

CHRONIC TOXICITY OF THE SYNTHETIC HORMONE 17 α -ETHINYLESTRADIOL TO
CHIRONOMUS TENTANS AND *HYALELLA AZTECA*ÈVE B. DUSSAULT,^{†‡} VIMAL K. BALAKRISHNAN,[‡] KEITH R. SOLOMON,[†] and PAUL K. SIBLEY*[†][†]Department of Environmental Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada[‡]Aquatic Ecosystem Protection Research Division, Environment Canada, 867 Lakeshore Road, Burlington, Ontario L7R 4A6, Canada

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Abstract—The chronic toxicity of the synthetic hormone 17 α -ethinylestradiol (EE2) was investigated in two benthic invertebrates, the midge *Chironomus tentans* and the freshwater amphipod *Hyalella azteca*, in life-cycle water-only assays. In *C. tentans*, a 50% decrease in emergence was observed at a concentration of 1.5 mg/L; emergence was a more sensitive endpoint than survival, growth, or biomass. Reproduction was not significantly affected by EE2 exposure until a concentration of 3.1 mg/L, where emergence, and therefore reproduction, did not occur. In contrast, reproduction was the most sensitive endpoint in *H. azteca* (50% decrease in reproduction observed at a concentration of 0.36 mg/L). The sensitivity of the F1 generation to EE2 was also investigated with *H. azteca*, but was not different from the F0 generation. The data from the present study were combined with those from previous 10-d toxicity assays, to derive acute to chronic toxicity ratios (ACRs) for EE2. The ACRs calculated for EE2 were 13 for *C. tentans* and 16 for *H. azteca*, indicating that the application factors currently used in ecological risk assessment for the derivation of chronic toxicity are protective and conservative for these organisms. The results of the present study suggest that chronic toxicity was not mediated by disruption of endocrine pathways. Using a hazard quotient approach, the risk associated with sublethal exposure to EE2 was $\ll 1$ for *H. azteca* and *C. tentans*, indicating that adverse effects are not expected, and that environmental exposure to EE2 likely poses a low risk to benthic invertebrates.

Keywords—17 α -Ethinylestradiol Chronic toxicity Benthic invertebrates *Chironomus tentans* *Hyalella azteca*

INTRODUCTION

Concerns about environmental exposure to anthropogenic chemicals capable of interfering with reproduction and development have existed for several years. In the early 1990s, a number of studies reported incidences of hormonal imbalance leading to sexual disruption in fish exposed to sewage effluents [1–3]. Two classes of contaminants, (1) degradation products arising from the alkylphenol polyethoxylates class of nonionic surfactants and (2) the steroidal hormones 17 β -estradiol, estrone, and 17 α -ethinylestradiol (EE2), were identified as probable causative agents. These compounds have since come under increased scrutiny due to their frequent detection in sewage effluents and their demonstrated capacity to disrupt sexual function in fish [2–5].

Natural and synthetic estrogens, whose primary role is to regulate the sexual function of vertebrates, exhibit high biological activities and have been detected at environmental concentrations elevated enough to explain the observed sexual disruption of wild and caged fish downstream of sewage treatment facilities [4,5]. Thus, the potential environmental effects of the synthetic hormone 17 α -ethinylestradiol, used in family planning and hormone replacement therapy, have been the subject of numerous investigations. Studies have shown that exposure to low concentrations of EE2 induced the production of the egg yolk precursor vitellogenin in male and juvenile fish and caused increased incidence of intersex and reduced fertilization success as well as feminization of male fish [6–8]. Recently, it has been demonstrated that chronic sublethal exposure to EE2 at an environmentally relevant concentration caused the collapse of a fish population [9].

The potential effects of EE2 on vertebrates such as fish have been the subject of extensive investigations, because the endocrine physiology of these organisms is comparable to that of humans and hence may exhibit similar responses. Far fewer studies have investigated effects of EE2 on invertebrates, in part because knowledge of basic invertebrate endocrinology is less understood, but also because sexual maturation and function in most invertebrates is mediated by hormonal systems that differ from vertebrates [10]. Based on their structural dissimilarity and different regulatory systems compared to vertebrate hormones, most invertebrates, especially ecdysozoans, in which unambiguous evidence of sex steroid receptors has not been reported [11], would not be expected to respond with the same sensitivity. For example, sex hormones are not involved in the development and differentiation of secondary sex characteristics of insects, which are determined genetically [12], and sexual differentiation in crustaceans is mediated via the androgenic hormone [10]. However, several invertebrate hormonal functions are under the control of other steroidal hormones (ecdysteroids, for example) that display structures similar to those of estrogens, and it has been hypothesized that their function could be affected by chronic exposure to EE2. In fact, a number of studies have suggested that this synthetic estrogen may be capable of disrupting invertebrate hormonal systems [13–15].

In a previous study, we investigated the effects of EE2 on the survival and growth of two benthic invertebrates, the midge *Chironomus tentans* and the freshwater amphipod *Hyalella azteca*, in 10-d assays [16]. The calculated median effects concentrations for these relatively short-term exposures ranged from 1.1 to 6.6 mg/L, indicating negligible risks to these benthic invertebrates in comparison with measured environmental concentrations (0.001–0.273 μ g/L) [17,18]. However, few

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studies have investigated the response of aquatic invertebrates to EE2 over long-term, chronic exposures, which may be more appropriate for assessing sublethal effects (for example, endocrine disruption). Since basic knowledge of invertebrate endocrinology remains largely unknown, the use of longer-term exposures that integrate effects across some or all life stages to investigate sublethal effects of known vertebrate endocrine disruptors has been recommended for determining potential risk [10]. In the present study, we investigated the chronic toxicity of the synthetic hormone 17 α -ethinylestradiol in the midge *C. tentans* (now *C. dilutus*) and the freshwater amphipod *H. azteca*, in two life-cycle assays. Survival, growth, emergence (*C. tentans*), and reproduction were assessed, to quantify the potential effect of EE2 on these organisms. Acute to chronic toxicity ratios were calculated for EE2 to compare with the uncertainty factors currently used in environmental risk assessments and to verify the suitability of current approaches used to derive chronic toxicity values. Finally, the data from the present study were combined with environmental exposure data from the literature, using a hazard quotient approach, to assess the environmental risk posed by EE2 to benthic invertebrates.

MATERIALS AND METHODS

Chemicals

Analytical grade methanol (MeOH) and ammonium carbonate were obtained from Caledon (Georgetown, ON, Canada), 17- α -ethinylestradiol ($\geq 98\%$) and isotopically labelled $^2\text{H}_2$ -17 β -estradiol (atomic purity $>98\%$) were acquired from Sigma-Aldrich (Oakville, ON, Canada), formaldehyde (37% wt) and glacial acetic acid were purchased from Fisher Scientific (Whitby, ON, Canada). Ethanol was obtained from Commercial Alcohols (Brampton, ON, Canada).

Chironomus tentans life cycle test

The chronic effects of EE2 were investigated in a flow-through assay, using 300-ml tall-form glass beakers containing 50 ml of sand and held within larger aquaria containing 16 L of water. Water circulation between the beakers and the water within the aquaria was ensured via two water-exchange ports. All beakers were aerated continuously, ensuring maintenance of adequate dissolved oxygen levels and water circulation. The screened water exchange ports were cleaned twice weekly to avoid clogging.

Test solutions were prepared daily by adding 24 ml of each EE2 solution in MeOH to 12 L of reconstituted water (solvent concentration of 0.02%). Solutions were aerated continuously, and dispensed into the appropriate aquarium by gravity, at a flow of approximately 10 ml/min, for a maximum volume of 12 L/d. Additional aeration at the point of entry into the tanks ensured proper mixing within each aquarium.

Toxicity testing was performed following the U.S. Environmental Protection Agency methods [19]. A total of seven treatments were tested: a water and solvent control (0.2% MeOH) and five EE2 treatments of 0.03, 0.1, 0.3, 1, and 5 mg/L in reconstituted water (water characteristics, mean \pm standard error of the mean, $n = 84$ –126; pH: 8.1 ± 0.02 , temperature: $22.4 \pm 0.05^\circ\text{C}$, dissolved oxygen: 7.7 ± 0.10 mg/L, conductivity: 360 ± 2.3 mS/cm). Each aquarium contained 16 beakers, four of which were initiated on day 10 (auxiliary beakers), because *C. tentans* emergence is known to exhibit a bimodal pattern, to ensure uninterrupted avail-

ability of males throughout the emergence period [19]. Upon test initiation, 12 larvae <24 h post-hatch were counted and transferred into each beaker, which were then randomly assigned to a position within their respective aquarium. On day 20, four replicates of each concentration were used to estimate survival, growth, and biomass normalized to the number of larvae added, using ash-free dry weight. Emergence traps were then added to the remaining beakers ($n = 8$), and emergence was monitored daily. Mating success (as indicated by the percentage of females ovipositing), number of eggs per egg mass, and hatching success were quantified [19]. Each treatment was independently terminated after no emergence had been observed for seven consecutive days, and the sand from each beaker was inspected for any remaining larvae and/or pupae.

Hyaella azteca life cycle test

Chronic toxicity of EE2 to the freshwater amphipod *H. azteca* was investigated during a 42-d static renewal assay in 300 ml tall-form beakers, based on the procedures outlined in the U.S. Environmental Protection Agency guidelines [19], using a randomized complete block design. However, the assay was prolonged for an additional 21 d to investigate the survival and growth of the second (F1) generation. A total of seven replicates of eight treatments, including a water and solvent control (0.2% MeOH) and six EE2 concentrations (10^{-5} , 10^{-4} , 10^{-3} , 0.01, 0.1, and 1 mg/L), were tested in reconstituted water (water characteristics, mean \pm standard error of the mean, $n = 70$ –86; pH: 7.9 ± 0.02 , temperature: $22.8 \pm 0.07^\circ\text{C}$, dissolved oxygen: 7.7 ± 0.08 mg/L, conductivity: 384 ± 7.2 mS/cm). Complete water renewal was performed twice weekly, and the beakers were aerated throughout the assay to maintain adequate levels of dissolved oxygen.

Upon test initiation, 15 animals, 7 to 14 d old (F0 generation), were transferred into each beaker. Survival, mating behavior (formation of an amplexus, or mating pair), and reproduction were monitored twice weekly, at the time of solution renewal. On day 42, adults were counted and preserved in Kahle's solution (5% formaldehyde, 5% glacial acetic acid, 27% ethanol). All preserved animals from the F0 generation were digitized, and animal length and length of the gnathopods of males were measured.

All young from the F0 generation produced during the last week of the assay (days 35–42) were pooled by treatment, and a subset of the animals were divided equally among four new replicates, to avoid potential differences in growth due to variations in animal density. These juveniles were exposed to the experimental conditions for an additional 21 d, to investigate second generation (F1) effects on survival and growth. However, because these animals had not reached complete sexual development after the 21-d exposure period, sex could not be determined with certainty, hence growth was estimated using dry weight for the pooled individuals from each beaker.

Chemical analysis

Water was sampled weekly and was preconcentrated by solid phase extraction and eluted in MeOH. Analyte recovery was $87 \pm 4.8\%$ (mean \pm standard error of the mean, $n = 6$) [16]. When the water concentration could be analyzed directly without preconcentration, an aliquot was filtered through a 0.45 μm syringe filter. Samples were frozen until analysis was completed. All samples were analyzed by liquid chromatography tandem mass spectrometry using an Alliance 2695 separation module (Waters, Milford, MA, USA) and a Quattro Ultima

tandem LC triple quadrupole mass spectrometer (Micromass, Manchester, UK), equipped with a Z-Spray electrospray ionization source and operated in negative ionization mode, as detailed in Dussault et al. [16]. A 50- μ l aliquot of a 10 mg/L solution of $^3\text{H}_2$ -17 β -estradiol was added to 250 μ l of each sample prior to injection and served to correct for variations in ionization efficiency. A postcolumn infusion of 0.5% ammonium carbonate was added, at a rate of 10 μ l/min.

Statistical analysis

Differences in the various endpoints estimated were verified using a multiple factor analysis of variance (ANOVA), including the variables concentration and position within the experimental chamber. When the ANOVA indicated a significant difference, a Tukey honestly significant post hoc test was performed to identify treatments that differed significantly from controls. Adherence to the assumptions of normality and homoscedasticity was verified using the Shapiro–Wilk statistic and by plotting predicted values against residuals. When data did not conform to the assumptions of normal distribution of residuals or homogeneity of variance, various data transformations were attempted (logarithmic, square root, arcsine square root). If none of the transformations attempted successfully overcame the violation of the assumptions, differences between treatments were tested using a Kruskal–Wallis test. Since pairwise comparisons were not possible with this procedure, verification of differences between the control and other treatments was performed using an additional series of Kruskal–Wallis tests involving each group of treatment and control separately. The level of significance selected for this series of tests was then adjusted using the Bonferroni method to $\alpha = 0.05/n$, where n is the number of comparisons made [20].

For the endpoints that were estimated several times during the assays (emergence in *C. tentans*, survival, cumulative number of mating pairs, and cumulative number of young in *H. azteca*), differences were tested using a repeated measures ANOVA, followed by a Tukey honestly significant post hoc test, to identify treatments and days on which significant differences from the control were observed. Verification of the assumption of the ANOVA was performed by inspection of a cumulative probability plot and a plot of predicted values against residuals.

Lethal median concentrations and effects concentrations were estimated by nonlinear regression, using four models (linear, exponential, Gompertz, and logistic), with the linear model being selected, because it yielded the best fit for each of the endpoints investigated. All calculations were based on measured concentrations. Computations were performed using SAS[®] (ver 9.1, SAS Institute, Cary, NC, USA). The selected level of significance was $\alpha < 0.05$.

Acute to chronic ratios

In the absence of chronic toxicity data, a common approach in ecological risk assessment has been to extrapolate from acute toxicity data, using acute to chronic toxicity ratios (ACRs). Studies have shown that the chronic toxicity of a number of chemicals can be relatively accurately estimated in fish and aquatic vertebrates from acute toxicity data, using an ACR of approximately 25 or less, although higher ACRs have been calculated for pesticides and metals [21,22]. Nevertheless, the use of this approach for the assessment of biologically active substances has been debated and may not be appropriate.

For example, ACRs of 10^4 to 10^6 for several endocrine disruptors have been reported in fish [23,24]. To evaluate the suitability of this approach for EE2 in benthic invertebrates, an ACR was calculated for each animal investigated, using the formula $\text{ACR} = \text{LC50}_{\text{acute}}/\text{EC10}_{\text{chronic}}$, where $\text{LC50}_{\text{acute}}$ was the concentration causing 50% mortality from previous 10-d water-only toxicity assays performed within our laboratory [16], and where $\text{EC10}_{\text{chronic}}$ was the concentration causing a 10% effect calculated in the present study. The benefit of using EC10 values rather than no-observed-effects concentrations (NOECs) is that in contrast with NOECs, the EC10 is estimated using all the concentrations of the data set and is independent of the concentrations tested.

Hazard quotient

The data from the present study were combined with environmental exposure data from the literature to estimate the risk associated with EE2 exposure to benthic invertebrates using a hazard quotient approach [25]. The hazard quotient (HQ) was calculated using the equation: $\text{HQ} = (\text{exposure}/\text{effect}) \cdot \text{UF}$, where exposure was the highest surface water concentration reported in the literature, effect was the lowest LC50 or EC50 value calculated from the present study, and UF was the uncertainty factor, used to account for the uncertainties associated with the extrapolation between measurement (*C. tentans* and *H. azteca*) and assessment (benthic invertebrates) endpoints, as explained in the Environment Canada guidance document [25]. Since our calculations included an effect measure for chronic toxicity, a UF of 10 was applied.

RESULTS AND DISCUSSION

Toxicity to *C. tentans*

Exposure to EE2 had significant effects on the survival of *C. tentans* (Fig. 1; Table 1). On day 20, only 20% of larvae had survived the exposure to 3.1 mg/L EE2. However, growth was not significantly affected by EE2 ($p = 0.06$). This may have resulted from the greater food availability for the surviving larvae, in comparison with treatments in which little to no mortality occurred. Because of this apparent negligible effect of EE2 on animal growth, it was not possible to calculate an EC50 for this endpoint. In contrast, biomass was a more sensitive endpoint ($\text{EC50} = 2.0$ mg/L; Table 1), because its calculation indirectly incorporates the effects of both survival and growth. Significantly lower larval biomass relative to the control was observed at the lowest concentration, 0.02 mg/L ($p < 0.0001$). Other studies have reported 10-d LC50s of 0.84 mg/L and 4.1 mg/L for *C. riparius* and *C. tentans*, respectively [16,26], yet the present study is the first to provide a quantitative estimation of the chronic toxicity of EE2 in benthic invertebrates.

As expected, a bimodal emergence pattern was observed in *C. tentans*, with males emerging 2.8 to 4.8 d earlier than females on average (Fig. 2). Significantly decreased emergence was observed in animals exposed to 0.14 mg/L EE2 and higher ($p \leq 0.02$). When animals from each sex were analyzed individually, decreased emergence was detected in males at a concentration of 0.14 mg/L on days 27 to 33 ($p \leq 0.03$; Fig. 2), but not later, when emergence levels were similar to other treatments. Exposure to 3.1 mg/L prevented the emergence of both males and females, which was significantly different from the control from day 26 and 31 and thereafter, respectively ($p \leq 0.003$). By the end of the assay, decreased emergence was

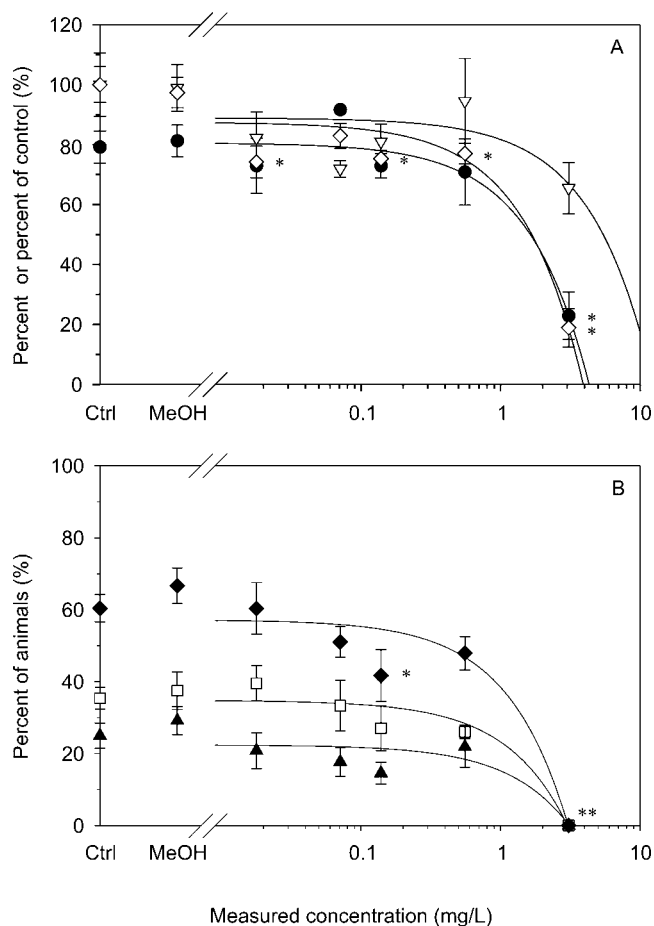


Fig. 1. (A) Survival (●), growth (▽), and biomass (◇) estimated after 20 d of exposure; (B) emergence of *Chironomus tentans* (males [□], females [▲], and all animals [◆]) after 42 d of exposure to the synthetic hormone 17 α -ethinylestradiol (EE2). Values are mean percent of the control (\pm standard error of the mean). Ctrl = control, MeOH = methanol control. * indicates values significantly different from the controls ($p < 0.05$). ** indicates significant differences in comparison with the control, for all three data points.

also observed in females at 0.14 mg/L EE2 ($p \leq 0.01$; Fig. 1). A slight delay was detected in the average time to emergence of females exposed to 0.14 mg/L ($p = 0.03$, Table 2), but not at 0.56 mg/L. Given the absence of delayed emergence at other concentrations, we attribute this delay to experimental variation. In contrast, there were no significant delays in the average time to emergence of males (with the exception of the transitory delayed emergence at 0.14 mg/L, on days 27–33).

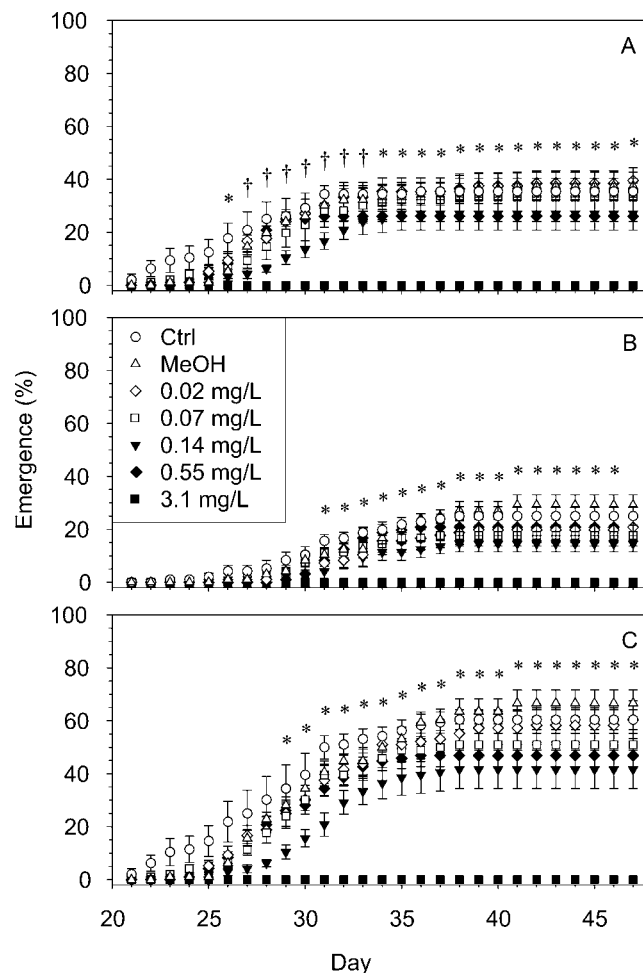


Fig. 2. Cumulative emergence (mean \pm standard error of the mean) of adult males (A), females (B), and all animals (C) in *Chironomus tentans*, after exposure to the synthetic hormone 17 α -ethinylestradiol (EE2). Ctrl = control, MeOH = methanol control. * indicates values significantly lower than control in treatments exposed to 3.1 mg/L. † indicates values significantly lower than control in treatments exposed to 0.14 and 3.1 mg/L.

Although larvae were occasionally observed at the sediment surface at EE2 concentrations of 3.1 mg/L, pupation was unsuccessful and no emergence was observed, thus no reproduction took place. Similar observations have been reported in studies investigating the sublethal effects of perfluorooctane sulfonic acid and perfluorooctanoic acid on *C. tentans* and lindane on *C. riparius* [27,28], suggesting this type of occur-

Table 1. Survival, growth, and emergence estimates (LC $_x$ /EC $_x$, the concentration causing $x\%$ mortality [LC $_x$] or effect [EC $_x$]; mean [95% confidence interval]; mg/L) and lowest observed effect concentration (LOEC) in the midge *Chironomus tentans* and the freshwater amphipod *Hyalella azteca* during a life-cycle assay with the synthetic hormone 17 α -ethinylestradiol (EE2)

		LC/EC50	LC/EC25	LC/EC10	r^2	Pr < W ^a	LOEC
<i>Chironomus tentans</i>	Survival	2.16 (1.616–2.700)	1.08 (0.808–1.348)	0.43 (0.322–0.539)	0.68	0.174	3.1
	Biomass	1.96 (1.554–2.362)	0.98 (0.780–1.181)	0.39 (0.311–0.472)	0.76	0.670	<0.02
	Emergence (males)	1.51 (1.121–1.897)	0.75 (0.561–0.949)	0.30 (0.224–0.379)	0.47	0.167	3.1
	Emergence (females)	1.55 (0.990–2.117)	0.78 (0.495–1.058)	0.31 (0.198–0.423)	0.31	0.139	3.1
	Emergence (all)	1.53 (1.243–1.810)	0.76 (0.621–0.905)	0.31 (0.249–0.362)	0.63	0.005 ^b	0.14
<i>Hyalella azteca</i>	Biomass ^c	0.77 (0.579–0.953)	0.38 (0.290–0.476)	0.15 (0.116–0.191)	0.53	0.022 ^b	0.74
	Reproduction	0.36 (0.234–0.493)	0.18 (0.117–0.247)	0.07 (0.047–0.099)	0.34	0.312	0.74

^a Pr < W = Shapiro–Wilk statistic.

^b None of the attempted data transformations resulted in a normal distribution of the residuals during the nonlinear regression.

^c Due to minor effects on biomass, EC50 values resulted from an extrapolation (0.77 vs 0.74 mg/L) and should be interpreted with caution.

Table 2. Mean reproductive statistics (mean \pm standard error of the mean) in the midge *Chironomus tentans* during a life-cycle assay with the synthetic hormone 17 α -ethinylestradiol (EE2)

Life history parameter	Treatment (mg/L) ^a						
	Ctrl	MeOH	0.02	0.07	0.14	0.56	3.1
Days to emergence (males)	26.0 \pm 1.24	27.4 \pm 0.53	27.0 \pm 0.57	26.8 \pm 1.10	28.5 \pm 0.53	26.6 \pm 0.38	—
Days to emergence (females)	28.0 \pm 1.05	29.5 \pm 0.85	30.9 \pm 0.70	30.5 \pm 1.04	32.4 \pm 0.92*	30.7 \pm 0.47	—
% Females	41 \pm 5.0	45 \pm 5.4	31 \pm 7.0	39 \pm 10.1	35 \pm 7.7	40 \pm 8.0	—
% Ovipositing	70 \pm 16.1	72 \pm 14.7	86 \pm 13.6	90 \pm 16.1	60 \pm 16.1	69 \pm 14.7	—
Days to oviposition	33.8 \pm 1.22	33.9 \pm 1.10	35.2 \pm 1.32	33.3 \pm 1.36	34.8 \pm 1.55	33.6 \pm 0.57	—
Egg masses/replicate	1.5 \pm 0.53	1.4 \pm 0.41	1.4 \pm 0.26	0.8 \pm 0.25	1.0 \pm 0.33	1.6 \pm 0.50	—
Eggs/egg mass	1,100 \pm 141.6	1,040 \pm 158.4	1,054 \pm 129.3	917 \pm 141.7	982 \pm 158.4	759 \pm 141.7	—
% Hatching	90 \pm 4.5	85 \pm 4.8	74 \pm 10.0	95 \pm 2.4	92 \pm 3.7	66 \pm 33.0	—

^a Ctrl = water control; MeOH = methanol control.

* Indicates values significantly lower than control ($p < 0.05$).

rence probably results from nonspecific toxicity rather than a receptor-mediated response.

A few other studies to date have investigated the chronic effects of EE2 on chironomids. Watts et al. [15] reported delayed molting and reduced weight in *C. riparius* exposed to 1 mg/L EE2, but not at lower exposure concentrations. These findings are consistent with the present study, in which exposure to 3.1 mg/L caused a complete failure to pupate in *C. tentans*, whereas emergence was successful in animals exposed to concentrations of 0.56 mg/L and lower. Another study investigated the effects of EE2 on the emergence of *C. riparius* over two generations in sediments spiked with nominal concentrations of 1 to 100 ng/L EE2, but did not show concentration–response relationships or life-cycle disruption [29]. Although to date, the direct effect of EE2 on the ecdysteroid activity of invertebrates has seldom been examined, these data indicate that EE2 is unlikely to directly interfere with this function. In a study investigating the effects of various chemicals on the ecdysteroid activity of *Drosophila melanogaster* cell lines, EE2 was reported to cause a weak antagonistic activity, however only at concentrations >74 mg/L [30], which is above the water solubility of this compound. Because the structure of ecdysone from crustaceans and insects is identical, based on this collective evidence, it appears unlikely that environmental concentrations of EE2 would cause direct interactions with the ecdysteroid receptor.

In contrast with emergence, reproduction in *C. tentans* was not significantly affected by exposure to EE2. No concentration–response relationship was observed up to 0.56 mg/L (Table 2). Reproduction could not be investigated in the highest treatment, since no emergence had taken place. There were no significant differences in the sex ratio, which varied between 31 and 45% females. The number of days to oviposition was also not significantly altered by EE2 exposure ($p = 0.96$), and no differences in the number of egg masses or eggs were detected ($p \geq 0.12$; Table 2). In contrast, studies have found reproduction to be particularly sensitive to environmental stress [31]. A reduction in food source resulted in delayed emergence, a decreased number of females ovipositing, and a decreased number of egg masses in *C. tentans* [32], while *C. tentans* exposed to zinc-spiked sediments exhibited concentration-dependent decreases in the mean number of eggs produced per female [31]. Hence, the absence of significant effects on reproduction at concentrations below 3.1 mg/L [32], where effects on survival, growth, and biomass were detected, indicate that toxicity is unlikely to have occurred as a result of disruption of reproductive hormones.

Toxicity to *Hyalella azteca*

Exposure to EE2 had significant effects on the survival and growth of the F0 generation *H. azteca* ($p \leq 0.03$), but only at the highest concentration investigated, 0.74 mg/L (Fig. 3). Although survival was not significantly affected in the first few weeks of the experiment, significant mortality was observed at the highest concentration after 28 d ($p \leq 0.02$; Fig. 4A). By day 42, only 52% of animals exposed to 0.74 mg/L had survived, and the size of males and females was significantly smaller than control animals (by 17 and 14%, respectively; $p \leq 0.002$; Fig. 3). Biomass was also only affected at the highest concentration ($p = 0.001$; Fig. 3A) and was re-

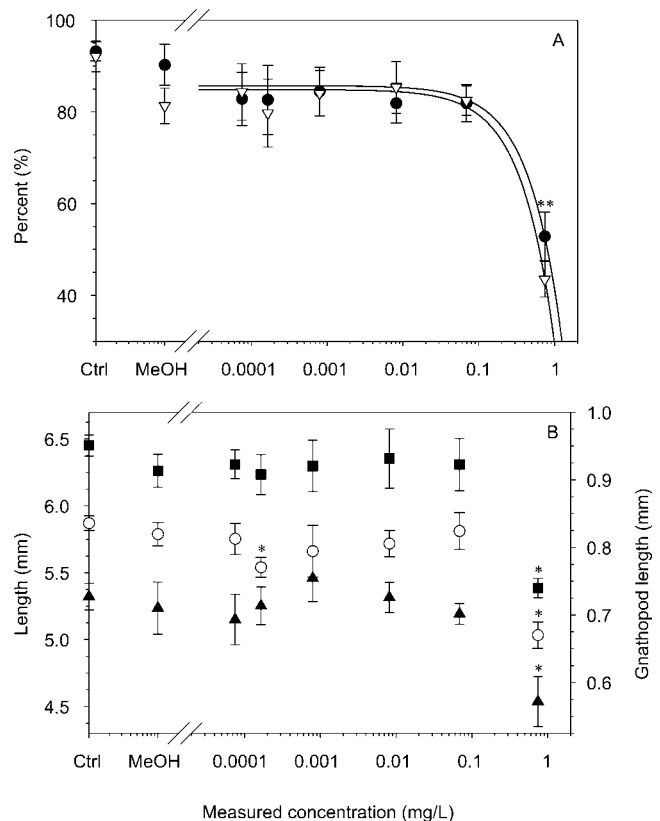


Fig. 3. (A) Survival (●) and biomass (▽); (B) length of males (■), length of females (▲), and gnathopod length of males (○) after a 42-d exposure of *Hyalella azteca* to the synthetic hormone 17 α -ethinylestradiol (EE2). Ctrl = control, MeOH = methanol control. Values are mean \pm standard error of the mean. * indicates significant differences compared to the control ($p < 0.05$).

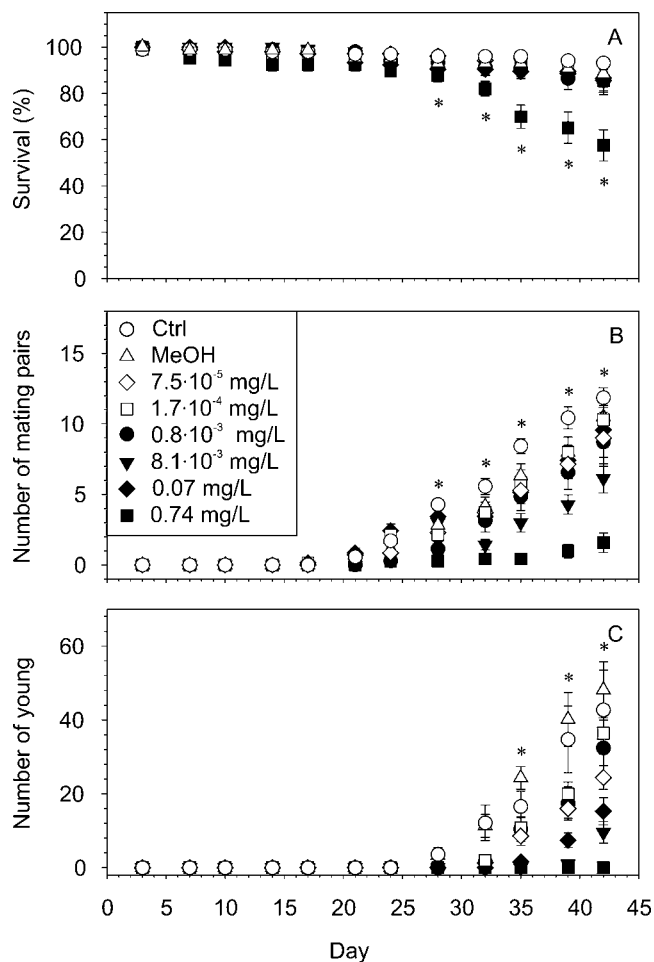


Fig. 4. (A) Survival, (B) cumulative reproduction, and (C) cumulative number of mating pairs of *Hyalella azteca* (mean \pm standard error of the mean) during a 42-d exposure to the synthetic hormone 17 α -ethinylestradiol (EE2). Ctrl = control, MeOH = methanol control. * indicates significant differences between control and the highest exposure concentration (0.74 mg/L; $p < 0.05$).

duced to 44% of control. Survival, growth, and biomass of animals from the F1 generation (survival, 72–97%; growth, 89–131% of control; biomass, 81–121% of control) generally mimicked the response observed in the F0 generation and were not significantly affected by EE2 exposure at concentrations as high as 0.07 mg/L ($p \geq 0.20$). Animals exposed to 0.74 mg/L did not reproduce, hence the highest exposure treatment was not represented in the F1 generation. Survival after 21 d was slightly lower in the F1 generation (87 vs 94%), which we attribute to differences in the age of the organisms at first exposure. Animals from the F0 generation were 7 to 14 d old at the beginning of the exposure, whereas animals from the

F1 generation were born during the assay and therefore exposed from an earlier age, characterized by higher mortality rates.

Due to the low magnitude of effects of EE2 on survival and growth (less than 50% decrease in each), it was not possible to calculate lethal median concentrations for these endpoints. The effects on biomass were more pronounced, which permitted the calculation of median effects concentrations. However, the resulting EC50 value was slightly beyond the range of concentrations investigated (0.77 vs 0.74 mg/L) and should therefore be interpreted with caution (Table 1).

After chronic exposure to EE2, significant differences in the length of the gnathopod of F0 males were detected ($p = 0.0001$). A significantly smaller gnathopod length was detected at 1.65×10^{-4} mg/L ($p < 0.05$), but not again until the highest concentration (0.74 mg/L) where the mean length of the gnathopod was also significantly smaller than in the control ($p < 0.0001$; Fig. 3). However, since the animals exposed to 0.74 mg/L were also significantly smaller than the control animals, it was suspected that the smaller size of the gnathopod was due to smaller animal size. To test this hypothesis we assessed the relationship between body length and gnathopod length that was found to follow the quadratic equation $y = -0.037x^2 + 0.597x - 1.471$ ($r^2 = 0.68$, $p < 0.0001$), where y is the length of the gnathopod and x is the body length of the animal. Since the appearance of secondary sex characteristics occurs within a relatively short period during the animal's development (in comparison with its growth, which occurs throughout most of its life), a nonlinear relationship between body length and gnathopod length is conceivable, especially if the latter incorporates a wide range of animal sizes. However, the presence of a nonlinear relationship precludes the use of a linear normalization to correct for animal body length. The multiple factor ANOVA performed using the variables length and concentration, revealed that once animal body size had been accounted for, concentration was no longer a significant variable ($p = 0.17$; Table S1, <http://dx.doi.org/10.1897/08-005.S1>), and that the length of the gnathopod of males was not significantly affected by EE2 concentration. In contrast with these findings, Vandenberg et al. [13] reported histological aberrations in male *H. azteca* in a two-generation study, as well as a significant decrease in the body-normalized length of the gnathopod of second-generation males exposed to 0.1 and 0.32 $\mu\text{g/L}$ EE2.

Mean reproduction statistics for *H. azteca* are presented in Table 3. The percentage of F0 females in each beaker was 35 to 57%, did not vary significantly between treatments ($p > 0.51$; Table 3), and was likely due to the random distribution of juveniles upon initiation of the experiment. Although mating behavior was first observed at approximately the same time in all treatments (days 17–21, Fig. 4B), a significantly smaller

Table 3. Life history parameters of *Hyalella azteca* during a life cycle exposure to the synthetic hormone 17 α -ethinylestradiol

Reproductive parameter	Treatment (mg/L) ^a							
	Ctrl	MeOH	7.5×10^{-5}	1.7×10^{-4}	0.8×10^{-3}	8.1×10^{-3}	0.07	0.74
% Females	51 \pm 2.7	46 \pm 6.0	35 \pm 7.0	51 \pm 5.3	57 \pm 4.6	40 \pm 7.6	40 \pm 5.3	48 \pm 7.9
Cumulative no. of mating pairs	11.9 \pm 0.70	10.5 \pm 1.18	10.7 \pm 1.89	12.7 \pm 1.30	10.4 \pm 2.00	11.0 \pm 1.85	11.3 \pm 1.41	1.9 \pm 0.67*
Young/female	6.3 \pm 1.56	8.5 \pm 0.48	7.6 \pm 1.15	9.2 \pm 2.40	5.2 \pm 1.14	6.9 \pm 1.13	5.7 \pm 0.92	0 ^a

^a Ctrl = water control, MeOH = methanol control.

* Indicates values significantly lower than the control ($p < 0.05$).

cumulative number of mating pairs was observed at the highest concentration, from day 28 onwards ($p \leq 0.01$). Reproduction was significantly affected by EE2 exposure ($p = 0.001$), as the animals exposed to the 0.74 mg/L treatment failed to reproduce (Fig. 4C). The cumulative number of young produced was significantly different between treatments on day 35 and thereafter ($p \leq 0.04$). Hence, reproduction was a more sensitive endpoint than survival or growth (EC50 = 0.36 mg/L; Table 1).

Despite the absence of reproduction at the highest exposure concentration, eggs were occasionally observed in the brood pouch of some exposed females during digitization (data not shown), indicating that reproduction may have been delayed due to smaller animal size, rather than have ceased altogether. Delayed growth is known to affect mating behavior and reproduction [33]; hence, we suspect that reproduction at 0.74 mg/L EE2 was delayed due to its chronic effects on growth. In contrast, Watts et al. [14] reported increased population growth and a sex ratio in favor of females after exposure to 0.1 to 10 μ g/L EE2, in a 100-d multigeneration assay with *Gammarus pulex*. In light of these findings, larger scale multigeneration studies may provide further information into potential long-term population effects.

Although a number of studies have suggested that exposure to EE2 could disrupt the endocrine function of amphipods, others have reported that other environmental factors, such as parasitism by *Microsporidia*, can also cause feminization of male crustaceans [34,35]. Hence, studies concluding cause-effect relationships between exposure to vertebrate endocrine disruptors and feminization of amphipods have been questioned. In contrast, the results of the present study agree with the investigations of Hutchinson et al. [36], in which EE2 had no significant effects on survival, development, sex ratio, or reproduction of the marine copepod *Tisbe battaglia*, at concentrations up to 0.1 mg/L. A 21-d EC50 of 0.15 mg/L was reported for the reproduction of *Daphnia magna* [37], while EE2 also had no effects on the precopulatory behavior of *G. pulex*, following a 10-d exposure to concentrations as high as 3.7 mg/L [26]. Finally, a recent study reported no significant effects on the concentrations of ecdysone in *D. magna* exposed to as much as 1 mg/L EE2, which suggests that its effects are not mediated by the ecdysone receptor [38].

Acute to chronic ratio

For each animal tested, an ACR was calculated, by comparing EC10 values for emergence (*C. tentans*) and reproduction (*H. azteca*) from the present study with the LC50 values from a 10-d toxicity study previously performed in our laboratory [16]. The ACRs calculated were 13 (*C. tentans*) and 16 (*H. azteca*), which are well within the value of 25 reported in fish and aquatic vertebrates for more than 90% of contaminants [21,22], but are much lower than the ACRs of 15,000 and 150,000 reported for the Japanese medaka *Orizyas latipes* [24]. A recent study reported ACRs of 2.6 to 5 for EE2 in the cladoceran *D. magna* [38]. Thus, despite the high biological activity and adverse effects of EE2 observed in vertebrates, these data confirm that the uncertainty factors currently used for the derivation of chronic toxicity values [25] are appropriate and conservative for *C. tentans* and *H. azteca*. As there is no clear evidence of sex steroid receptors in ecdysozoan invertebrates, the low ACRs obtained in the present study, in conjunction with those estimated by Clubbs and Brooks [38], strongly indicate that the toxicity of EE2 to these

ecdysozoan invertebrates was not caused by the disruption of hormonal pathways. Further studies will be required to determine whether these findings can be extrapolated to other invertebrate classes, such as mollusks, in which functional sex steroid receptors have been reported.

Hazard quotient

The data collected in the present study were used to evaluate the environmental risk posed by the presence of EE2 to benthic invertebrates, using a hazard quotient (HQ) approach, where $HQ = (\text{exposure}/\text{effect}) \cdot UF$ [25]. The highest environmental exposure reported was from a U.S. stream survey (0.273 μ g/L) [17], while the lowest effect data from the present study was the EC50 calculated for the reproduction of *H. azteca* (0.36 mg/L). Since the effects data used were from chronic toxicity studies, an uncertainty factor of 10 was selected, which serves to account for uncertainties associated with extrapolating between measurement endpoints for *C. tentans* and *H. azteca* and the assessment endpoint (protection of benthic invertebrate communities), as outlined in the Environment Canada guidance document [25]. This yielded a hazard quotient of 0.01, indicating that environmental exposure to EE2 poses a small risk to benthic invertebrates. This estimate substantiates the findings of a previous assessment performed in our laboratory, in which 10-d toxicity data yielded a hazard quotient of 0.25, leading to the conclusion that a small risk existed for benthic invertebrates exposed to EE2 [16]. It should, however, be noted that an UF of 10, as recommended in the Environment Canada guidelines, may not be protective of all benthic invertebrates. Functional sex steroid receptors have been detected in mollusks, which may be more sensitive to EE2 than ecdysozoan invertebrates, including the organisms investigated in the present study. For example, Jobling et al. [39] reported that exposure of the freshwater snail *Potamopyrgus antipodarum* stimulated embryo production at concentrations of 25 ng/L.

CONCLUSIONS

The results of the present study indicate that chronic exposure to EE2 had minor effects on the life-cycle of *C. tentans* up to a concentration of 0.14 mg/L, at which the first detectable signs of altered emergence were observed. With an EC50 value of 1.5 mg/L, emergence was a slightly more sensitive endpoint than survival or growth. Similarly, adverse effects of EE2 to the freshwater amphipod *H. azteca* were observed at 0.74 mg/L, and survival, growth, and biomass were decreased. Reproduction was the most sensitive endpoint investigated in *H. azteca*, with a calculated EC50 of 0.36 mg/L. The data from the present study represent the first quantitative estimate of the chronic effects of EE2 on benthic invertebrates and were subsequently used to derive an ACR of 13 and 16, thus indicating that the uncertainty factors currently used in ecological risk assessment for the derivation of chronic toxicity data are protective of the organisms investigated and likely most ecdysozoans, in which estrogen receptors have not been identified to date.

Although a few studies have suggested that EE2 may elicit an effect on invertebrate hormonal systems [13–15], other investigations, including the present study, have not reported disruption of endocrine systems [15,26,29,36,38]. Since environmental factors such as parasitism have also been demonstrated to cause feminization of male crustaceans, their effects would need to be investigated further before a conclusive

assessment of the endocrine effects of EE2 on crustaceans could be made [35]. Hence, unambiguous cause–effect relationships between exposure to estrogens and invertebrate endocrine disruption, if any, have yet to be established. Investigations on the interaction between EE2 and the ecdysteroid receptor have indicated that no adverse effects are expected at environmental concentrations [30,38]. The risk associated with chronic exposure to EE2 was investigated for benthic invertebrates, using a hazard quotient approach, and further revealed that adverse effects are unlikely to occur at environmental concentrations.

SUPPORTING INFORMATION

Table S1. Summary statistics for the analysis of variance investigating the effects of length and concentration on the size of the gnathopod in *Hyalella azteca*, during a lifecycle exposure to the synthetic hormone 17 α -ethinylestradiol ($r^2 = 0.68$, $p < 0.0001$).

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