POPULATION RESPONSES OF THE FRESHWATER AMPHIPOD GAMMARUS PULEX (L.) TO AN ENVIRONMENTAL ESTROGEN, 17α-ETHINYLESTRA DIOL

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(Received 4 April 2001; Accepted 10 August 2001)

Abstract—The effects of the environmental estrogen 17α-ethinylestradiol (EE) on mixed populations of 90 individual Gammarus pulex were examined following a 100-d exposure in a flow-through system. Counts of total animal numbers revealed that, in all treatment groups, population size dramatically increased due to recruitment, with neonate and juvenile gammarids the most abundant. At concentrations of 1 and 10 μg/L EE, the recorded mean population sizes of 385 and 411, respectively, were significantly greater (p = 0.018) than the control (169). Mean population sizes in the solvent control (257) and 100 ng/L EE treatment (267) were not significantly different (p > 0.05) from the control. In addition to total counts, detailed image analysis of each individual animal allowed the assessment of length–frequency distributions, adult sex ratio, number of precopula pairs/ovigerous females, and measurement of secondary antenna and gnathopod length (secondary sex characteristics). The sex ratio of adults at 100 ng, 1 μg, and 10 μg/L EE was greater than 2:1 (female:male), and significantly more females (p = 0.008) were recorded at these concentrations compared with the control. The number of male adults, precopula guarding pairs, and ovigerous females did not differ between treatments (p > 0.05). Secondary antennal and gnathopod length in males was consistently greater than in females (p ≤ 0.001), but comparison between groups revealed no difference in these parameters.

Keywords—17α-Ethinylestradiol Gammarus pulex Population Increased recruitment Sex ratio

INTRODUCTION

One of the fundamental aims of aquatic ecotoxicology is to establish how pollutants affect organisms at the population level [1], but despite the obvious importance of such pollutant-induced changes, direct laboratory study of populations is rarely carried out [2]. The preferred approach involves examination of toxicity at the individual level, using survival and various sublethal responses (growth, development, reproduction, behavior, etc.), which are then used in an attempt to predict effects at higher levels of biological organization. Although this approach has many advantages, validation of the predictions made by relatively short-term, simple bioassays does require testing at the population level. For the invertebrates, the favored method of assessing effects on populations is the life table or demographic study, in which the age-specific mortality and fecundity of individuals is recorded for the life span of a single cohort and used to provide a measure of the intrinsic rate of population increase. These studies are largely limited to relatively short-lived organisms such as daphnids and copepods [3,4], presumably due to cost and logistical difficulties. However, alternative strategies including measures of age/length composition, reproductive behavior/status, sex ratio, and fertility for the assessment of population effects on relatively long-lived macroinvertebrates with complicated life histories (such as the amphipod crustacea) have been described by several authors [5–8].

The successful use of amphipods, such as Gammarus pulex, Hyalella azteca and Corophium volutator, to assess population-level effects is attributable to a number of factors, including their ecological importance, ease of laboratory culture, relatively short generation time, and sensitivity to pollutants [9–11]. In addition, all three species are well established as test organisms and consequently a large toxicity database is available. In Europe, G. pulex has been extensively used in acute and chronic freshwater bioassays utilizing a wide range of response criteria such as survival, growth, and feeding behavior [12–14]. This species also incorporates a period of precopulatory guarding behavior into its reproductive biology whereby the male claps and swims with the female until she molts, thus ensuring successful sexual contact during the postecdyosis period when mating can occur. The prevention or disruption of precopulatory guarding can be a sensitive indication of environmental stress, with laboratory [15] and in situ field studies [16,17] demonstrating that the time taken for precopula animals to separate is indirectly correlated with the concentration of pollutant to which they are exposed.

We have recently reported [18] on a series of short-term bioassays where the effects of the environmental estrogens 17α-ethinylestradiol and bisphenol A on the reproductive behavior of adult G. pulex were assessed. Endocrine disrupting chemicals present in surface waters are currently a cause of great concern, and much research effort has been centered on this topic in recent years. For invertebrates, where knowledge of endocrinology is limited, integrative response criteria relating to the disturbance of known endocrine-mediated processes (growth, reproduction, etc.) have been recommended for the assessment of these chemicals [19]. The results obtained in a previous investigation showed that precopulatory guarding was not affected at environmentally realistic concentrations of ethinylestradiol (EE) and bisphenol A. Disturbance of the precopulatory behavior of individual pairs as a result of short-term exposure could potentially lead to decreased recruitment at the population level, but these earlier results [18] predict no affects at higher levels of biological organization (i.e., populations). However, chronic exposure to endocrine-disrupting...
The purpose of the present investigation was to establish if the failure of 17α-ethinylestradiol to influence reproductive behavior of *G. pulex* in a short-term assay [18] predicted a similar lack of response at the population level.

### MATERIALS AND METHODS

**Toxicant solution**

A stock solution of 17α-ethinylestradiol was prepared from the solid compound (Sigma-Aldrich, Dorset, UK), which was dissolved in analytical-grade ethanol to provide a concentration of 100 mg/L. Solutions used in tests (100 ng, 1 μg, and 10 μg/L EE) were made up in dechlorinated mains tap water at ≤0.01% ethanol. This was also the final percentage of ethanol present in the solvent control.

**Test organisms**

*Gammarus pulex* were obtained from a local stream by kick sampling in early summer (May/June) and were transported to the laboratory on the same day as collection and maintained as described by McCahon and Pascoe [9]. They were allowed to acclimatize to laboratory conditions for at least 10 d. Prior to the start of the study, gammarids were sorted according to body length to give 16 mixed populations each comprising 12 neonates (1.5–3 mm), 40 juveniles (3–6 mm), 30 adults (15 × 6–8 mm, 15 × 8–10 mm), and 4 precopula guarding pairs—a total of 90 animals. This composition was designed to maximize the potential for recruitment throughout the course of the study by providing a population structure that would allow normal size assortative mating [23] to occur. Each population was held for 48 h in separate 1-L aquarium filled with 500 ml of aerated dechlorinated water and conditioned [24] horse chestnut leaves (*Aesculus hippocastanum*) as food and cover.

**Experimental design**

The effects of EE on populations of *G. pulex* were examined in a temperature-controlled room (16 ± 1°C) with a 16:8-h light:dark regime. Animals were exposed in 30 × 20 × 20-cm glass aquaria (~vol 11 L) with flow-through dosing controlled by peristaltic pumps. To prevent the loss of animals, a mesh screen (≤0.5-mm pore size) was fixed over the exit tube from each aquarium. Each treatment group (control, solvent control, 100 ng, 1 μg, and 10 μg/L) was assigned three replicates of the glass aquaria (15 aquarium and 1,350 animals in total), which were filled with 9.5 L of the relevant test solution.

Of the 16 *G. pulex* populations, 15 were randomly assigned to separate test aquaria as described above. The one remaining population was preserved in 70% ethanol to provide an initial population profile. Food and cover for the animals were provided by the addition of 70 g dry weight of horticultural grade silver sand (sieved to ≤1 mm) and several conditioned [24] horse chestnut and alder (*Alnus glutinosa*) leaves to each aquarium. Food was subsequently provided at regular intervals, typically every 5 to 7 d, when most of the previous ration had been consumed. Any leaves with excessive white thread-like fungal growth were removed from the system. The water column was continuously aerated in order to maintain dissolved oxygen at ≥80% of the air saturation value. To minimize disruption, test populations were not sampled before the end of the study.

**Dosing regime**

Exposure of *G. pulex* to EE was regulated using a flow-through system comprised of two multichannel Watson-Marlow (Cornwall, UK) 202/U peristaltic pumps and five 2-L glass beakers as separate toxicant/control-water reservoirs. This system was chosen so that water quality and chemical concentration could be maintained over the extended test period (100 d). One pump was loaded with nine cassettes to dose the three replicate control, solvent control, and 100-ng/L treatments and the second with six cassettes to dose the 1- and 10-μg/L treatments. All cassettes were fitted with 460-mm lengths of 1.85-mm bore polyvinylchloride (PVC) manifold tubing (Watson-Marlow, Green/Green), which were replaced every three weeks in accordance with the manufacturer’s instructions to prevent irregularities in flow caused by tube damage. Both pumps were set to run at 20% of their maximum speed, corresponding to a delivery volume of 60 ml/h into each test aquarium. The flow rate was continually monitored during the test by the addition of a single cassette to each pump, which drew dechlorinated water from a 1-L beaker and deposited it into a measuring cylinder. The test solutions (and control water), held in 2-L glass beakers, were delivered into the appropriate aquarium via the manifold tubing, which was positioned above the surface of the water overlying the gammarids. To ensure thorough mixing of the test solution prior to delivery, a 35-mm stir bar was added to each beaker, which was then magnetically stirred for the duration of the dosing period. The pumps were set to dose for 4 h daily, corresponding to a delivery volume to each aquarium of 240 to 250 ml/d. With three replicates in each treatment group, the 2-L reservoir of test solution had to be replaced every 2 to 3 d.

**Population counts**

At the end of the study (100 d), the contents of each aquarium were poured through a series of sieves (2.0–0.25 mm) and preliminary counts made of total population size and the number of precopula pairs/ovigerous females in each replicate. The entire population was then preserved in 70% ethanol for later detailed investigation using calibrated image-analysis software (Kings College, Cambridge, UK, Measurement Package, written by Tony Brain, run on BBC Master Series Microcomputer). The body length of each individual was measured, as described by Blockwell et al. [6], from behind the eye to the tip of the third uropod along the curve of the dorsal surface. These measurements provided detailed length–frequency distributions of each replicate population and allowed the classification of individuals as neonates, juveniles, or adults. In the case of the adults (>6-mm length), animals were sexed, based on the morphology of the antennae, gnathopods, and uropods [25], and additional measurements were made of the secondary antennae and gnathopods, which are recognized as secondary sexual characteristics. However, it proved difficult to accurately sex animals less than 10 mm in length; therefore, animals in the 6- to 10-mm range (young adults) were not included in the statistical analyses of adult characteristics and sex ratio.
Population responses of *Gammarus pulex* to 17α-ethinylestradiol

**Table 1. Measured concentrations of 17α-ethinylestradiol (EE) recorded at the start of the *Gammarus pulex* population study**

<table>
<thead>
<tr>
<th>Sample identification</th>
<th>Nominal concn. EE</th>
<th>Measured concn. EE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dosing solution</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. 1</td>
<td>100 ng/L</td>
<td>132 ng/L</td>
</tr>
<tr>
<td>No. 2</td>
<td>1 µg/L</td>
<td>0.71 µg/L</td>
</tr>
<tr>
<td>No. 3</td>
<td>10 µg/L</td>
<td>7.6 µg/L</td>
</tr>
<tr>
<td><strong>Test aquarium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. 1</td>
<td>100 ng/L</td>
<td>104 ng/L</td>
</tr>
<tr>
<td>No. 2</td>
<td>1 µg/L</td>
<td>0.7 µg/L</td>
</tr>
<tr>
<td>No. 3</td>
<td>10 µg/L</td>
<td>7.5 µg/L</td>
</tr>
</tbody>
</table>

**Data analysis**

All statistical analysis of the data was carried out using Minitab for Windows (Ver 10, State College, PA, USA). One-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparison test (α = 0.05), was performed on total population counts, precopula pair/vigorous female number, size of secondary sexual characters, and adult sex ratios in order to identify any differences attributable to EE exposure. The relationship between body length and size of secondary antennae and gnathopods was examined using simple linear regression with subsequent comparison of the fitted regression lines. Comparison of mean antennal and gnathopod lengths was achieved using paired *t* tests.

**Toxicant analysis**

Analysis of EE was carried out by colleagues at the Fraunhofer-Institut für Umweltchemie und Ökotoxicologie IUCT (Schmallenberg, Germany) using gas chromatography-mass spectrometry-mass spectrometry (GC-MS-MS) techniques as described by Bohmer and Kurzawa [26]. Due to restrictions on the number of samples that could be analyzed, the concentration of EE present in the dosing solutions and test aquaria (analyzed separately to ensure correct dilution) could only be determined at the start of the study, prior to the first 4-h dosing cycle. However, these values confirmed good agreement with nominal concentrations (Table 1), and the same stock solution (100 mg/L EE in ethanol) was used throughout the study to formulate the test solutions, providing confidence that the appropriate concentration range was attained.

**RESULTS**

**Population size and structure**

The data show that total population size and the number of neonates, juveniles, and adults all exhibited a marked increase compared with the numbers at the start of the study (Fig. 1). However, at 100 ng/L, 1 µg/L, and 10 µg/L of EE, population size increased more than was recorded in the control. In all cases, population growth was attributable to recruitment, with neonate and juvenile gammarids most abundant, accounting for ≥70% of animals recovered. For example, in the control, neonates and juveniles contributed 122 animals to a mean population size of 169, whereas at 10 µg/L EE, 319 (77%) out of a total of 411 animals were immature. Detailed length–frequency distributions (Fig. 2) confirm this pattern, with most animals from the test populations falling within the 1.5- to 6-mm size class (neonates and juveniles). Although there were differences in the mean number of neonates and juveniles between treatments, the percentage of animals in this size class was remarkably consistent at approximately 70%, with fluctuations typically <10% of the total population size. Since the population profile was similar in all cases, only the initial population, control, and 10 µg/L EE are shown in Figure 2.

Data analysis revealed that the number of animals in separate size classes and total population size were significantly affected by exposure to EE. At test concentrations of 1 and 10 µg/L, the respective mean population sizes of 385 and 411 *G. pulex* were significantly greater (p = 0.018) than the control.

**Fig. 1.** Mean population size and composition for *Gammarus pulex* exposed to 17α-ethinylestradiol (EE) for 100 d. Vertical bars represent SE (standard error). The asterisk indicates a significant difference (p ≤ 0.05) from control.

**Fig. 2.** Length–frequency distribution recorded at the start (initial population) and end of the *Gammarus pulex* population study. (a) initial population; (b) control; (c) 10 µg/L 17α-ethinylestradiol (EE).
Table 2. Summary of adult response criteria recorded following a 100-d exposure to 17α-ethinylestradiol (EE); SE = standard error

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Mean (± SE) no. precopula pairs</th>
<th>Mean (± SE) no. ovigerous females</th>
<th>Mean (± SE) adult sex ratio, male : female</th>
<th>Mean (± SE) percentage of adults in population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4 (1)</td>
<td>3 (2.1)</td>
<td>0.891 (0.08)</td>
<td>11.10 (1.80)</td>
</tr>
<tr>
<td>Solvent control</td>
<td>2.3 (0.3)</td>
<td>5 (0.6)</td>
<td>0.91 (0.31)</td>
<td>9.87 (1.83)</td>
</tr>
<tr>
<td>100 ng EE/L</td>
<td>2.3 (1.2)</td>
<td>8 (1)</td>
<td>0.361 (0.06)</td>
<td>16.74 (2.78)</td>
</tr>
<tr>
<td>1 μg EE/L</td>
<td>2.3 (0.9)</td>
<td>9 (3.2)</td>
<td>0.371 (0.10)</td>
<td>11.86 (1.41)</td>
</tr>
<tr>
<td>10 μg EE/L</td>
<td>2.7 (0.7)</td>
<td>8.3 (2.2)</td>
<td>0.481 (0.06)</td>
<td>11.52 (1.98)</td>
</tr>
</tbody>
</table>

In addition to sex ratio, the percentage of the population comprised of females (Table 2) was significantly greater ($p = 0.001$) than the percentage of males in each of the three groups exposed to EE. Although adults that could not be accurately sexed (generally <10 mm) were excluded from this analysis, it seems unlikely that this would have affected the overall result since this criterion was applied equally to each treatment group.

The possible effects of EE on sexual development of *Gammarus pulex* were assessed by comparing measurements of the secondary sexual characters (antennae and gnathopods) for adults of both sexes in the different treatment groups. The use of linear regression confirmed the existence of a strong linear relationship between the length of the secondary antennae or gnathopods and body length. This relationship was noted for male and female adults in each of the treatment groups examined, although only control data are presented (Figs. 3 and 4) since the trend was applicable in all cases. Comparison of the regression lines within a particular treatment revealed significant differences between the sexes in relation to the slope of the fitted line for antennal length, with this character larger in males than females. Similar comparison of the lines relating to gnathopod length did not show any significant differences. Comparison of mean antennal length within each treatment group revealed that this character was significantly greater ($p \leq 0.001$) in males than females (Fig. 5). Further analysis demonstrated that there was no difference in this parameter between treatments, with male and female antennal length consistent at approximately 3.5 and 2 mm, respectively. This trend was also evident in comparisons of mean gnathopod length; male gnathopods were larger than those of the females ($p \leq 0.001$), but there were no differences between treatments.

**DISCUSSION**

The data obtained in this investigation indicated that chronic exposure to EE resulted in a significant increase in popu-
A second factor that may have contributed to the increases in population growth is the significantly higher number of female adults recovered from the EE-treated aquaria. The sex ratio of adults in these three treatments (100 ng, 1 μg, 10 μg/L) was female biased by a ratio greater than 2:1. Although fluctuations in sex ratio have been observed in wild populations of *G. pulex* [34], the 1:1 ratio reported in the control and solvent control suggest that the differences seen in the present study were related to chemical exposure. Increased recruitment in the exposed populations could be a logical consequence of the existence of a higher proportion of female adults, and the natural conclusion would be that EE affected sexual differentiation in such a way as to promote females over males. However, this view is not supported by the literature in relation to sexual differentiation in malacostracan crustacea since this process is thought to be controlled by androgenic steroids [31] produced in the androgenic gland. The evidence for this, reviewed by Hasegawa et al. [35], is that removal of the androgenic gland from young male malacostraca results in the development of a functional female, and conversely, implantation of an androgenic gland into a young female causes it to develop into a male. In light of this information, the effects on sexual differentiation recorded in the present study cannot be easily explained, but the data are statistically significant, providing confidence in the reliability of the result. Despite the lack of evidence that estrogenic chemicals can affect crustacean sexual differentiation, in the absence of more detailed endocrinology, this mode of action cannot be discounted as a partial explanation for the results recorded.

In the same way that accelerated female maturation might be expected to correspond to a greater number of ovigerous females, the effects noted on sexual differentiation could be associated with effects on the secondary sexual characteristics. Evidence that secondary sexual development in crustacea can be affected by estrogenic chemicals is provided by Omlstead and LeBlanc [36], where the length of the female abdominal process in *Daphnia magna* increased in response to diethylstilbestrol exposure. However, Brown et al. [32] provided a contrasting view and reported increased male antennal length of *C. volutator* exposed to 4-nonylphenol. In the present study, neither secondary antennal nor gnathopod length were affected by exposure to the test chemical. The lack of any association between these response criteria and the conflicting evidence provided by previous investigations serve to make interpretation of the results more difficult and reflect the complex nature of the interaction between invertebrate steroid receptors and chemicals known to affect vertebrate endocrine systems.

**CONCLUSION**

The results of this investigation showed that populations of *G. pulex* exhibited increased growth following exposure to EE. An increase in the rate of sexual maturation and a shift in the adult sex ratio combined with increased female numbers may explain the results, but only partial support for these findings is available from previous investigations. Although the effects seen in this study cannot be wholly explained, they nevertheless represent an important data set in relation to the assessment of endocrine-disrupting chemicals with invertebrates. The study has identified several response criteria related to reproduction and development that are sensitive at environmentally relevant concentrations to the effects of EE. In addition, the investigation has shown that the effects of this particular chemical on *G. pulex* cannot be predicted from a
short-term bioassay [18]. Chronic tests that examine integrated, population-level responses provide the most suitable means for assessing the potential risk of exposure.

Acknowledgement—The authors thank the European Union for financial support (contract ENV-4-CT97-0509) and colleagues at the Fraunhofer-Institut für Umweltchemie und Ökotoxikologie IUET, Schmallenberg, for analyzing the EE.

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