Evidence Suggesting that Di-*n*-butyl Phthalate has Anti-androgenic Effects in Fish

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Abstract–Phthalate ester plasticizers are anti-androgenic in mammals. High doses of certain phthalates consistently interfere with the normal development of male offspring exposed in utero, causing disrupted sperm production, abnormal development of the genitalia, and in some cases, infertility. In the environment, phthalates are considered ubiquitous, and are commonly measured in aquatic ecosystems at low ng to μg per litre concentrations. Given the similarity between mammalian and teleost endocrine systems, phthalate esters may be able to cause anti-androgenic endocrine disruption in fish in the wild. In the present study, adult male three-spined sticklebacks (*Gasterosteus aculetaus*) (*n*=8) were exposed to di-*n*-butyl phthalate (DBP) (0, 15, and 35 μg DBP/L) for 22 d and analyzed for changes in nesting behavior, plasma androgen concentrations, spiggin concentrations, and steroidogenic gene expression. Plasma testosterone concentrations were significantly higher in males from the 35 μg DBP/L group compared to the solvent

control, while plasma 11-ketotestosterone concentrations were not significantly affected. Expression of steroid acute regulatory protein and 3β-hydroxysteroid dehydrogenase remained unchanged. Spiggin concentrations were significantly lower in the males exposed to 35 µg DBP/L. Nest-building appeared to be slower in some males exposed to DBP, but this was not statistically significant. These results suggest that DBP has anti-androgenic effects in fish. However, further research is required to firmly establish the consequences of chronic DBP exposure in fish.

Keywords–Phthalates, Stickleback, Spiggin, Anti-androgen, Plasticizer

Introduction

Phthalate esters (or phthalates) are oily liquids, most commonly used as plasticizers in polyvinylchloride plastics. They are also frequently used in the production of pesticides, medications, cosmetics, and a number of other widely used products [1, 2]. As plasticizers, phthalates impart flexibility [3]. A significant proportion of the end product of a plastic may be phthalate: up to 40% of the final weight in some instances. As phthalates are not chemically bound to the plastic, they leach out over time [4]. Phthalates are synthesized in very large amounts; in western Europe, approximately 1 million tonnes of phthalates are produced each year (www.phthalates.com).

In general, studies have found that the widespread production and use of phthalates, combined with their level of solubility, means that they are frequently detected in environmental samples. Once present, they mainly accumulate in organic phases such as soil and suspended particulate matter, but dissolve in water as well. From surface waters, phthalates can readily bioconcentrate in fish [5].

Phthalates are considered ubiquitous in the environment, and have been identified in soil, atmospheric, and aquatic samples worldwide [6, 7]. They have been detected in surface waters in Australia, Canada, the United States, Portugal the Netherlands, France, the United Kingdom, China, Nigeria, and Malaysia, and have generally been measured in the low ng/L to µg/L range [8-10]. At times, concentrations have been reported to reach tens to hundreds of µg/L, and in some cases, high mg/L concentrations [11, 12].

For a long time, phthalates were considered innocuous environmental contaminants with no specific biological activity. However, the discovery that some phthalates were weakly estrogenic in vitro [13] led to an intense period of study of

phthalates as potential endocrine disruptors. After much research, it is now firmly established that di-ethylhexyl phthalate (DEHP), DBP, benzyl-butyl phthalate (BBP), di-*iso*-butyl phthalate, di-*iso*-nonyl phthalate, di-hexyl phthalate, and di-pentyl phthalate are anti-androgenic endocrine disrupters in mammals [14, 15]. However, it appears that they do not act directly as androgen receptor antagonists, or interfere with the genetic expression of the androgen receptor [14, 16]. Further, they are not considered to be estrogenic in vivo, despite the finding that DBP binds to the estrogen receptor at very high concentrations in vitro [17]. The precise mechanism by which phthalate esters exert anti-androgenic effects is currently unknown. Despite this, several studies have demonstrated that when DBP, DEHP, BBP, and di-*iso*-nonyl phthalate are administered to pregnant female rats during a critical window of testicular development (between gestation days (GD) 12-21), they induce a spectrum of effects in male offspring characteristic of in utero androgen disruption, similar to *Testicular Dysgenesis Syndrome* in humans. Such symptoms in male rat offspring include downregulation of several steroidogenic genes and reduced testicular and serum testosterone concentrations [14-16, 18].

The down regulation of several genes in steroidogenesis is thought to be central to phthalate ester-induced anti-androgenic disruption, resulting in reduced testicular testosterone production. There are several steroidogenic genes that have been observed to be down regulated following phthalate exposure. Steroid acute regulatory protein (StAR), which regulates the transport of the steroid precursor, cholesterol, into the mitochondria is one of the most commonly down-regulated genes following phthalate exposure at high doses in mammals (500 mg/kg/d). 3β-Hydroxysteroid dehydrogenase

(3β-HSD), a catalytic enzyme involved in converting pregnenolone to progesterone among other reactions has also been found to be down-regulated following phthalate exposure, and is one of the most sensitive genes to phthalate exposure in rats (<50 mg/kg/d). Other genes commonly affected include cholesterol side-chain cleavage enzyme, and 17α-hydroxylase [15, 18].

Since testosterone is critical to the normal development and masculinisation of the male reproductive tract in utero, its reduced production is thought to result in the abnormal development of the internal and external genitalia, symptoms of which include reduced anogenital distance, nipple retention, dysgenic testicular tissue, reduced spermatogenesis, cryptorchidism, and hypospadias [14-16].

Due to the presence of phthalate esters in surface waters and the highly conserved vertebrate endocrine system, we aimed to investigate the potential anti-androgenic activity of the phthalate ester, DBP, in the adult male three-spined stickleback (*G. aculeatus*), in order to determine if phthalate esters may be contributing factors to the endocrine disruption observed in wild fish populations [19, 20]. Previous studies of the potential effects of phthalates in fish have been conducted, including specific investigations into their effects on reproduction. However, they have shown varied and inconsistent results [21-24]. Despite being anti-androgenic in mammals, very few of these studies have investigated whether or not phthalates have similar effects in fish.

The aim of the present experimental study was to assess whether or not DBP exposure would alter the onset of breeding condition, nesting behaviors, plasma androgen and spiggin concentrations, and steroidogenic gene expression in adult male three-spined sticklebacks in a 22 d study.

Methods

The design of the present study was based on previously conducted nesting studies [25]. In brief, quiescent male three-spined sticklebacks were placed into individual tanks (3.8 L) for three weeks and exposed to DBP via a flow-through system. Reproductive behavior (nest building) was monitored during the exposure period, and various biochemical and molecular endpoints were measured at the end of the experiment. The fish were maintained in the laboratory under British Government Home Office Licence, as specified under the 'Animals (Scientific Procedures) Act 1986'. Consent for the work was granted by the local ethics committee.

Animal husbandry

Three-spined sticklebacks (n≈80) were obtained from the wild on August 19th, 2008, from a series of ponds near Hook, Hampshire, U.K. (51°18' N 0°55'W) (Moore & Moore Carp Suppliers, UK). The fish were divided by sex; males were subjected to *winter* conditions with a photoperiod of 8:16 h light:dark, a light intensity of approximately 50 lux, and temperatures of approximately 18 to 20°C. These conditions would be expected to lead to sexual immaturity.

Exposure system

The experimental system consisted of three treatments: a solvent (methanol) control, and nominal concentrations of 50 and 100 μg DBP/L (Fisher Scientific, UK). The experiment was conducted over 22 d (March 9th to 31st, 2009, n=8 fish per

treatment group). Stock solutions of methanol, 2.5 g DBP/L and 5 g DBP/L (dissolved in methanol) were pumped into distribution trays at a rate of 0.01 ml/min, where they were mixed with carbon-filtered, dechlorinated water flowing at 500 ml/min. The DBP-treated water from each distribution tray then flowed into nine separate tanks, eight tanks containing fish and another empty tank receiving solvent control or DBP-treated water, termed the analytical chemistry tanks (8 L). These three analytical chemistry tanks were used to collect water samples from each treatment group for analytical chemistry without disturbing the fish. The system was set up 3 d prior to the beginning of the experiment to allow the concentrations of DBP to stabilize before adding the fish. The retention time of the water in the fish tanks was approximately 1 h.

On day 0 of the experiment, single male fish from the stock tank were randomly added to each of the 24 experimental fish tanks. The photoperiod was extended to 16:8 h light:dark per day, and the light intensity was increased to approximately 1000 lux. The temperature was maintained at 20°C, and each tank was aerated. These environmental conditions were implemented in order to bring the fish into full sexual maturation.

Fish were fed three times daily, twice with frozen bloodworm and once with ZX 400 Fish Food (ZM Systems, UK). The males were left to acclimatize to these new conditions for 10 d. On day 10, nest-building material was added (50 ml of clean gravel, 30 ml of silica sand, a handful of black and green polyester thread, and one red thread), and males were visually isolated from one another. Subsequently nest building was documented in each tank and recorded with a photograph every second day. Males typically added the red thread to the entrance of the nest, which helped to indicate nest completion. On days 16 and 20, the males were visually exposed to a gravid female

contained in a beaker for 5 min, to encourage nest maintenance and/or building [25]. On day 22, the experiment was terminated.

Fish were anaesthetized and blood was sampled from the caudal peduncle using a heparinized capillary tube and stored on ice until further processing. The gonads were removed, weighed, and snap frozen in liquid nitrogen for subsequent analysis of gene expression. The kidney was also excised for the measurement of spiggin concentration and stored first on ice, and at -20°C thereafter. The blood samples were spun in the centrifuge at 12,500 x *g* for 5 min, and blood plasma was drawn off and decanted into clean vials and stored at -20°C.

Water chemistry

Water samples were collected from the solvent control, 50 and 100 µg DBP/L analytical chemistry tanks for analysis of DBP concentrations every 3 to 4 d. Five hundred millilitres of water was collected from each of the solvent control and 50 µg DBP/L analytical chemistry tanks, and 250 ml was collected from the 100 µg DBP/L tank. All samples were kept at 4°C until they were spiked with 100 µl BBP as the internal standard (2.5 g BBP/L methanol) and liquid-liquid extracted three times with dichloromethane at a 1:10 ratio (dichloromethane:water). Quality control samples of the DBP stock solution and BBP internal standard were also prepared in triplicate for gas chromatography/mass spectrometry (GCMS) analysis by aliquoting 10 and 100 µl of each, respectively, into glass vials. All samples were dried under a stream of nitrogen gas at room temperature, reconstituted in 1 ml hexane, and stored at 4°C until analyzed by GCMS (Perkin-Elmer, UK). Thus, the solvent control and 50 µg DBP/L samples were

concentrated 500-fold, and the water samples from the 100 µg DBP/L were concentrated 250-fold for analysis on the GCMS. The samples were analyzed twice; once chronologically by treatment group, and a second time in random order.

A volume of 1.0 µl of each sample was injected in the split mode (1:2 ratio) at 250°C into a 30 m x 0.25 mm x 0.25 µm BPX5 column (SGE Analytical Science, Australia). The helium carrier gas flowed at a rate of 1 ml/min. The following temperature programme was used: 50°C for 1 min, increased at 10°C/min to 285 °C, and maintained at 285°C for 10 min. The quadrupole mass spectrometer operated in electron impact ionization mode at 70 eV. The transfer line and the injector were set up at 250°C and the source was 180°C. Measurements on the GCMS were performed in the single-ion monitoring (SIM) mode: 163 *m/z* for dimethyl phthalate, and 149*m/z* for diethyl phthalate, DBP, BBP, DEHP, and di-*n*-octyl phthalate (to ensure no contamination was occurring). Each phthalate was identified by its specific elution time. The peak areas of each of the phthalates in the extracted water samples were quantified by comparing them to the serially diluted standards with identical elution times (Phthalate Esters Mix, 2000 µg/ml in hexane, Sigma-Aldrich, USA) diluted in hexane (2.5, 5, 10, 20, and 40 mg/L) (stored at 4°C). Standards were run at the beginning and end of each run, with one standard repeated every five samples. The limit of quantification (LOQ) was set to 20% of the peak area of the lowest standard concentration (2.5 mg/L). Hence, concentrations as low as 1 µg/L in water could be quantified after concentrating the water sample 500-fold.

Blood plasma and kidney sample processing

11-Ketotestosterone (11-KT) and testosterone concentrations were measured in the blood plasma of the fish using radioimmunoassay [26]. Spiggin concentrations of kidney samples were measured using an enzyme-linked immunosorbent assay (ELISA) [27].

RNA extraction

Testes were homogenized and RNA was extracted using the Rneasy Kit according to the manufacturer's instructions (Qiagen, UK). The purity of each RNA sample was measured by spectrophotometry (Nanodrop™ , Fisher Scientific, UK). All samples were stored at -80°C.

cDNA transcription

mRNA was converted to cDNA with the Superscript II First-Strand Synthesis Kit (Invitrogen™ , Paisley, U.K.) according to the manufacturer's instructions, and stored at -20°C. cDNA sample quality was verified by reverse transcriptase polymerase chain reaction (RT-PCR) (94°C for 5 min, 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, followed by 72° for 5 min, and 4°C until removed) with β-actin primers (Forward 5' – GAT ATG GAG AAG ATC TGG C- 3' and Reverse 5' – GTT GGC TTT GGG GTT CAG G – 3') (T. Runnalls, Institute for the Environment, personal communication) using Accuprime Taq DNA Polymerase System (Invitrogen™). These sequences were a 100% match for homology with the three-spined stickleback partial β-actin sequence (DQ018719.1) using the NCBI Basic Local Alignment Search Tool (BLAST). The predicted product size (101 base pairs) was also confirmed by gel electrophoresis (1%).

Real-time RT-PCR (qPCR)

cDNA was diluted to 1:10 in water for qPCR. Real-time PCR reactions (50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min) were prepared in triplicate in 96-well plates (Microamp® Optical Reaction Plates, Applied Biosystems, USA) using SYBR Green Master Mix (Applied Biosystems). Amplification was monitored with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) and then analyzed by the ABI™ software program, SDS 2.1. Forward and reverse primers for qPCR for 3β-HSD and StAR (ENSGACT00000001847 and ENSGACT00000015623, respectively) were designed using the Primer Quest program (eu.idtdna.com). Primer sequences for 3β-HSD were Forward 5' – TCA GTT ACT CGG ACT TCA ACC ACG – 3' and Reverse 5'– ACC TCC ATC AGG AAG CAG AAT GAG – 3', and for StAR were Forward 5' – GGT GGG ACC GAG GGA CTT TG – 3' and Reverse 5'– GTT GGT GTG CTG AGT TGA CAT T – 3'. Product sequences were confirmed to be >80% homologous with both target genes. Dissociation curves were run after each qPCR run to ensure no interference by primer-dimers or contamination had occurred. The expression of the target gene transcripts were quantified relative to the expression of the β-actin gene using the Relative Expression Software tool (REST©). Specifically, the formula $E_{target}^{(\text{control Ct} - \text{treated Ct})} / E_{ref}^{(\text{control Ct} - \text{treated Ct})}$ was used for each target sample, where the cycle thresholds (Ct) were the mean of the three replicate samples and E was the efficiency of each primer pair. The efficiencies of the StAR, 3β-HSD, and β-actin were each determined by running them in a qPCR assay with serially diluted cDNA (ranging from 1 to 10^6 times) [28].

Statistical analysis

Normality of the data was tested using a Kolmogorov Smirnov test. One-way analysis of variance was used to analyze normally distributed data with Holm-Sidak test for pair-wise comparison. Kruskal-Wallis one-way analysis of variance (ANOVA) on Ranks with Tukey Test was used to analyze all nonparametric data. Kaplan-Meier Survival Analysis Log-Rank was used to determine if there were any significant differences in the timing of nest-building. Pearson product moment correlation was used to compare testosterone and 11-KT concentrations in the plasma. Statistical differences in gene expression between fish from the solvent control and those exposed to 50 or 100 μg DBP/L was calculated by REST© software with two thousand random allocations performed [28]. Significance was set at α=0.05.

Results

Water chemistry

Concentrations of DBP in each water sample were calculated based on the percent recovery of the BBP internal standard. One hundred percent recovery was assigned as the mean BBP concentration of the quality controls (27.12 ± 2.53 mg/L, mean ± standard deviation (SD) of a nominal concentration of 25 mg/L).

The solvent control water samples appeared to be free from significant phthalate contamination. Three of twelve solvent control samples had detectable traces of DBP but they were all below the LOQ, and thus are considered negligible. Two of these samples

appeared to be the result of carry over and none of the duplicate samples confirmed these results on the same day.

Five of 36 samples had DEHP contamination above the LOQ, at concentrations of 2.46 ± 0.08 μg DEHP/L (mean ± SD). These were collected from the solvent control (two of 12 samples), 50 μg DBP/L (one of 12 samples), and 100 μg DBP/L treatment tanks (two of 12 samples). Again, none of the duplicate samples confirmed these concentrations on the same day. Five other samples were found to have trace level DEHP contamination, but these were well below the LOQ. Again, two of these samples appeared to be due to carry-over on the GCMS.

Overall, DBP was detected at quantifiable concentrations in all DBP-treated tanks, although the measured concentrations of DBP were lower than their nominal values (**Figure 1**). The mean measured concentrations of the 50 and 100 μg DBP/L tanks during the experiment were 15.23 ± 6.28, and 35.20 ± 8.03 μg DBP/L, respectively. Thus, the nominal concentrations were considered to be inaccurate, and mean measured concentrations will be used throughout the remainder of the manuscript.

Length, weight, and gonadosomatic index

No significant differences in weight, length, or gonadosomatic index were noted ($p>0.05$, degrees of freedom (*df*) 2, ANOVA on Ranks).

Plasma androgen concentrations

The concentrations of 11-KT in the plasma of DBP-exposed males were slightly higher in the fish exposed to 35 μg DBP/L but this was not significantly different from

those in the solvent control group (p =0.372, df 23, ANOVA) (**Figure 2a**). However, plasma testosterone concentrations were significantly higher in the fish exposed to 35 μg DBP/L compared with those from the solvent control group (p =0.007, df 23, ANOVA) (**Figure 2b**). Due to the slightly higher variation in the testosterone concentrations in the fish exposed to 35 μg DBP/L, the data were also log-transformed. Again, the log-transformed plasma testosterone concentrations were significantly higher in the fish exposed to 35 μg DBP/L (p =0.014, df 2, ANOVA) than in the fish in the solvent control (data not shown). Concentrations of testosterone and 11-KT in the plasma were also significantly positively correlated with one another in all groups (p<0.01, Pearson Product Moment correlation, data not shown).

Kidney spiggin concentrations

There was one outlier in the spiggin data: a very low spiggin concentration in one of the solvent control males. This was considered to be an outlier because it was more than two standard deviations from the mean. Further, it was both the first kidney sample to be collected by the laboratory technician and the first sample in the spiggin ELISA run. Thus, it is possible that either inaccurate sample collection or an anomaly at the beginning of the ELISA may have resulted in its low spiggin concentration. Once omitted, the results showed a strong negative correlation between spiggin concentration and DBP treatment, which was significant at the highest DBP concentration (p =0.011, df 22, ANOVA) (**Figure 3**).

Nest-building behavior

Nest building appeared to be slightly delayed in the males exposed to the highest concentration of DBP. All eight males in the solvent control group had completed their nests by day 15. In contrast, three males in the highest treatment concentration of DBP (35 μg DBP/L) did not build nests until day 22, the last day of the experiment. However, there were no statistically significant differences between the rates of nest-building in males exposed to DBP compared to rates of the solvent control group (p =0.355, df 2, Kaplan-Meier Survival Analysis). Five males from both the solvent control and 15 μg DBP/L groups, and four from the 35 μg DBP/L treatment group, had all built nests within 24 h of the addition of nesting material (**Figure 4**).

Real-time RT-PCR

The expression of the β-actin reference gene was found to be stably expressed across all treatment groups. Mean ± standard error of the mean (SEM) Ct values for this gene were 17.14 ± 0.14, 17.28 ± 0.17, and 17.40 ± 0.19 in the solvent control, 15, and 35 μg DBP/L groups, respectively.

The expression of the steroidogenic genes, StAR and 3β-HSD, relative to β-actin, did not appear to be affected by exposure to DBP. There were no significant differences between the expression levels of either gene in the testes of the males exposed to any concentration of DBP compared to controls (p = 0.915 for StAR, and p =0.975 for 3β-HSD) (both df 23, ANOVA).

Discussion

The use of wild fish for experimentation

The use of wild fish in the present experiment was a potential risk, due to potential differences in age and life history of the fish used. For example, it is possible that some, or all of the fish used in the current experiment had been exposed to contaminants during critical stages in development and this that may have later affected their behavior and/or physiology. However, since the fish spent 201 d in the laboratory prior to the experiment, we assume that chemicals bioconcentrated in tissues would likely have been eliminated by the beginning of the experiment. Further, the random distribution of the males within the treatment groups should have mitigated any potential confounding factors between individuals. It must then be assumed that any differences between the treatments are attributable only to the effects of DBP.

Water chemistry

Phthalates are particularly difficult to administer in water at stable concentrations [23, 29]. Further, the ubiquity of phthalates made contamination of the water a potential problem during the exposure period. The finding of low levels of DBP and DEHP in some of the control water samples may be considered by some to be cause for concern. However, DBP was not detected above the LOQ in the solvent control samples and the small amounts that were present were likely to have emanated from carry-over on the GCMS, not from the tank water itself.

Di-ethylhexyl phthalate, however, is much more ubiquitous in the environment. While DEHP was detected at measurable levels (2.61 ± 0.08 μg/L) in five samples, this was not confirmed by the duplicate water samples. Thus, it is thought that the contamination arose during the extraction/analysis procedure and not from the tank water.

Regardless, it is the authors' opinion that the contamination was at such low concentrations and in so few samples as to be considered insignificant in biological terms. These findings do, however, underline the care with which both an experiment itself and the analytical chemistry must be undertaken to eliminate all sources of contamination when carrying out ecotoxicological research using phthalates.

Overall, the analytical results suggest that the fish in the (nominal) 50 and 100 μg DBP/L tanks were exposed to distinct ranges of concentrations of DBP throughout the experiment and that the solvent control was relatively free of phthalates (Figure 1). While the measured concentrations of DBP were not from water sampled directly from the fish tanks (in order to minimize disturbance to the fish) they are considered to be accurate representations of the concentrations of DBP to which the fish were exposed.

Physiological effects of DBP on the adult male three-spined stickleback

DBP exposure did not have any effect on fish length, weight, or gonadosomatic index during this relatively short-term experiment. This supports the findings of the previous experiments, in which the lengths, weights, and gonadosomatic indices of both the fathead minnows and three-spined sticklebacks exposed to various concentrations of phthalate ester were unaffected by exposure [24].

Plasma hormone concentrations, spiggin concentrations, and real-time RT-PCR

The results of the real-time PCR analysis, hormone concentrations, and spiggin assay suggest a somewhat complex picture. DBP-exposure was associated with increases in the plasma concentrations of 11-KT and testosterone, which was significant for

testosterone only (Figure 2), steroidogenic gene expression appeared to be unaffected, and spiggin concentrations were significantly reduced (Figure 3).

In fish, only one in vivo exposure study has examined the effects of phthalates on plasma hormone concentrations [21]. In that study, an unknown number of Common carp (*Cyprinnus carpio*) were exposed to 0, 5.5, 10.5, 15.5, and 20.5 mg DEHP/L in a recirculated system for 48 h. Strong concentration-related increases in the plasma concentrations of 11-KT and testosterone in the pooled blood samples of DEHP-treated fish were observed compared to the solvent control group. This increase was significant in the 15.5 and 20.5 mg/L concentration groups (n=1, p<0.05). However, in that study water chemistry was not conducted to confirm exposure concentrations, and more importantly, all reported concentrations far exceeded the saturation concentration of DEHP in water, which is only 3 μg/L. It is possible that such responses are genuine; however, those data should be treated with some caution.

Although several mammalian studies have also reported elevated plasma androgen concentrations following phthalate exposure at lower doses [30, 31], the majority of studies report significant decreases in plasma testosterone concentrations at doses of >500 mg/kg/d [32, 33]. It is currently unclear why apparently contradictory effects occur. Interestingly, differences in steroidogenic gene expression and plasma hormone concentrations have also been found. Reports of significantly increased expression of steroidogenic genes, including StAR, have been reported in phthalate-exposure studies in rats when plasma testosterone concentrations were found to be significantly reduced [32, 33]. Complicating interpretation of the results reported here is the fact that the control of steroidogenic gene expression is still poorly understood in fish.

Previous studies with fathead minnows (*Pimephales promelas*) found that the expression of both StAR and 3β-HSD were significantly altered by both age and GSI [34]. Even when these factors were taken into account, the exposure of fathead minnows to the anti-androgen ketoconazole (4, 25, 100, and 400 μg/L) resulted in significantly increased expression of StAR in the testes of male fathead minnows exposed to concentrations of 6 and 400 μg/L. In that study, 20β-hydroxysteroid dehydrogenase and cytochrome b5 were also significantly upregulated (at 400 μg/L), but the expression of other genes including 3β-HSD and 11β- hydroxysteroid dehydrogenase were unchanged [34]. It is possible that in the present study other steroidogenic genes, such as cholesterol side-chain cleavage enzyme may have been altered in their expression. It is also possible that higher concentrations of DBP are required to elicit an effect.

It is also important to note that in the present study β-actin was used as a reference gene although its use in this capacity is controversial. A study of the fathead minnow found that expression of β-actin in the gonads was significantly downregulated in response to 17α-ethinylestradiol (EE2) exposure [35]. However, a similar study of zebrafish (*Danio rerio*) found that β-actin was one of the most stably expressed genes in the gonads following chemical exposure [36]. In the present study, β-actin expression appeared to be unaffected by DBP-exposure, and thus it is considered to be an appropriate reference gene for the analysis of StAR and 3β-HSD expression.

In contrast to plasma androgen concentrations, spiggin concentrations were strongly reduced, an effect that was significant at the highest DBP concentration (Figure 3). Spiggin is a sensitive biomarker of androgenic disruption, and concentrations are able to undergo 100,000-fold increases in response to androgen exposure [27, 37]. The half-

life of spiggin in the three-spined stickleback is unknown, but it is estimated that it requires one to three months for it to be degraded to background levels in sexually mature males (I. Katsiadaki, personal communication). Several studies have shown that spiggin concentrations are consistently reduced following anti-androgenic endocrine disruption in both in vitro and in vivo assays [37, 38] and can be measured within 10 d of induction in vivo [27]. Estrogenic chemicals are unable to induce spiggin production [38, 39], but at very high concentrations 17β-estradiol, bisphenol A (10^{-6} M each), and nonylphenol (10^{-8} M), have been found to reduce spiggin concentrations in vitro by antagonizing the activity of dihydrotestosterone [38]. The authors hypothesized that this effect may have been due to interference with the androgen receptor, which has been observed previously with these chemicals at high concentrations in vitro [38]. While DBP has been found to be very weakly estrogenic in vitro (10^{-7} times less potent than 17β-estradiol), there is little sound evidence to support an estrogenic mechanism of action in vivo [17] . Based on its low estrogenic potency, paired with the high concentrations of 17β-estradiol required to reduce spiggin production in vitro [38], it is highly unlikely that the reduction observed in spiggin concentration in fish exposed to 35 μg DBP/L in the present study was due to an estrogenic mode of action. Rather, the concentration-related reduction in kidney spiggin concentrations reported here is indicative of an anti-androgenic mode of action of DBP in fish as is observed in mammals.

It remains unclear as to why the plasma androgen concentrations in the three-spined sticklebacks exposed to DBP in the present study were found to increase (significantly, in the case of testosterone), while spiggin concentrations were significantly decreased. This may be due to the unpredictable responses of plasma androgens to

phthalates, to anti-androgens in general, and/or to the inherent fluctuation of plasma concentrations both in general and in response to spawning stage [40].

Despite the variable and unpredictable effects of phthalates on plasma androgen concentrations in both fish and mammals, what remains clear is that endpoints dependent on androgen concentrations are substantially more consistent in their response to anti-androgenic chemicals than the androgen concentrations themselves. For example, in male rats exposed to phthalates in utero, reduced anogenital distance, nipple retention and agenesis of the Wolffian ducts are consistently observed [14, 15]. While these effects do not occur with 100% frequency, their dependence on androgens supports the suggestion that phthalates cause anti-androgenic effects in vivo. In a similar manner, the result of reduced spiggin concentrations in DBP-exposed fish in this experiment provides strong support for the hypothesis that DBP acts as an anti-androgen in fish, despite increases in plasma testosterone concentrations and the absence of changes in steroidogenic gene expression. However, this conclusion is possibly countered by a lack of effect on other androgen-dependent parameters in the present study, such as nest-building (Figure 4). While some delay appeared to have occurred in the nest building activity of a few males in the 35 μg DBP/L group, overall the males across all groups built nests at a similar rate and were successful in maintaining them (except one male in the 15 μg DBP/L group). This suggests that phthalate exposure at concentrations of 15 and 35 μg/L does not significantly impede the nest building behaviors of the male three-spined stickleback, at least in the short-term.

It remains unknown whether or not adult male three-spined sticklebacks exposed to DBP would have been able to spawn successfully, and also if they would have been

capable of competing for females. Considering that the kidney spiggin concentration was significantly reduced in males exposed to these low phthalate concentrations, it is possible that higher concentrations of DBP may interfere with the ability of these fish to reproduce completely. For example, at higher concentrations DBP may reduce spiggin production to such an extent that males may not be able to build nests and, ultimately, to spawn. This merits further investigation.

Conclusions

Di-*n*-butyl phthalate exposure significantly increased plasma testosterone concentrations, and significantly reduced spiggin concentrations in male three-spined sticklebacks exposed to 35 μg DBP/L after 22 d. Thus, DBP appears to be able to act as an endocrine disruptor in sexually mature three-spined sticklebacks at concentrations that have been measured in the aquatic environment. The results suggest that DBP is anti-androgenic to fish, as it is to mammals. Further research is warranted to confirm the mechanism of action and to determine the full repertoire of anti-androgenic effects of phthalates on fish.

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Figure Legends

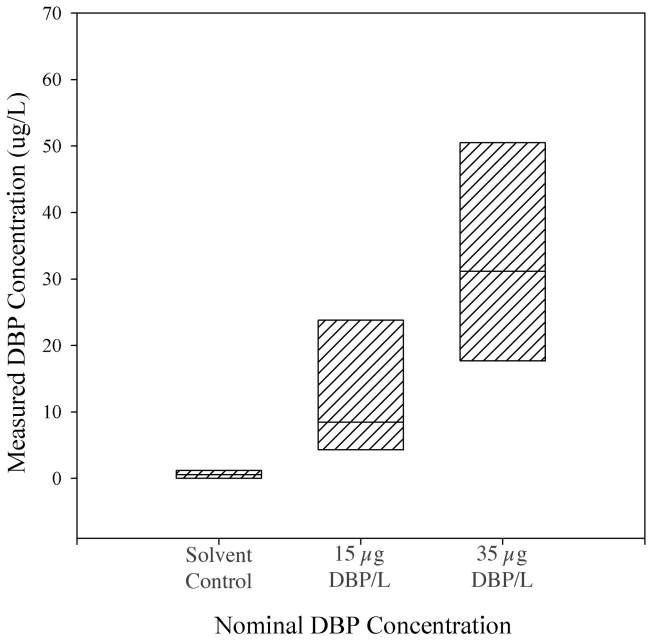
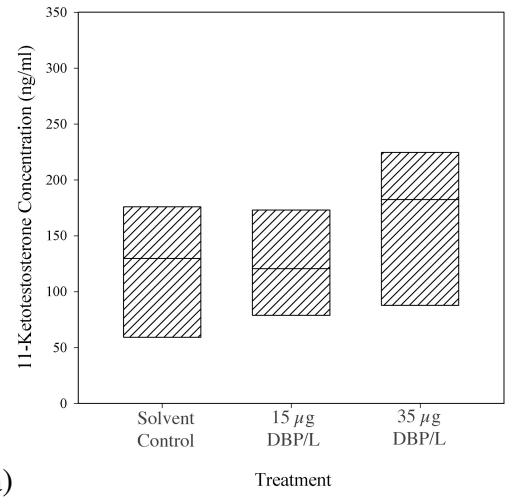
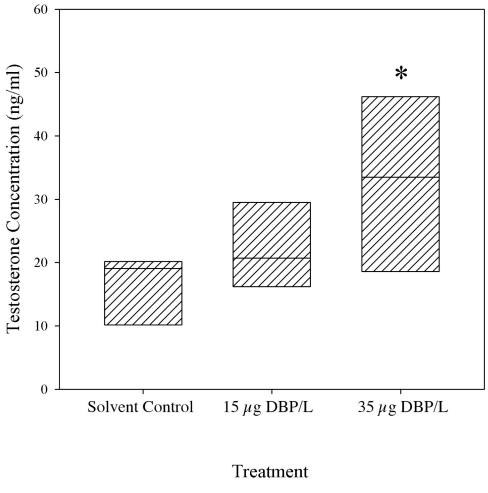


Figure 1. Box plot (25th and 75th percentiles about the median) of the measured di-*n*-butyl phthalate (DBP) concentrations in the water samples collected from the experiment exposing adult male three-spined sticklebacks to DBP for 22 d.



(a)



(b)

Figure 2. Box plots (25th and 75th percentiles about the median) of the plasma concentrations of (**a**) 11-ketotestosterone, and (**b**) testosterone in male three-spined sticklebacks exposed to various concentrations of di-*n*-butyl phthalate (DBP) for 22 d (*n*=8, * $p<0.05$ compared to solvent control group).

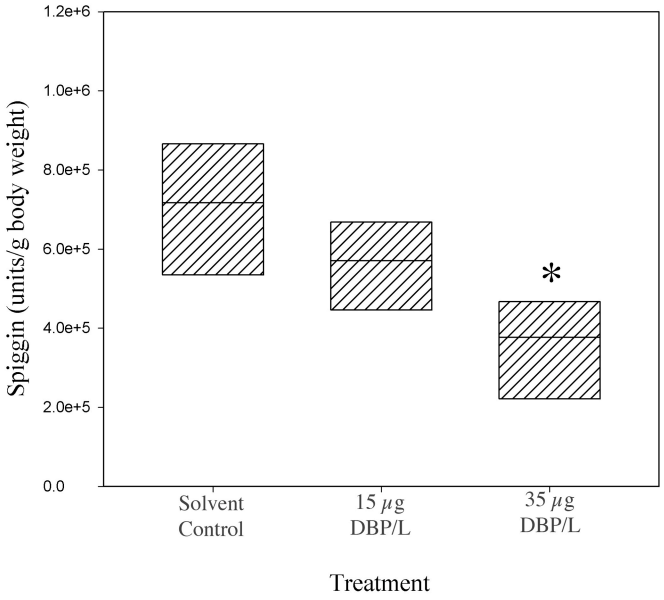


Figure 3. Box plot (25th and 75th percentiles about the median) of spiggin concentrations in male three-spined sticklebacks exposed to various concentrations of di-*n*-butyl phthalate (DBP) for 22 d (*n*=8, *$p<0.05$ compared to the solvent control group).

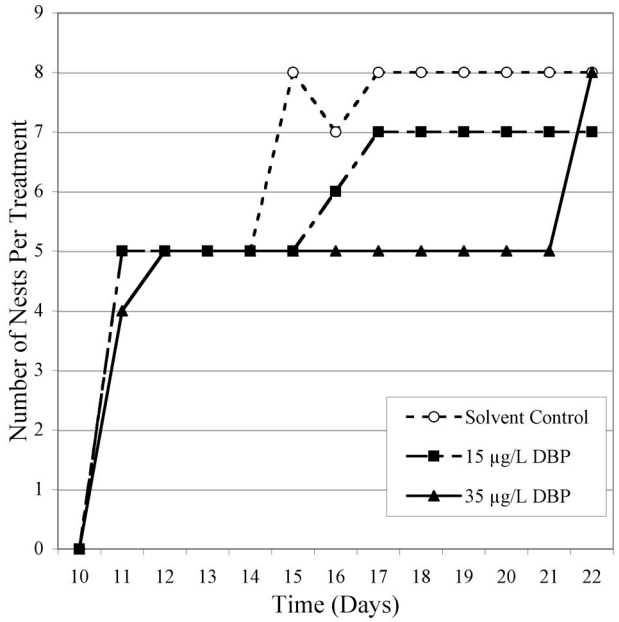


Figure 4. Cumulative number of nests in each treatment group over time, during an experiment exposing adult male three-spined sticklebacks to various concentrations of di-*n*-butyl phthalate (DBP) for 22 d (*n*=8). Nest building was monitored starting day 10 of the experiment when nest building material was added.