to suggest that snails are more sensitive to BPA at the lower temperature, as has been previously claimed. The present results indicate that the sensitivity of *M. cornuarietis* to BPA is similar to that of other aquatic invertebrates for which data are available.

Keywords—Endocrine disruption Life history Mollusk Reproduction Risk assessment

INTRODUCTION

The present study presents the culmination of a multiyear and multilaboratory study designed to quantify the effects of bisphenol A (BPA) on the gastropod *Marisa cornuarietis*. Because *M. cornuarietis* is not a standard test species and relatively little was known about its husbandry requirements, much initial effort was devoted to establishing basic knowledge of its biology under laboratory conditions, during which its performance was tested under different temperature, light, water hardness, water turnover, and feeding regimes. An important feature of this initial work, which involved three independent laboratories, was to quantify sources of intra- and interlaboratory variability in baseline values for key life history traits [1–3]. Once appropriate conditions for maintaining multiple-generation populations of *M. cornuarietis* in the laboratory were established, a preliminary toxicity test with BPA was performed in order to determine the appropriate concentration range of BPA to test as well as to provide guidance on the needed levels of replication for detecting effects of BPA [4].

The reason that such great efforts were put into establishing the effects of BPA on *M. cornuarietis* is that this species was identified during the European Union (EU) risk assessment of BPA [5] as potentially the most sensitive aquatic invertebrate of those tested, although it was acknowledged that there was enough uncertainty in the published results that no predicted no effect concentration could be derived from the available *M. cornuarietis* data. Enhanced egg production and morphological deformities in this species at concentrations as low as 0.015 μg/L had been reported [6–8], though the studies had been heavily criticized for flaws in their design and statistical analyses [9]. Given the importance of *M. cornuarietis* to the outcome of the EU risk assessment, it was thus critical to establish whether the effects reported for this species could be confirmed. This issue became of particular importance when it was determined that the previous studies on *M. cornuarietis* used for the EU risk assessment [6–8] suffered from a number of deficiencies in statistical design and analysis as well as uncertainties in the actual concentrations of BPA to which snails were exposed [4,9].

In the present study we provide the results of a carefully designed and statistically robust study that quantifies the effects of BPA on adult fecundity, egg hatchability, and juvenile growth of *M. cornuarietis*. Our results fail to confirm previous reports and suggest that sensitivity of this species to BPA is similar to that of other aquatic invertebrates that have been studied.

MATERIALS AND METHODS

Culture establishment and conditions

Wild specimens of *M. cornuarietis* were collected from Lake Guajataca, Puerto Rico (see Aufderheide et al. [1], for site details and collection methods) and transported to ABC Laboratory (Columbia, MO, USA), at which the present experiments were performed. The snails were cultured under

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conditions similar to those specified for testing purposes. A 12:12-h light:dark photoperiod with two 30-min transition periods was used with illumination provided by fluorescent lights at an intensity range of 400 to 800 lux. The cultures were set up on a single-pass flow-through system to maintain adequate water quality (i.e., dissolved oxygen, temperature, and pH). Water temperature of cultures was maintained at 25°C. Snails were fed fresh, commercially purchased organically grown romaine lettuce (*Lactuca sativa*, romaine) and commercial algal wafers (supplied by Hikari, Hayward, CA, USA, http://www.hikariusa.com/algae_wafers.htm).

Experimental design

The present study was designed and performed according to the Organization for Economic Cooperation and Development (Paris, France) principles of good laboratory practice [10]. The endpoints we chose for the present study are adult fecundity, egg hatchability and time to hatch, and juvenile growth rate since these endpoints are considered to be the most biologically meaningful, covering the main stages of the life cycle, and they can be combined to give a measure of effect at the population level [11]. In addition, they are less subjective and more amenable to statistical analysis than morphological or histological endpoints. The overall design and level of replication employed were determined on the basis of extensive information on baseline values of the selected endpoints under control conditions [3] and a preliminary BPA-exposure study [4]. The latter was used both to select appropriate exposure concentrations and to provide information for a statistical power analysis on the basis of which levels of replication in the present study were decided.

The adult snails used to initiate the BPA trials were cultured at ABC Laboratories (for approximately five generations). Males were in the size range of 28.5 to 32.6 mm in diameter (mean diameter = 30.1 ± 1.1), and females were in the range of 30.0 to 36.3 mm in diameter (mean diameter = 32.4 ± 1.5 mm). Age at study initiation was 132 d posthatch. Previous work has shown snails in this size/age range to be reproductively active [1]. Snails were impartially allocated among replicate aquaria and chambers.

Each adult replicate consisted of a glass aquarium (approximate base dimensions 60 × 30 cm) containing a working volume of 25 L of test solution. Each replicate was segregated into 10 equal size chambers using perforated glass partitioning and received 10 volume additions of water per day. The water delivery system was split between the 10 chambers so that water addition was similar among the 10 chambers. The test chambers were immersed in a circulating water bath adjusted to maintain the water temperature at 25 ± 1°C, which was measured continuously using a multiscan temperature probe (IOTech, Cleveland, OH, USA) positioned centrally in one of the centrally located replicate aquaria. One breeding pair of *M. cornuarietis* was placed into each chamber, resulting in a density of approximately 0.8 snails/L.

Approximately 2 g of lettuce and one-half of an algal wafer (~0.13 g) were fed to each breeding pair three times per week. Each replicate was cleaned by siphoning feces and uneaten food particles three times per week just prior to the addition of new food. Aquarium walls were wiped once per week, and glass partitions were removed and cleaned individually as needed.

Dilution water and test chemical

The dilution water was prepared by blending naturally hard well water with well water that was demineralized by reverse osmosis. These two freshwater types were blended to yield a total hardness of 130 to 160 mg/L as $CaCO_3$ and a calcium content of approximately 30 mg/L. Calcium chloride was mixed with the dilution water prior to delivery into the diluter system in order to increase the total calcium content to within a range of 60 to 90 mg/L. Prior to the initiation of the studies and at least once during the course of the studies, calcium content was measured from duplicate samples of dilution water collected near the drain of one of the control replicate chambers. The direct calcium content measurements were equated to total hardness values, and test solution hardness was monitored on a weekly basis to demonstrate adequate calcium addition. Calcium analysis was conducted by inductively coupled plasma mass spectroscopy (PerkinElmer ELAN 6000, Wellesley, MA, USA) according to standard methodology (U.S. Environmental Protection Agency method 200.8 [12]). The dissolved oxygen concentrations were kept at ≥60% of saturation in the test solutions throughout the test. Test replicates were aerated to maintain dissolved oxygen concentrations above this level. The test solution temperature, dissolved oxygen, and pH content were measured once per week.

The BPA test concentrations were prepared in the following nominal concentrations: 0 (control), 0.10, 1.0, 25, and 640 μg BPA/L. A modified proportional diluter system [13] was used to intermittently introduce test substance and dilution water into the test chambers. The diluter system was volumetrically calibrated before test initiation and inspected at least twice each day during the tests for function and stock usage, and the metering pumps were checked weekly for delivery volumes. A stock solution was prepared every 7 to 10 d and added to the stock bottle on the diluter daily or as needed. The diluter system was set up to deliver dilution water or test solutions to each test vessel for a minimum equilibration period of 14 d prior to addition of the test organisms.

Analytical confirmation of the test solution BPA concentrations was performed by high-performance liquid chromatography (Agilent 1100; Agilent Technologies, Palo Alto, CA, USA) with ultraviolet fluorescence detection (excitation wavelength = 230 nm; emission wavelength = 280 nm). A Zorbax Eclipse XDB C8 column (Agilent Technologies) (15 cm × 4.6 mm), a mobile phase (Isocratic) of 65:35 methanol:deionized water, a flow rate of 1 ml/min, and an injection volume of 900 μl were used. The method quantitation limit was 0.0600 μg/L for the adult fecundity and hatchability exposures and 0.0593 μg/L for the juvenile growth exposure.

The BPA concentrations were measured twice during the equilibration period, at test initiation, weekly during the adult fecundity and juvenile growth trials, twice a week during the hatchability trials, and at test termination. The analytical samples were composites of equal-volume subsamples collected from each replicate aquarium.

Adult fecundity trial

An adult fecundity trial was performed for a 26-week period. Replicate test vessels with 10 breeding pairs were tested at each test concentration. There were 12 replicate vessels used for the control and six replicate vessels for each of the BPA treatments (i.e., 120 pairs for the control and 60 pairs for each treatment level = 360 adult pairs in total). Chambers were checked three times weekly for the presence of eggs. Both

numbers of egg masses and numbers of eggs per egg mass were recorded. When preparing to collect eggs for hatchability trials (see the following discussion), adults were checked daily so that the exact date of egg deposition used in the hatchability trials could be determined.

To quantify the effect of BPA exposure on egg production, nested analysis of variance (ANOVA) was used [14] in which replicate aquaria were nested within BPA treatments, and the dependent variable was eggs/female/month (averaged for each four-week period for each female over the entire trial; $n = 10$ per replicate aquarium). Pairwise comparisons between the control and each BPA treatment were performed by calculating the mean fecundity for each replicate vessel and then testing the replicate means against the control using a two-sided Dunnett's test (i.e., tests for treatment effects that are both greater than and less than the control).

Egg hatchability trial

A hatchability trial was started two months after the initiation of the adult fecundity trial. Hatchability trials were initiated by the selection of five females from each of six replicate vessels for the control and five females from each of three replicate vessels for each BPA treatment (randomly selected). Selection was made when at least five females produced an egg mass in that replicate over as short a time period as possible, ideally 24 h. If more than five females produced an egg mass in the time period, then a random selection process was used to select the five females.

Five consecutive egg masses were collected from each of the selected females, resulting in 25 egg masses per replicate and 75 egg masses per treatment level (for six of the 180 females the number of clutches was less than five). The number of eggs was counted for each of these egg masses. Each egg mass was placed separately in a glass and nylon mesh basket submerged in the test solutions. The time, in days, to first hatch and approximate time to 50% hatch were recorded. Viable eggs were defined as those eggs that appeared intact with a noncloudy appearance and with a visible actively developing snail within the egg. Egg masses were observed for up to two weeks if viability was low or if it was difficult to distinguish viable versus nonviable eggs.

Effects of BPA on egg hatchability were tested by calculating average percent hatch for each mother (mean of five egg masses), transforming the data to odds ratios (logit transformation, following Sokal and Rohlf [14]). The logit transform Y of the hatching frequency was calculated as

$$Y = \ln[(H + 0.5)/(n + 0.5 - H)],$$

where H is the number of hatched eggs of a clutch of size n. This transformation was applied to each of the clutches. The transformed data were analyzed using a nested ANOVA, with replicate aquaria nested within treatments. Nested ANOVA was also used to test for BPA effects on the mean day of first and 50% hatch. Since some clutches never reached 50% hatch, the data for this endpoint were transformed using the inverse transformation (which transforms an infinite value to a limited value, i.e., 0). Pairwise comparisons between the control and each BPA treatment were performed by calculating the mean (percent hatch, day to first hatch, or day to 50% hatch) for each replicate vessel and then testing the replicate means against the control using a two-sided Dunnett's test.

Juvenile growth trial

A juvenile growth trial was performed for an exposure period of three months. The growth trial was initiated by collecting clutches from each of five females per replicate vessel (six replicate vessels for the control and three from each BPA treatment). The clutches were transferred to hatching baskets as above. At 32 d posthatch, five juvenile snails were selected from the clutches, blotted dry, individually identified by numbering the shell using a fine-point permanent marker, and weighed [3]. For one of the breeding pairs in the 1-μg BPA/L treatment, no young were available, and for this replicate, six young were selected from the remaining four breeding pairs. Subsequently, the juvenile snails (five from each of the five breeding pairs per replicate vessel) were placed in an aquarium measuring 78 × 22 × 30 cm (length × width × height) with a test solution depth of 19 cm, yielding a working volume of 32 L (at a density of 0.8 snails/L). Snail wet weights were measured weekly to the nearest 0.1 mg on a laboratory balance after gently blotting excess water from snails. At the end of the trial, all snails were sexed, and growth rates were estimated for males and females separately.

Juvenile growth rates (g/d) were calculated by fitting a third-degree polynomial of the form $y = c_1x^3 + c_2x^2 + c_3x + c_4$, where c represents a constant, to the data set for each individual (i.e., using nine wet-weight measurements per individual) and calculating the growth rate from the slope of the curve at 60 d posthatch (i.e., midway during the trial) from the equations. Growth rate and wet weight on day 60 posthatch, both estimated from the equation for the growth curve, were compared by nested ANOVA (with replicate aquaria and breeding pairs nested within treatments). Residual variance estimates and the degrees of freedom were corrected for the difference in the number of observations per replicate and breeding pair using the method described by Satterthwaite [15]. Pairwise comparisons between the control and each BPA treatment were performed by calculating the mean growth and wet weight for each replicate vessel and then testing the replicate means against the control using a two-sided Dunnett's test.

Additional adult fecundity trial at 22°C

An additional adult fecundity trial was performed at 22°C over a period of 12 weeks using a control treatment and a single BPA exposure treatment of 25 μg/L with four replicate test vessels each for the control and the BPA treatment. Adult snails were allowed to acclimate to the test temperature for approximately 30 d prior to exposure. All other details are as described previously for the 25°C trial.

RESULTS

Analytical chemistry

In general, measured concentrations of BPA were close to nominal concentrations (Table 1). Mean measured concentrations of BPA ranged from 74 to 135% of the nominal concentrations in the adult fecundity trial, from 69 to 159% in the egg hatchability trial, and from 62 to 67% in the juvenile growth trial. Given the ready biodegradability of BPA [16], the adherence of the measured concentrations to the nominal concentrations is remarkably close and exceeds that in other published studies of BPA and *M. cornuarietis* [8].

Table 1. Measured concentrations of bisphenol A during the adult fecundity, egg hatchability, and juvenile growth trials with *Marisa cornuarietis*. The method quantitation limit was 0.0600 μg/L for the adult fecundity and hatchability exposures and 0.0593 for the juvenile growth exposure. Sample size = *n*, and SD = standard deviation

	Nominal treatment concentrations			
Trial	0.10 μg/L	1.0 μg/L	25 μg/L	640 μg/L
Adult fecundity				
Mean (*n*, SD)	0.135 (27, 0.093)	0.91 (27, 0.188)	18.5 (27, 3.29)	554 (27, 141.2)
% of nominal	135	91	74	87
Egg hatchability				
Mean (*n*, SD)	0.159 (15, 0.073)	0.99 (15, 0.126)	17.3 (15, 6.66)	696 (15, 82.4)
% of nominal	159	99	69	109
Juvenile growth				
Mean (*n*, SD)	0.062 (15, 0.089)	0.66 (16, 0.085)	15.5 (16, 2.36)	429 (16, 66.2)
% of nominal	62	66	62	67

Adult fecundity trial

One male snail was found dead in a single chamber of one of the control replicates, and one female snail was found dead in a single chamber of one of the 0.1-μg/L replicates after approximately four months of exposure. These were the only mortalities during the adult fecundity trial. The female partner of the dead control male snail continued to produce clutches for the remaining two months of the study. No significant effect of BPA on adult egg production was observed at any of the tested concentrations ($p = 0.21$) (Fig. 1); however, there was a significant difference among replicates within treatments ($p = 0.003$). The two-sided Dunnett's test did not detect any significant differences for any BPA treatment compared to the control.

Snails produced an average of 643 (standard deviation [SD] = 172.8) eggs/female/month (averaged over all treatments and pairs) (Table 2). The contribution of BPA concentration to the total variance in egg production was estimated by treating the effect of BPA levels as a random factor, just as the replicate effect. This analysis indicated that only 1.6% of the total variance in egg production could be attributed to BPA treatment, whereas 8.2% was due to differences among replicate tanks, and 90.2% was due to variability among breeding pairs. The within-replicate (aquarium) coefficients of variation for this endpoint provide an estimate of the size of among breeding pair variability. These ranged from 10 to 45% and averaged 25%.

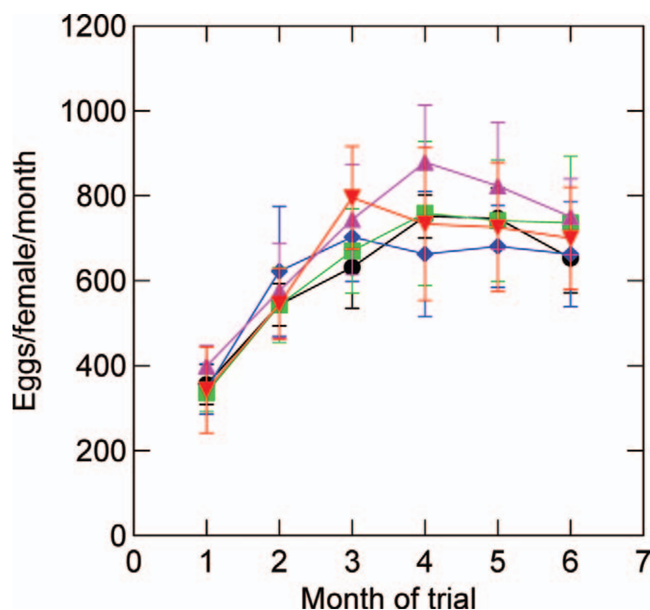


Fig. 1. Adult fecundity trial. Average reproductive output plotted against month of the trial for the different treatment groups ($n = 120$ breeding pairs for control; $n = 60$ breeding pairs for bisphenol A treatment groups). Black circles = control; blue diamonds = 0.1 μg/L; green squares = 1.0 μg/L; pink triangles = 25 μg/L; red inverse triangles = 640 μg/L. Error bars represent 95% confidence intervals around treatment means.

Egg hatchability trial

Clutch sizes in the hatchability trial ranged from 15 to 280 eggs/clutch (overall mean 97.3). Percent hatch varied from 5 to 100% (overall mean 93.7). No effect of BPA exposure on percent hatch was observed ($p = 0.40$) (Fig. 2), as was no significant difference in percent hatch among replicate aquaria within treatments ($p = 0.14$). The Dunnett's test did not detect any significant differences in mean percent hatch between the control and any of the BPA treatments. Analysis of the contribution of BPA concentration to the total variance in hatchability traits indicated that approximately 1% of the total variance in percent hatch was due to BPA treatment, 9% was due to differences among replicate tanks, and 90% was due to differences among breeding pairs.

Time to first hatch varied from 8 to 13 d. The BPA exposure had a significant effect on time to first hatch ($p = 0.008$) as did replicate ($p = 0.048$) (Fig. 3a). A two-sided Dunnett's test did not show any significant differences between any of the treatments and the control. A Tukey's test (which compares all pairs against each other) found that the 0.1-μg/L treatment had a significantly shorter hatching time than the 640-μg/L treatment. Approximately 29% of the variance in time to first

Table 2. Eggs and clutches produced during the adult fecundity trial with *Marisa cornuarietis*. One month = four weeks, and SD = standard deviation. It should be noted that there were twice as many replicates for the control compared to the bisphenol A-exposed groups

Treatment (μg/L)	Total clutches	Total eggs	Mean eggs per clutch	Eggs/ female/ month (mean, SD)	Clutches/ female/ month (mean, SD)
0	5,254	485,379	92	622 (165)	6.7 (1.43)
0.1	2,546	240,782	95	617 (161)	6.5 (1.39)
1.0	2,635	251,400	95	645 (173)	6.8 (1.39)
25	2,816	274,672	98	704 (195)	7.2 (1.46)
640	2,745	252,661	92	648 (185)	7.0 (1.68)

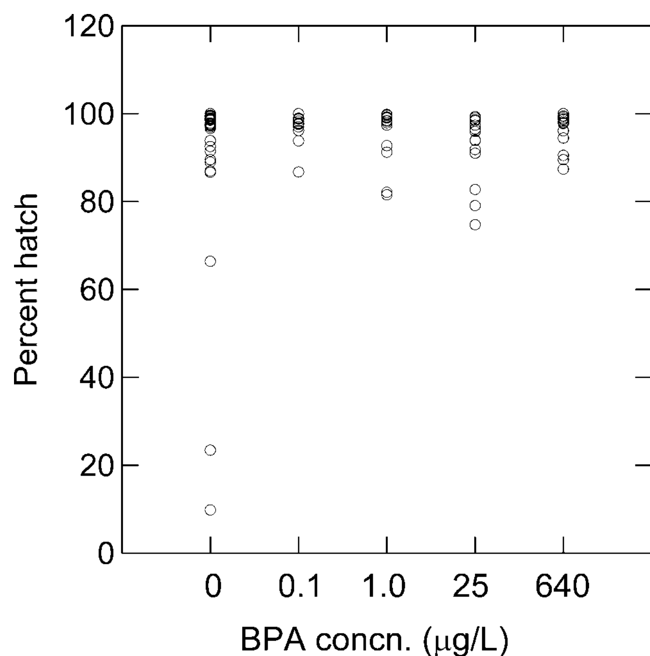


Fig. 2. Egg hatchability trial. Circles represent individual values (i.e., the average hatchability of five clutches for each female) for each treatment, not separated into replicates (n = 30 clutches for control; n = 15 clutches for bisphenol A [BPA] treatments).

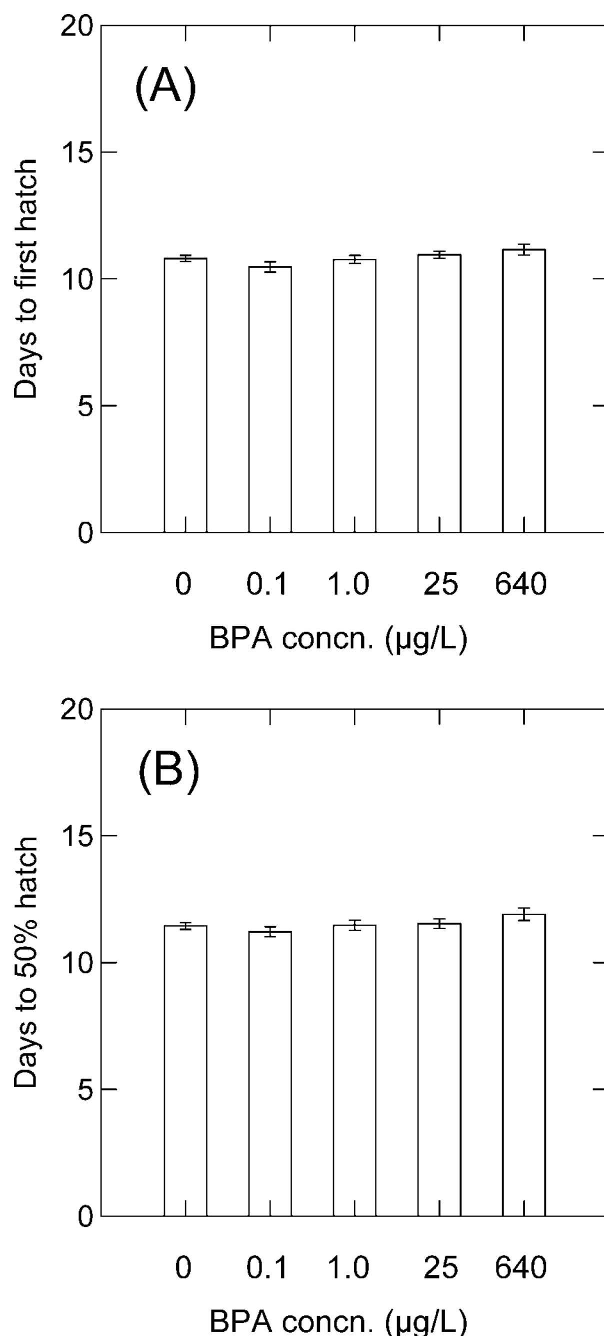


Fig. 3. Egg hatchability trial. Bars represent overall averages for each treatment, not separated by replicates (n = 30 clutches for control; n = 15 clutches for bisphenol A [BPA] treatments). Error bars represent 95% confidence intervals around treatment means. (**A**) Time to first hatch. (**B**) Time to 50% hatch.

hatch was due to BPA treatment, 11% was due to differences among replicate tanks, and 60% was due to variance among breeding pairs.

No significant effect of BPA exposure (p = 0.46) or replicate (p = 0.45) on time to 50% hatch was observed (Fig. 3b). The amount of variance attributed to BPA treatment and replicate together was less than the amount of variance attributed to the replicate alone, and therefore the amount of variance due to BPA treatment is estimated to be 0%. Approximately 0.3% of the variance could be attributed to variance among replicates; the remainder was due to differences among breeding pairs.

Juvenile growth trial

Of the 449 juveniles tested in this trial (~25 juveniles per replicate), all survived until the end of the trial, giving a survivorship of 100% in all treatments. Snails increased rapidly in size in the control and all BPA treatments. Wet weights as a function of age in the different treatments are shown in Figure 4; growth rates (g/d) and estimated wet weights at 60 d posthatch (i.e., halfway through the trial) are shown in Figure 5. Results of the nested ANOVA (with BPA concentration as a fixed effect and the replicates and breeding pairs as random effect factors) found significant effects of BPA on female growth (p = 0.013), female wet weight at 60 d posthatch (p = 0.042), and male growth rate (p = 0.024) and marginally significant effects on male wet weight at 60 d posthatch (p = 0.054). Replicate had a significant effect on male growth (p = 0.014) and wet weight (p = 0.038) and a marginally significant effect on female growth (p = 0.081) and wet weight (p = 0.090). However, by far the most significant effect on juvenile growth rate (for all endpoints) was due to differences among breeding pairs ($p \leq 0.00002$ for all endpoints; Table 3).

Two-sided Dunnett's tests, comparing replicate means between each of the BPA concentrations with the control for each of the four growth endpoints individually, found a significant decrease in female growth (p = 0.045) and a marginal effect on female wet weight (p = 0.053) in the 640-μg/L treatment compared to the control (Fig. 5). In addition, a significant increase in male growth rate (p = 0.045) and a marginal increase in wet weight (p = 0.083) were found in the 1-μg/L treatment compared to the control. The contribution of BPA treatment, replicate, breeding pair, and interjuvenile variability to the total variance in juvenile growth rate and estimated wet weight at 60 d posthatch is summarized in Table 4. From 15 to 21% of the variance in the four growth endpoints was due to BPA treatment, 8 to 13% was due to differences among replicate tanks, 24 to 49% was due to variability among

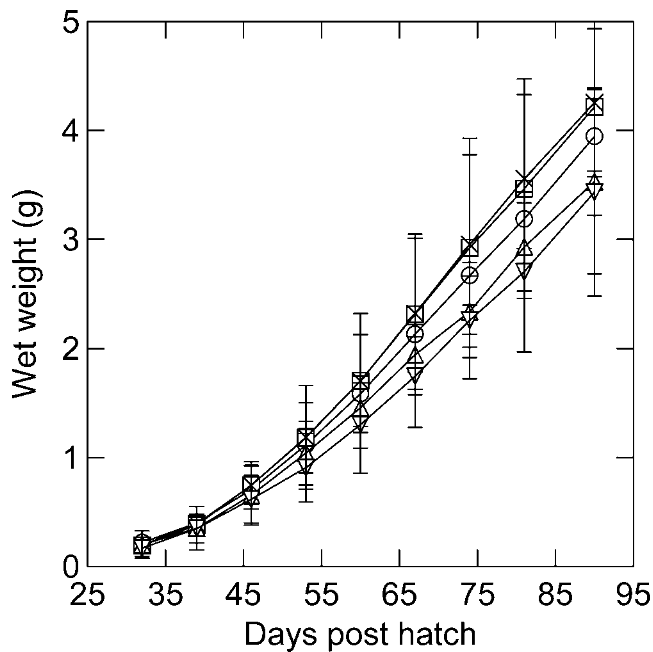


Fig. 4. Juvenile growth trial. Symbols represent mean wet weights for each treatment as a function of snail age. Error bars represent 95% confidence intervals around treatment means. The symbols represent the different bisphenol A treatments: ○ = control; × = 0.1 μg/L; □ = 1.0 μg/L; △ = 25 μg/L; ▽ = 640 μg/L.

breeding pairs, and 27 to 42% was due to variability among siblings from the same breeding pair (i.e., residual or error variance). Thus, the variability in growth associated with BPA treatment was less than the variability associated with either breeding pair or interjuvenile variability.

Additional adult fecundity trial at 22°C

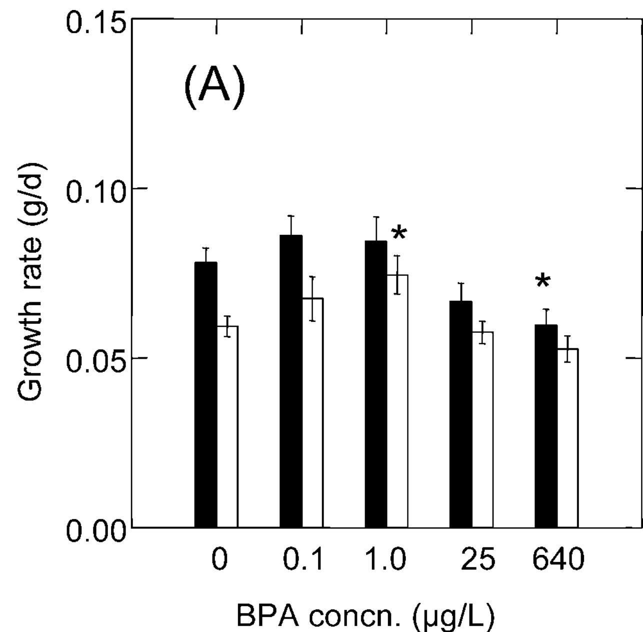
One hundred percent survival of adult snails was observed in all treatments during the course of the 12-week trial. No significant effect of exposure to 25 μg/L BPA on adult egg production was observed ($p = 0.22$), nor was there a significant difference among replicates within treatments ($p = 0.22$). Snails produced an average of 612 (SD = 109.9) eggs/female/month (averaged over both treatments and all pairs). The contribution of BPA concentration to the total variance in egg production was estimated by treating the effect of BPA levels as a random factor, just as the replicate effect. This analysis indicated that only 2.9% of the total variance in egg production could be attributed to BPA treatment, whereas 3.9% was due to differences among replicate tanks, and 93.2% was due to variability among breeding pairs. The within-replicate (aquarium) coefficients of variation for this endpoint provide an estimate of the size of the among breeding pair variability. These ranged from 12 to 28% and averaged 16%.

Table 5 compares egg production rates in the control and 25-μg/L treatment at 25 and 22°C. The table indicates that the percent difference in fecundity between control and the BPA-exposed snails was twice as high (though still not statistically significant) at the higher compared to the lower temperature (13 vs 7%). The three-degree difference in temperature resulted in a difference in fecundity of 5 and 12% in the control and BPA treatment, respectively.

DISCUSSION

Interpreting the results of toxicity tests for use in environmental risk assessment requires a thorough understanding of the biology and ecology of the test species, sufficient knowledge of baseline values for key test endpoints as well as the sources and degree of intra- and interlaboratory variability in them, and the ability to extrapolate the responses measured in the toxicity tests to likely impacts to populations in the field.

The present study represents the culmination of an extensive research program that was designed to eliminate uncertainties in previously published results of the effects of BPA



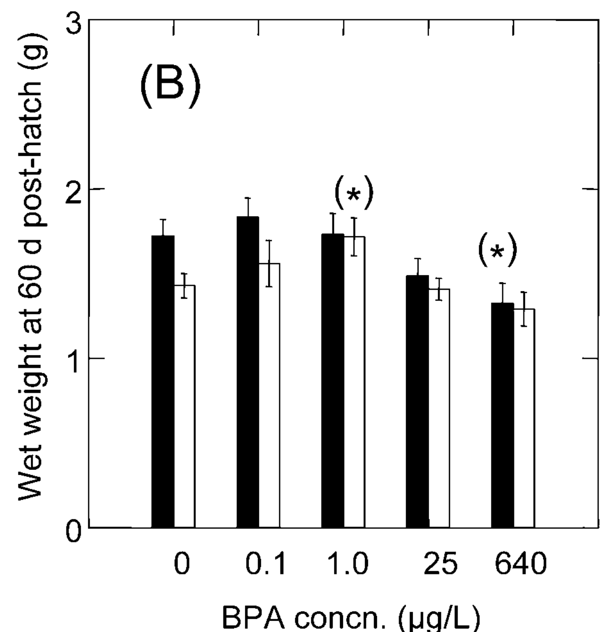


Fig. 5. Juvenile growth trial. Bars represent overall averages for each treatment, not separated into replicates (n = ~75 males and 75 females for control; n = ~37 males and 37 females for bisphenol A [BPA] treatments). Error bars represent 95% confidence intervals around treatment means. (**A**) Growth rate of juveniles estimated at 60 d post-hatch. (**B**) Wet weight of juveniles estimated at 60 d posthatch. Filled bars are females and open bars are males; * indicates significant difference from control; (*) indicates marginally significant difference from control.

Table 3. Juvenile growth trial. The *p* values for the test of the hypothesis that breeding pairs, replicates, and treatment did not affect the wet weight and growth rate for males and females

p Value for test of no effect of:	Males on 60 d posthatch		Females on 60 d posthatch	
	Wet weight (mg)	Growth rate (mg/d)	Wet weight (mg)	Growth rate (mg/d)
Treatment	0.054	0.024	0.042	0.013
Replicate	0.038	0.014	0.09	0.081
Breeding pair	6E−08	0.000019	<1E−11	6E−11

Table 4. Percent of total variance in growth endpoints explained by bisphenol A (BPA) treatment, replicate vessel, breeding pair, and interjuvenile variability

% Variance explained by:	Males		Females	
	Wet weight at 60 d posthatch	Growth rate	Wet weight at 60 d posthatch	Growth rate
BPA treatment	15	21	15	21
Replicate	12	13	9	8
Breeding pair	34	24	49	35
Intersnail variability	39	42	27	37

on *M. cornuarietis* [6,7]. Results of those studies indicated a much greater sensitivity to BPA by *M. cornuarietis* when compared to all other tested aquatic invertebrates, which was critical in deriving a predicted no-effect concentration for aquatic species for use in the European risk assessment of BPA. Chronic no-observed-effect concentrations (NOECs) for BPA for a range of invertebrate species have been compiled by Staples et al. [17] and are in the range 42 (*Hydra vulgaris*) to 3,160 μg/L (*Daphnia magna*). In contrast, Oehlmann et al. [8] reported chronic NOEC values as low as 0.0079 μg/L for increased egg and clutch production in *M. cornuarietis* exposed at 22°C; 10% effective concentration values of 0.015 μg/L (egg production) and 0.018 μg/L (clutch production) for snails exposed at 20°C, and 10% effective concentration values of 1 μg/L (egg production) and 2.1 μg/L (clutch production) for snails exposed at 27°C.

The present research program was initiated on the basis of recommendations from a multistakeholder expert group (including EU regulators, industry, and snail experts) set up by the BPA risk assessment rapporteur (UK). The expert group identified a series of tasks for further study that included identifying an appropriate source of *M. cornuarietis* to develop breeding cultures suitable for ecotoxicological testing; research characterizing the influence of water quality parameters (pH, dissolved oxygen, water hardness, water flow rate), seasonally varying factors (light cycle and temperature), snail density, diet, and feeding regime on the performance of snails under controlled conditions; research characterizing the reproductive cycle of *M. cornuarietis* under constant laboratory conditions; and identifying appropriate endpoints to be assessed in toxicity tests of the effects of BPA on *M. cornuarietis*.

Aufderheide et al. [1] and Selck et al. [2] provide extensive information on the biology and ecology of *M. cornuarietis* and address issues related to interlaboratory variability in snail performance. Forbes et al. [3] provide an analysis of the sources and degrees of intralaboratory variability in key test endpoints, whereas Forbes et al. [4] present the results of a preliminary toxicity test with BPA that were used to inform and improve the design of the present study.

The results of the present study support preliminary tests performed by ABC Laboratory [3,4] and show good intralaboratory repeatability. Forbes et al. [4] observed no effects of BPA exposure on reproductive output of *M. cornuarietis* during a 12-week exposure period at 25°C to concentrations in the range 0.1 to 640 μg/L. Average egg production rates (560 ± 255 eggs/female/month; averaged over all pairs and treatments) measured in the previous study were similar to those measured here (643 ± 173) and were markedly higher than those observed by Oehlmann et al. (peak rates were ~30–180 eggs/female/month [6,8]). The additional trial at 22°C showed no evidence that snails were more sensitive to BPA at the lower temperature as previously claimed [8]. After a temperature acclimation period of one month, reproductive output (eggs/female/month) was 5 to 12% lower at 22 than at 25°C. Average eggs/female/month was 13% higher in the 25-μg/L BPA treatment compared to the control at 25°C and 7% higher at 22°C, although neither difference was statistically significant. As in our previous studies, we observed substantial intersnail variability in this endpoint. Statistical power analysis using actual variances from the present results increased slightly the power estimated from the preliminary test [4]. Thus, despite the large intersnail variability in reproductive output, our design was such that we would have been able to detect a difference (increase or decrease) in egg production of approximately 22% or more with a power of 80% and a type I error rate of 5%.

We observed no effects of BPA exposure on egg hatchability, and clutches in all treatments showed high hatchabilities that averaged over 90%, similar to Forbes et al. [4]. Eggs within and between clutches hatched nearly synchronously with approximately a day between first and 50% hatch. No difference was observed between time to first or 50% hatch between the control and any of the BPA treatments.

In contrast to Forbes et al. [4], we detected significant effects of BPA on juvenile growth, with males showing higher growth rates and wet weights in the 1-μg/L treatment compared to the control and females showing lower growth rates and wet weights in the 640-μg/L treatment compared to the control (no other treatment levels showed differences compared to controls for either endpoint or sex). In Forbes et al. [4], growth effects were analyzed for male and female snails together. If the data from the present study are analyzed in the same manner (i.e., sexes combined), the effect of BPA on juvenile growth is not significant ($p = 0.12$ for growth rate and $p = 0.14$ for wet weight at 60 d posthatch). If we reanalyze the results from Forbes et al. [4] separately for males and females, as in the present study, we find that BPA exposure had a significant effect on male wet weight at 60 d posthatch ($p = 0.023$) but no effect on male growth rate ($p = 0.15$), female wet weight at 60 d posthatch ($p = 0.74$), or female growth rate ($p = 0.19$). A two-sided Dunnett's test found no significant difference in male wet weight at 60 d posthatch between the control and any of the BPA treatments. The lowest male wet weight was obtained in the 160-μg/L treatment, whereas the highest male wet weight was obtained in the 0.1-μg/L treatment. Thus, we observed a low repeatability of the BPA effect on juveniles between studies, and, despite the fact that the BPA effect on juvenile growth was statistically significant in the present study, the percent of the total variance in growth associated with BPA treatment was less than the

Table 5. Comparison of fecundity (eggs/female/month) in control and 25-μg/L bisphenol A (BPA) treatments at 25 and 22°C. Values are shown as means of replicates where sample size = n and SD = standard deviation. The far right column indicates the BPA effect at each temperature [100 × (BPA − Control)/Control], whereas the bottom row indicates the temperature effect in each of the two treatments [100 × (25 − 22°C)/22°C]

	Control	25 μg/L BPA	% Difference
25°C	622 (SD = 75.4, n = 12)	704 (SD = 54.7, n = 6)	+13.2%
22°C	592 (SD = 43.4, n = 4)	631 (SD = 37.0, n = 4)	+6.6%
% Difference	+5.1%	+11.6%	

percent variance associated with either breeding pair or interjuvenile variability. Therefore, due to both the low repeatability of the observed BPA effects on juvenile growth and the high variability in growth among experiments, among breeding pairs, and among offspring from the same breeding pair, we do not feel that this endpoint is sufficiently robust for use in risk assessment.

Summarizing the BPA effects on the test endpoints suggests a NOEC of ≥640 μg/L for reproduction, egg hatchability, and egg development rates, which is consistent with a preliminary study [4]. Effects on juvenile growth are less conclusive (suggesting a NOEC of 25 μg/L for negative effects on female growth and 0.1 μg/L for positive effects on male growth). As described previously, the effects on juvenile growth observed in the present study are not consistent with previous work [4] and would require further confirmation before they could be used in risk assessment.

Extrapolation of the results of laboratory toxicity tests on individual-level endpoints to effects on populations in the field is one of the greatest challenges in ecotoxicology [18]. Forbes et al. [11,19] showed that effects of toxicants at the population level are likely less than or equal to effects on the most sensitive individual life cycle traits and that risk assessments based on the latter are likely to be protective of effects on population-level impacts. Reproduction has shown to be relatively sensitive to toxicant exposure in many invertebrate species [11]. However, because population growth rate has been shown to be relatively insensitive to changes in reproductive output across a wide range of taxa from plants to large vertebrates [20], impacts of chemicals (and other environmental variables) on reproduction do not necessarily result in effects on population growth [21]. When effects on population growth do occur, they are often less than expected on the basis of changes in reproductive output [21–23]. Thus, it is unlikely that potential effects of BPA on reproduction of *M. cornuarietis* less than those detectable in the present study (i.e., <22%) would be of relevance for the population dynamics of this species. Achieving a more realistic extrapolation of laboratory toxicity test results to likely impacts on field populations requires the application of population models that take into account all the relevant biological and environmental variables that influence a population's responses to chemicals. However, more work is needed before such models become of widespread use in environmental risk assessment protocols.

CONCLUSIONS

We conclude that effects of BPA in the exposure range of 0.1 to 640 μg/L on *M. cornuarietis* are minimal and unlikely to be of significance for field populations of this species. Our results indicate that the sensitivity of *M. cornuarietis* to BPA is similar to other aquatic invertebrates for which results are available and do not support previous reports of a much greater sensitivity of this species. Because the study (and the research program on which it is a part) is based on thorough knowledge of the husbandry requirements of this species, understanding of baseline variability in key test endpoints, and a statistically sound design and analysis, it provides robust and scientifically sound data for use in risk assessment.

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