Long-term exposure to bis(2-ethylhexyl)phthalate (DEHP) inhibits growth of guppy fish (Poecilia reticulata)

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ABSTRACT: Bis(2-ethylhexyl)phthalate (DEHP) is a widely used plasticizer that is a commonly found contaminant of aquatic environments. However, little is known about the long-term effects of DEHP on fish development, as previous studies yielded conflicting results and mostly investigated the effects of concentrations higher than those found in natural habitats. We thus aimed to investigate the effects of DEHP (i) at concentrations present in the environment, and (ii) under conditions that might accentuate any deleterious consequences (larvae rather than adult fish, use of higher temperature). Different concentrations of DEHP (0.1–10 \( \mu \)g l\(^{-1}\)) applied continuously for 91 days were tested on guppy fish that were less than one week old at the beginning of the treatment. As early as 14 days after the start of exposure, guppies treated with 10 \( \mu \)g l\(^{-1}\) DEHP showed significantly reduced body length as compared with control fish. The inhibitory effect of DEHP was concentration-dependent and increased with time, leading to a maximal reduction in body length of 15 and 40% at 1 and 10 \( \mu \)g l\(^{-1}\) DEHP, respectively. The effect was even more pronounced for body weight, which was diminished by up to 40 and 70% at 1 and 10 \( \mu \)g l\(^{-1}\) DEHP, respectively. The reduction in growth was still significant at 91 days of DEHP treatment, whereas the Fulton’s condition factor was significantly reduced body length as compared with control fish. The inhibitory effect of DEHP was concentration-dependent and increased with time, leading to a maximal reduction in body length of 15 and 40% at 1 and 10 \( \mu \)g l\(^{-1}\) DEHP, respectively. The effect was even more pronounced for body weight, which was diminished by up to 40 and 70% at 1 and 10 \( \mu \)g l\(^{-1}\) DEHP, respectively. The reduction in growth was still significant at 91 days of DEHP treatment, whereas the Fulton’s condition factor was unaffected. While DEHP significantly blocked growth in both male and female guppies, no shift in the sexual development was observed. These data show that DEHP, at concentrations present in aquatic environments, can profoundly affect development in fish. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: DEHP; bis(2-ethylhexyl)phthalate; growth inhibition; guppy; fish; plastic softener; plasticizer; phthalate

INTRODUCTION

Bis(2-ethylhexyl)phthalate (DEHP) is a commonly used plastic softener and, therefore, is contained in many plastic products. Because DEHP is not covalently bound to the plastic material, it is able to diffuse into the environment over time (Takehisa et al., 2005), causing ubiquitous contamination. For example, DEHP concentrations of ~0.85 \( \mu \)g l\(^{-1}\) have been found in the Rhine, and up to 220 \( \mu \)g l\(^{-1}\) in rivers near sewage plants of the industrial region Nordrhein Westfalen in Germany (Furtmann, Landesamt für Wasser und Abfall Nordrhein-Westfalen, 1993).

Up to now, only little information has been obtained about any long-term effects of DEHP on the development of fish. In one study with a non-typically high DEHP concentration (554 \( \mu \)g l\(^{-1}\) in a flow-through system), the exposure of <3-day-old Medaka (Oryzias latipes) during 168 days diminished their weight by 13% (Defoe et al., 1990). Chikae et al., using more typical concentrations (0.1, 1 and 10 \( \mu \)g l\(^{-1}\)), also found a weight reduction at 5 months after treating Medaka eggs with DEHP until they hatched (Chikae et al., 2004a) and fry for 3 weeks (Chikae et al., 2004b). In contrast to these studies showing an effect when DEHP was applied to very young fish, the treatment of 3–4-week-old guppy fish (Poecilia reticulata) for 35 days with 320 \( \mu \)g l\(^{-1}\) DEHP did not show any effect on their growth (Adema, 1981). In the present study, we thus aimed to determine whether the continuous exposure of larval (<1 week old) rather than older guppy fish over several weeks with DEHP concentrations found in the environment (0.1, 1, and 10 \( \mu \)g l\(^{-1}\)) would affect their development. Guppy fish originate from South America and were chosen as they are easy to keep, have a chromosome-linked sexual development (Winge and Ditlevsen, 1947), and support higher temperatures, which may facilitate any DEHP effects (Barron et al., 1987).

MATERIAL AND METHODS

A total of 155 less than one week old guppies (P. reticulata) were equally divided into five different groups and added to five 10 l full glass aquaria, filled with fresh tap water. We did not use any water pumps, filters, soil or plants to avoid the DEHP concentrations being influenced by these factors. DEHP (Sigma; industrial grade >98% pure) was first diluted to 100 \( \mu \)g ml\(^{-1}\) in dimethylsulfoxide (DMSO), and then on to 10 and 1 \( \mu \)g ml\(^{-1}\) in DMSO. From each one of these stock solutions, 1 ml was then added into a different aquarium containing 10 l of tap water, thus achieving final concentrations of 0.1, 1 and 10 \( \mu \)g l\(^{-1}\) DEHP, respectively, and a final DMSO concentration of 0.01% (v/v), which was also used for the DMSO control. Another control aquarium (H\(_2\)O control)
control for 0 μg l⁻¹ DEHP). Tukey–Kramer HSD post-hoc tests were used for the pair-wise comparisons between the different DEHP treatments and the respective DMSO control. As in each tank the fish were not completely unrelated to each other (Hurlbert, 1984), additionally to the ANOVA, which theoretically requires totally independent individuals, we also applied a linear regression model using as single data points the average fish length and weight for each aquarium (y-values). The model assumes no influence at 0.05 μg l⁻¹ DEHP (y = α), but at higher amounts an effect that is linearly proportional to the logarithm of the DEHP concentration according to the following equation: \( y = \alpha + \beta \log_{10}(DEHP) \mu g l^{-1} \). With this model and a Student’s t-test, the H₀ hypothesis \( \beta = 0 \) (i.e. no DEHP influence) was tested. Survival rates were statistically assessed with a Pearson’s chi-square \( (\chi^2) \) test by comparing the number of individuals at the beginning to the number at the end of the experiment (14 vs 91 days).

RESULTS

To examine any effect of DEHP on guppy fish development, we measured both the length and weight of the fish at defined time points upon continuous DEHP exposure. These measurements were compared to the ones from fish kept in the absence of DEHP both with and without the solvent DMSO (DMSO control and H₂O control, respectively). While the solvent DMSO by itself inhibited neither the length nor weight of the fish significantly, DEHP caused a dose-dependent reduction in both growth parameters (Figs 1 and 2). The DEHP-mediated inhibition in growth was statistically significant as early as 14 days after beginning the exposure to DEHP \( (P_{\text{ANOVA}} = 0.002 \text{ for the length}) \) and increased with time (Figs 1 and 2; \( P_{\text{ANOVA}} < 0.001 \) from 28 days on for both the length and weight of the fish). In addition, the DEHP-mediated growth inhibition was dose-dependent as revealed by pair-wise comparisons between fish kept in the presence of DEHP (0.1, 1 and 10 μg l⁻¹), and control fish in DMSO (DMSO control). While at 14 days, only the highest DEHP concentration (10 μg l⁻¹) significantly inhibited the fish length (Fig. 2A), at 28 days and any later time points both 1 and 10 μg l⁻¹ DEHP significantly blocked the length as well as the weight of the fish (Fig. 2A and B). The lowest DEHP concentration of 0.1 μg l⁻¹ led at two time points (42 and 56 days) to a significantly reduced fish length (Fig. 2A), but only once (at 28 days) to a significantly smaller weight (Fig. 2B).

Longer treatment (≥28 days) with higher DEHP concentrations, as compared with 0.1 μg l⁻¹ DEHP, always restrained the growth of the fish. Thus, guppies treated with 1 and 10 μg l⁻¹ DEHP, e.g. for 49 days, were 15 and 30%, respectively, shorter than the DMSO control fish (Fig. 2A). The DEHP effect was even more pronounced for the weight, where the 49 day treatment gave a highly significant 40 and 70% inhibition of growth for 1 and 10 μg l⁻¹ DEHP, respectively (Fig. 2B). At 91 days, i.e. when we ended our experiment, fish treated with 1 and 10 μg l⁻¹ DEHP were 10 and 26% shorter, respectively, and 32 and 61% lighter, respectively, as compared with the DMSO control, while fish treated with 0.1 μg l⁻¹ DEHP were 4% shorter and 15% lighter, but this difference was not statistically significant (Fig. 2). Taken together, our results show that the continuous exposure of guppy fish to DEHP at 28 °C inhibits their growth in a time- and dose-dependent manner, with an overall significant effect being exerted by as little as 1 μg l⁻¹ DEHP.
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The Fulton's condition factor is useful to assess the stoutness of fish (Knaepkens et al., 2003). To further examine the DEHP effect, we thus determined for each guppy fish treated with DEHP its Fulton's condition factor and compared it with the ones from the DMSO control fish. As shown in Fig. 3, the Fulton's condition factor was relatively similar for control fish and guppies treated with DEHP; significant differences ($P_{\text{ANOVA}} \leq 0.01$) were only found for four time points (35, 49, 63 and 70 days) and post-hoc tests revealed a significant DEHP effect once for 1 µg l$^{-1}$ DEHP (35 days), and twice for 10 µg l$^{-1}$ DEHP (49 and 63 days; Fig. 3B). Therefore, DEHP significantly reduced both the length and weight of guppy fish without changing their Fulton's condition factors in most cases (particularly for 1 µg l$^{-1}$ DEHP).

Figure 2C indicates that the survival of guppy fish treated with 10 µg l$^{-1}$ DEHP appeared lower than in the remaining groups. At the end of our experiment (91 days), 79 and 85% of the H$_2$O and DMSO control fish, respectively, had survived. By contrast, the survival rate for fish exposed to 0.1, 1, and 10 µg l$^{-1}$ DEHP was 78, 70 and 35%, respectively. A statistical analysis using a $\chi^2$ test showed a significant DEHP effect ($P_{\chi^2} = 0.001$), and the $\chi^2$ residual values revealed that the mortality was increased for 10 µg l$^{-1}$ DEHP but neither for 0.1 nor 1 µg l$^{-1}$ DEHP.

As shown in Fig. 2, we found a dose-dependent influence of DEHP, both on the length and weight of the guppies. For each DEHP concentration, a separate fish tank had been used; the guppies within each aquarium were thus, by statistical means, not completely independent of each other (Hurlbert, 1984), which is not ideal for an ANOVA. To examine our data in a statistically more robust form, we applied a linear regression (cf. Material and Methods) for 91-day-old fish and found a highly significant DEHP effect, thus rejecting the $H_0$ hypothesis $\beta = 0$ (no influence of DEHP) both for the length and weight of the fish ($P = 0.001$ and $P = 0.003$, respectively). Similar data were obtained for all other time points, with $P$-values from 0.0002 to 0.03. Taken together, these data thus corroborate our conclusion that DEHP reproducibly and dose-dependently inhibits the growth of young guppy fish.

To assess whether DEHP differently affects male and female guppies, we analyzed the fish more closely at 49 days of treatment, i.e. a time point when there were still $\geq$20 guppies for...
each DEHP treatment (Fig. 2C). Male and female guppies – differentiated based on their coloring, shape of the anal fin (gonopodium) and presence or absence of a gravid spot – were identified and measured. As shown in Fig. 1, both male and female guppies responded to the DEHP treatment with a dose-dependent reduction in length. Interestingly, the DEHP-mediated inhibition in fish growth was more prominent for the female guppies, where 0.1 µg l⁻¹ DEHP already caused a highly significant reduction, which also occurred at 1 and 10 µg l⁻¹ DEHP (Fig. 1). A significant effect on male guppies, by contrast, was only observed at 1 and 10 µg l⁻¹ DEHP. The sexual development was not shifted towards a specific gender as the ratio of male vs female guppies was reverse for 1 and 10 µg l⁻¹ DEHP (Fig. 1). All together, our data show that DEHP exerts an inhibitory effect on both male and female guppies, with female guppies possibly being more susceptible than male guppies.

DISCUSSION

Our study shows a prominent inhibitory effect of DEHP on the growth of guppy fish. There are only few other long-time incubation studies assaying an effect of DEHP on the growth of fish. While one publication using an environmentally unrealistic high DEHP concentration (554 µg l⁻¹) describes a growth inhibition in larval Japanese Medaka (Defoe et al., 1990), other reports testing DEHP concentrations that have been used in our study (≤10 µg l⁻¹) for 10–126 days detected no effect either on larval rainbow trout (Oncorhynchus mykiss) (Mayer et al., 1977; Cohle and Stratton, 1992) or on juvenile fathead minnows (Pimephales promelas) (Adema, 1981). Interestingly, Adema (1981), exposing 3–4-week-old guppies for 4 weeks to an abnormally high DEHP concentration (320 µg l⁻¹), could not find any significant effect on the fish growth. We thus conclude that the age of the fish – larvae (<1 week) vs more adult (3–4 weeks) – at the start of the DEHP treatment may play a crucial role in determining whether DEHP inhibits their growth or not. Alternatively, as DEHP effects may be temperature-dependent (cf. below), the fact that Adema (1981) conducted the experiments at 23 °C (whereas our experiments were carried out at 28 °C), might have masked any DEHP effects. In any case, under ‘natural’ conditions DEHP will be present from the early embryonic stage on.

The mechanism by which DEHP inhibits the growth of guppies remains unknown. DEHP is a member of the phthalate group of plasticizers and has been shown to interfere with various hormone systems (Oehlmann et al., 2009). In our study we found that 1 µg l⁻¹ DEHP significantly reduced both the length and weight of guppy fish over a period of 14–91 days without affecting the Fulton’s condition factor in nine out of 10 time points (Fig. 3B). We thus conclude that DEHP delays the development of guppy fish rather than it changes their general health, which is consistent with a hormonal activity of DEHP.

After 91 days of treatment, our data show an increased guppy fish mortality with 10 µg l⁻¹ DEHP only. This concentration is below the toxic DEHP concentration determined for various fish species (Swedish Chemicals Agency, 2008) and may have various reasons. For example, smaller fish may generally have been more affected by our treatment including regular anesthetization once per week, and its combination with 10 µg l⁻¹ DEHP and might have had a particularly detrimental effect. Also, because the solubility limit of DEHP in water is 3 µg l⁻¹ (Staples et al., 1997), DEHP could have separated and floated as a thin film on top of the aquarium water, thereby limiting the oxygen gas exchange at the surface.

The rather strong DEHP influence occurring in our experiment, as compared with other studies using embryonic and larval (<1 week-old) fish (Mayer et al., 1977; Defoe et al., 1990; Cohle and Stratton, 1992) can be explained by the previously described temperature-dependent bioaccumulation of DEHP (Barron et al., 1987), showing that much more DEHP is retained in the fish body at higher rather than lower temperature. In accordance with this finding, we conducted our study at 28 °C, which is higher than the 10 °C used for rainbow trouts (Mayer et al., 1977; Defoe et al., 1990; Cohle and Stratton, 1992). In agreement with the facilitation of DEHP effects by increasing temperature (Barron et al., 1987), other studies – not considered robust enough by the European Risk Assessment (Swedish Chemicals Agency, 2008) – conducted at 25 °C also found an inhibitory effect of DEHP (Chikae et al., 2004a, b; Defoe et al., 1990). Taken together, these and our data indicate that any DEHP effects may be more prominent in warmer rather than cold water.

The solubility of DEHP in water amounts to approx. 3 µg l⁻¹ (Staples et al., 1997). To avoid DEHP being absorbed by any plastic or other items, we used full glass aquariums with nothing else but water. The highest DEHP concentration in our study was aimed at 10 µg l⁻¹, but it is possible that this concentration was not fully reached due to the saturation of water with DEHP. In any case, as compared with our data obtained with 1 µg l⁻¹, a maximally 10-fold higher DEHP concentration resulted in a more pronounced inhibitory effect. Because DEHP degenerates in water over time (Staples et al., 1997), the DEHP concentration in each one of our aquariums may have been somewhat below the theoretically calculated DEHP values. Even more so, the dramatic effect with regard to DEHP inhibiting the growth of guppies at concentrations occurring in our environment is remarkable.

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REFERENCES

Adema DM. 1981. Onderzoek naar een geschikte combinatie toets-methoeden ter bepaling van de aquatische toxiciteit van milieu-gevaarlijke stoffen. RIVM Report no. CL81/100, RIV 627905 001.
Cohle P, Stratton J. 1992. Early life-stage toxicity of DEHP (CAS no. 117–81–7) to rainbow trout (Oncorhynchus mykiss, Walbaum 1792) in a
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