

TESTOSTERONE METABOLISM IN *NEOMYSIS INTEGER* FOLLOWING EXPOSURE TO BENZO(A)PYRENE

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Abstract

Cytochromes P450 (CYPs) are important enzymes involved in the regulation of hormone synthesis and in the detoxification and/or activation of xenobiotics. CYPs are found in virtually all organisms, from archae, and eubacteria to eukaryota. A number of endocrine disruptors are suspected of exerting their effects through disruption of normal CYP function.

Consequently, alterations in steroid hormone metabolism through changes in CYP could provide an important tool to evaluate potential effects of endocrine disruptors. The aim of this study was to investigate the potential effects of the known CYP modulator, benzo(a)pyrene (B(a)P), on the testosterone metabolism in the invertebrate *Neomysis integer* (Crustacea; Mysidacea). *N. integer* were exposed for 96h to 0.43, 2.39, 28.83, 339.00 and 1682.86 $\mu\text{g B(a)P L}^{-1}$ and a solventcontrol, and subsequently their ability to metabolize testosterone was assessed. Identification and quantification of the produced phase I and phase II testosterone metabolites was performed using liquid chromatography coupled with multiple mass spectrometry (LC-MS²). Significant changes were observed in the overall ability of *N. integer* to metabolize testosterone when exposed to 2.39, 28.83, 339.00 and 1682.86 $\mu\text{g B(a)P L}^{-1}$ as compared to the control animals.

Keywords

benzo(a)pyrene, biomarker, cytochrome P450 modulator, *Neomysis integer*, testosterone metabolism

1. Introduction

Endocrine disruption has become a widely investigated and politically charged issue (Colborn, 1996, Krimsky, 2000). Many definitions exist for an endocrine disruptor. The International Programme for Chemical Safety, for example, defines endocrine disruptors as exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations (WHO/PCS/EDC/02.2). You (2004) recently mentioned that a change in bioavailability of steroids through alteration of the steroidogenesis or biotransformation rates, leads to changes in endocrine function.

Endocrine disruption in invertebrates has received increasing attention from the late '90s onwards (deFur et al., 1999). From a workshop organized in 1998, it became clear that most of the knowledge on invertebrate endocrinology, at that time, was limited to arthropods. In general, arthropods rely on peptides, terpenoids, biogenic amines, and steroids as signaling molecules (Quackenbush, 1986). While vertebrate-type steroids (e.g., androgens and estrogens) have been detected in several arthropod species (Verslycke et al., 2002, Janer et al., 2005), a true functional role remains to be discovered (deFur et al., 1999). A number of studies have assessed steroid metabolism in several invertebrate model species. These studies provide novel insights into our understanding of steroid metabolic pathways among invertebrate phyla, but also evaluate species differences in their susceptibility to chemical disruption of steroid metabolism (Verslycke et al., 2003; Janer et al., 2005).

In both vertebrates and invertebrates, enzymatic biotransformations are important processes responsible for the detoxification and elimination of xenobiotics (Baldwin et al., 1994a). By far, the most important enzymes involved in the metabolism and detoxification of xenobiotics are the cytochrome P450s (CYPs), in both vertebrates and invertebrates (James and Boyle, 1998). It has been proposed that differences in chemical susceptibility between animal species can be correlated with differences in the types of CYPs present and their expression (Guengerich, 1997, Sivapathasundaram et al., 2003). James reported that crustaceans have functional CYPs, but that the detailed mode of action of these enzymes might differ from that in vertebrates (James, 1989). Since then, numerous studies have provided an increased understanding of the degree of similarity between enzyme systems in invertebrates and vertebrates (Snyder, 1998). The oxidative metabolism of several invertebrate species has been successfully evaluated using vertebrate-type steroids - generally

testosterone - as a substrate (Baldwin et al., 1994a, Baldwin et al., 1994b, Verslycke et al., 2002, De Wasch et al., 2002, Janer et al., 2005). Recent studies have also used chemically-induced alterations in steroid metabolism as a biomarker for evaluating endocrine-disrupting effects in invertebrates (Baldwin et al., 1994a, 1994b, Baldwin et al., 1997, Oberdörster et al., 1998, Morcillo et al., 1998, Verslycke et al., 2003, 2004).

Neomysis integer (Leach 1814) is an opossum shrimp, belonging to the subphylum of Crustacea and the order Mysidacea. It is one of the most common mysids inhabiting estuarine and brackish water environments along the European coastlines (Tattersall and Tattersall, 1951). *N. integer* has been proposed as an appropriate European alternative to the standard American mysid test species, *Americamysis bahia*, which is not relevant to the lower salinity and colder waters generally found in European estuaries (Emson and Crane, 1994, Roast et al., 1998, Verslycke et al., 2003).

Recently, we reported on the steroid metabolism in *N. integer* (Verslycke et al., 2002), and its alteration through chemical exposure (Verslycke et al., 2003, 2004). Benzo(a)pyrene is a polycyclic aromatic hydrocarbon (PAH), a known CYP modulator, and a known pollutant of aquatic environments (Atienzar et al., 2004). Specifically, B(a)P accumulates and persists in estuarine/marine sediments (Schuler et al., 2003, Rust et al., 2004). The aim of this study was to evaluate the effects of B(a)P on the testosterone metabolism of *N. integer*.

2. Material and methods

2.1. Reagents and chemicals

Benzo(a)pyrene (B(a)P), dimethylsulfoxide (DMSO), testosterone, β -boldenone and methyltestosterone (MT) (the latter used as an internal standard) were obtained from Sigma-Aldrich (St-Louis, MO, USA). Androstenedione, androstadienedione and the different testosterone metabolites (2 α -, 6 α -, 7 α -, 11 α -, 11 β -, 15 α - and 16 α -hydroxytestosterone) were purchased from Steraloids (Newport, RI, USA). All chemicals used for extraction and as eluent during LC-MS analysis were of analytical grade from Merck (Darmstadt, Germany). The solvents and reagents used for the B(a)P analysis were all ultra resi-analyzed[®] quality obtained from J.T. Baker (Deventer, Holland).

2.2. B(a)P analysis in water

B(a)P was extracted using solid phase extraction with Bakerbond Speedisk[®] C18 extraction cartridges (J.T. Baker, Deventer, The Netherlands). The cartridges were rinsed with

10mL ethyl acetate and conditioned with methanol and Milli-Q[®] water (10mL each) for about 1min. The water samples were forced through the disk under vacuum. After passing the water through the cartridges, 5mL of ethyl acetate was added to the cartridge and allowed to soak for 1min, followed by 5mL methylene chloride and two 3mL aliquots of ethyl acetate: methylene chloride (1/1:v/v). The extract was dried by addition of anhydrous sulphate and further cleaned by passing through a column containing 4g of aluminium oxide (5% deactivated). Finally the extract was concentrated to 1mL under a stream of nitrogen gas.

The extracts were analysed on a GC-MS Ion Trap PolarisQ (ThermoFinnigan, Austin Texas, USA) in single ion monitoring mode (SIM). The gas chromatograph was equipped with a Rtx[®]-5Sil MS column (30m, 0.25mm id) (Restek, Bellefonte, PA, USA). Samples (3µL) were injected with a programmable temperature vaporizing injector in the splitless mode with a splitless time of 1.5min. Injection time was 0.1min at an injector temperature of 109°C, followed by a ramp of 10°C min⁻¹ to 350°C. The temperature program of the oven was as follows: initial temperature of 104°C, hold for 2min, ramp to 330°C at 40°C min⁻¹, hold for 10min. Carrier gas and damping gas was Helium with a flow rate of respectively 1.5mL min⁻¹ and 2.5mL min⁻¹.

In order to quantify, an internal standard method was used. To that end, benzo(a)pyrene-D12 was added to the sample before extraction. For identification of the peaks the retention time and ion mass were recorded (mass B(a)P: 252, mass B(a)P D-12: 264). The limit of detection of this method was 1µg L⁻¹.

2.3. Mysid assay

Initial *Neomysis integer* populations were collected by hand net in the Braakman, a brackish waterbody near the Scheldt river in Hoek (The Netherlands). Maintenance of laboratory cultures has been described previously (Verslycke et al., 2003).

Juvenile mysids (4.5mg ± 2.0mg) were taken from the laboratory cultures and randomly placed in 400mL glass beakers (4 animals/ B(a)P concentration, 3 replicates beakers per B(a)P concentration), each containing 200mL of the required B(a)P concentration. The following treatments were tested: a solventcontrol, 0.43, 2.39, 28.83, 339.00 and 1682.86µg B(a)P L⁻¹. The culture and dilution medium was based on commercially available seawater salts (Instant Ocean[®]) dissolved in and diluted with aerated deionised tap water to a final salinity of 5psu. B(a)P was delivered to the exposure solutions in the solvent carrier dimethylsulfoxide (DMSO). DMSO concentrations in the solvent control and the different

B(a)P treatments was 0.05%. Exposures were performed in a temperature-controlled chamber (Liebher®, Laborimpex, Brussels) at 15°C and test solutions were continuously aerated during the experiment. Test solutions were renewed after 48h and animals were fed twice daily with 24h to 48h old *Artemia nauplii*.

Following 96h exposure to B(a)P, mysids were transferred individually into 5mL glass tubes containing 2mL of culture medium. Two µg of testosterone dissolved in 10µL of ethanol was added to the tubes and allowed to incubate for 6h at 15°C.

2.4. Extraction and Clean-up procedure

Both the resulting test medium and the organisms were extracted to evaluate the presence of testosterone metabolites.

Organisms were collected from the incubation medium, dried on a tissue, transferred into a previously weighed 1.5mL eppendorf vial and shock frozen in liquid nitrogen. The weight of the organism was recorded. 100µL of milliQ water was added to facilitate homogenisation with a motor-driven teflon homogeniser. Following homogenisation, 12ng of internal standard - methyltestosterone - and 1mL of ethyl acetate were added to perform the extraction. The mixture was vortexed and centrifuged (5min, 14000g) and the supernatant was transferred to a conical glass tube. This extraction step was repeated once. Next, both extracts were subsequently combined and vacuum-evaporated to dryness. The dry residue was reconstituted in 30µL of methanol and 90µL of 0.02M formic acid. Fifty µL were injected into the LC-MS system.

The medium was extracted twice with 2mL of ethyl acetate after adding 12ng of methyltestosterone. Evaporation to dryness, reconstitution and injection on column were similar to those described above.

2.5. LC-MS² detection

The HPLC apparatus comprised of a HP 1100 series pump, autosampler and degasser (Agilent, Palo Alto, CA, USA). Chromatographic separation was achieved using a Symmetry C18 column (5µm, 150 x 2.1mm, Waters, Milford, MA, USA). To separate the different compounds, a linear gradient was used consisting of a mixture of 60% 0.02M formic acid and 40% methanol. The methanol percentage increased from 40 to 80% in 25min. The flow rate was 0.3mL min⁻¹. Between each sample the column was allowed to equilibrate at initial conditions. Analysis was carried out using an LCQ^{DECA} Ion Trap Mass Analyzer

(ThermoQuest, San José, CA, USA) equipped with an atmospheric pressure chemical ionisation (APCI) interface and Xcalibur 1.2. software. The components were detected in positive ion mode MS/MS full scan.

2.6. Statistical analysis

Statistical analysis was performed with Statistica[®] software (Statsoft, Tulsa, OK, USA). All data were tested for normality and homogeneity of variances using Kolmogorov-Smirnov and Levene's test, respectively. The effect of the treatment was tested for significance using a one-way analysis of variance (Dunnett's test). If the assumptions were not met, the non-parametric Mann-Whitney U-test was used. Statistical significance is described at $p < 0.05$ (*), or $p < 0.01$ (**).

3. Results and discussion

3.1. Solvent toxicity of DMSO

DMSO was used as vehicle for the water-insoluble chemical benzo(a)pyrene. Therefore, we first determined the maximum concentration of DMSO which did not affect *Neomysis integer* survival during the 96h exposure period. Two beakers, each containing 3 juvenile mysids per DMSO concentration (0, 0.1, 0.5, 1, 5 and 10%) were exposed for 96h at 15°C. Like in the final B(a)P assay, the test solutions were renewed after 48h. Survival as a function of DMSO concentration was measured daily.

Increasing concentrations of DMSO resulted in time and concentration-dependent toxicity to the mysid *N. integer*. No mortality was observed in the controls, and in animals exposed to 0.1, 0.5 and 1.0% (vol/vol) DMSO. However, 24h exposure to 5% DMSO resulted in 50% mortality, and no mysids survived exposure to 5% DMSO for 48h. In addition, no survival was observed in the 10.0% DMSO exposures after 24h. Based on the results of this exposure and those of previously published studies (Okumura et al., 2001, Ura et al., 2002), the use of DMSO in final concentrations $\leq 1.0\%$ appear to be appropriate for performing 96h-acute toxicity testing. In this study with B(a)P we applied a much lower solvent concentration, i.e. 0.05% for our subsequent experiments, similar to guidelines for acute toxicity testing as set by USEPA and ASTM (USEPA, 1975; ASTM, 1996). This corresponds with 0.5mL L^{-1} DMSO.

3.2. Measured B(a)P concentrations

In the aquatic environments B(a)P strongly absorbs to sediments and particulate matter. Levels of B(a)P biodegradation are low in most waters and sediments and significant photodegradation of B(a)P only occurs near the surface of water bodies (www.speclab.com). This results in high B(a)P sediment concentrations, as well as accumulation in aquatic organisms. Uptake of PAHs by aquatic organisms is dependent on the bioavailability of the PAHs and on the physiology of the organism (Juhasz et al., 2000).

B(a)P concentrations measured during the exposure (at 0, 8, 24, and 48h) are depicted in Figure 2. An exponential decrease of the exposure concentrations was noted from the addition of the chemical until the renewal of the media. This decrease is likely due to the lipophilic character and to photodegradation of the substance. Time-weighted averages (TWA) were calculated based on the fitted exponential curves. These average values are representative of the exposure concentrations in the different treatments. As seen in Table 1, the measured TWAs are lower than the nominal concentrations, and this was most obvious in the highest treatment (5000 $\mu\text{g B(a)P L}^{-1}$ nominal concentration: 1682.86 $\mu\text{g B(a)P L}^{-1}$ TWA concentration). The TWAs were used as actual concentrations throughout the results and discussion section and on the X-axis (concentration axis) of the figures.

Table 1. Calculated TWA (time-weighted average) concentrations in $\mu\text{g B(a)P L}^{-1}$.

Nominal concentration ($\mu\text{g B(a)P L}^{-1}$)	Calculated TWA concentration ($\mu\text{g B(a)P L}^{-1}$)
0.5	0.43
5	2.39
50	28.83
500	339.00
5000	1682.86

3.3. Testosterone metabolism

When *N. integer* is exposed to testosterone in the water, it produces a range of testosterone metabolites that can be categorized into phase I metabolites, formed via introduction of a functional group (reduced and hydroxylated derivatives) and phase II metabolites, produced via attachment of a polar moiety to the group (conjugated derivatives) (Verslycke et al., 2002). Changes in testosterone metabolism in *N. integer* can be used to

detect exposure to endocrine-disrupting chemicals (Verslycke et al., 2003, 2004). In this study, mysids that were exposed for 96h to different B(a)P concentrations and subsequently for 6h to testosterone, were evaluated for their ability to eliminate testosterone as various metabolic derivatives.

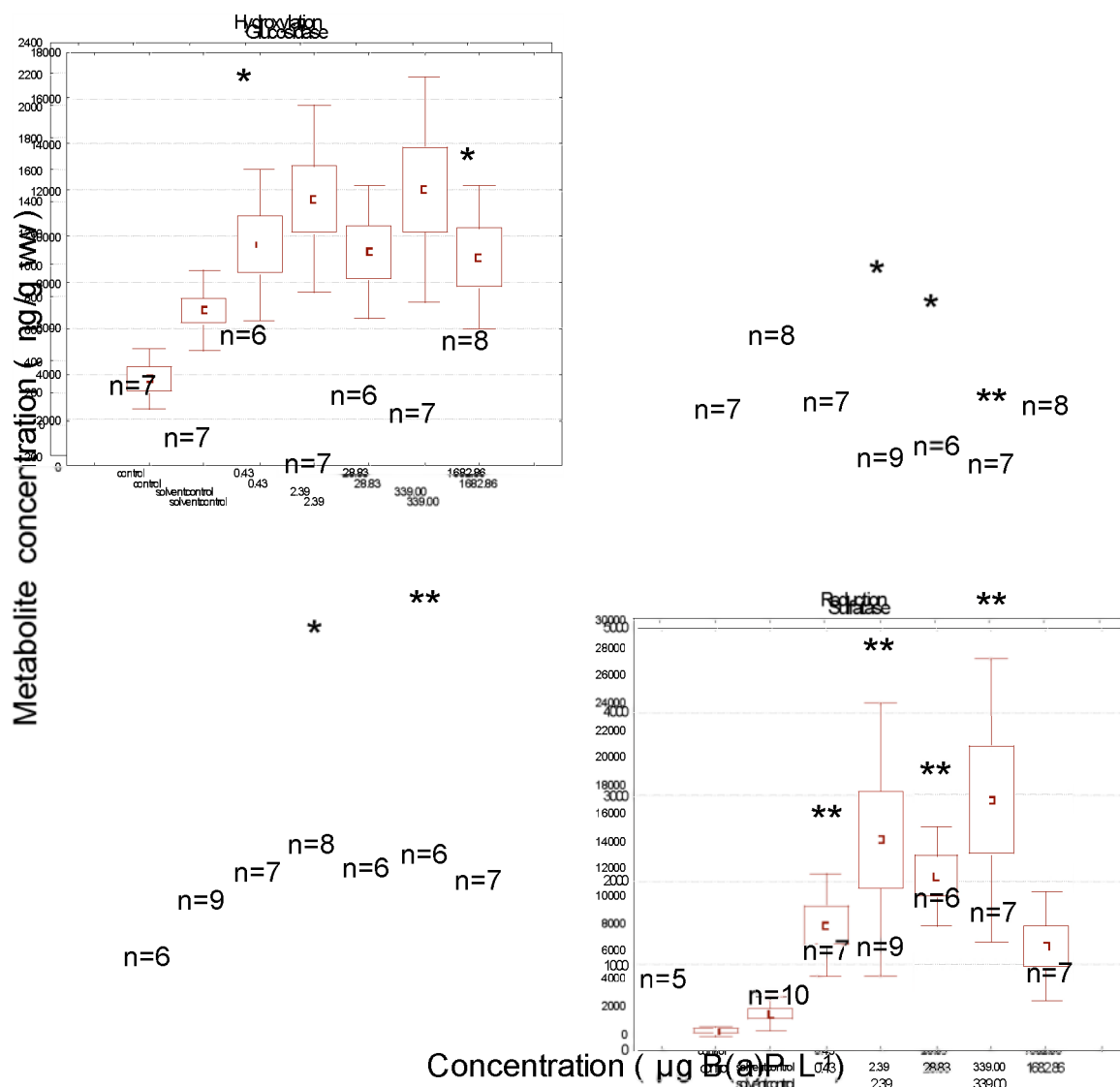


Figure 1: Metabolic elimination of testosterone by *Neomysis integer* following a 96h of exposure to different concentrations of benzo(a)pyrene as hydroxylated, reduced, glucose-conjugated and sulfate-conjugated testosterone metabolites. Number below the whisker indicates the number of observations.

Figure 1 shows the metabolic elimination of the testosterone by *N. integer* following exposure to different concentrations of B(a)P. The exposure medium was analysed for phase I and phase II testosterone metabolites. No significant differences could be noted between the

control and the solvent control. For the interpretation of the data, the metabolic elimination of testosterone after exposure to different concentrations of B(a)P were compared to the solvent control.

The polar hydroxylated phase I metabolites 6 α -hydroxytestosterone, 11 β -hydroxytestosterone and β -boldenone were less abundant present in the medium than androstenedione (AED) and androstadienedione (ADD), both reduced phase I testosterone metabolites. It needs to be noted that the detection method used (LC-MS²) is selective and very specific. Because of this specificity, not all possible testosterone metabolites were quantified, but only those incorporated into our LC-MS procedure. This LC-MS procedure, however, is based on our earlier work with [¹⁴C]-labelled testosterone and thin-layer chromatographic approaches, which identified the most important testosterone metabolites produced by *N. integer*. For the cladoceran *Daphnia magna*, Baldwin and LeBlanc previously described the preferential excretion of polar metabolites and the retention of the non polar metabolites of testosterone, following exposure to [¹⁴C]-testosterone and quantification via autoradiography (Baldwin and LeBlanc, 1994a). Although, AED was found in high concentrations in the medium, the major compounds detected in *N. integer* in this study were testosterone and the non polar metabolite AED. No hydroxylated metabolites were found in the organisms. As such, similar to daphnids, the polar hydroxylated metabolites were more efficiently excreted by the mysids into the medium, whereas the non polar metabolites were retained inside the animal.

Phase II testosterone metabolites were excreted into the medium as glucose-conjugated and sulfate-conjugated metabolites. The major conjugated metabolite is testosterone, both as glucosylated testosterone and sulfated testosterone. Glucosylated AED and ADD were found in the solvent control and glucosylated ADD was detected in the lowest exposure treatment (0.43 $\mu\text{g B(a)P L}^{-1}$). Overall, glucosylation was more important than sulfation. This is similar to our previous studies with mysids (Verslycke et al., 2004), and also corroborates earlier studies by Baldwin (Baldwin and LeBlanc, 1994a, Baldwin, 1997).

We are not aware of any previously published studies on the influence of B(a)P on the testosterone metabolism of invertebrates. In mammals PAHs are known to be CYP1A inducers (Mimura and Fujii-Kuriyama, 2003), and the role of the aryl hydrocarbon receptor (AHR) as a regulator of CYP1A induction has been studied extensively in many vertebrates (Hahn, 2002). CYP1A is not only involved in the biotransformation of many PAHs but CYP1A is, at least in rats, also involved in the metabolism of steroids like testosterone and

androstenedione (Smeets et al., 2002). This implies that B(a)P could influence testosterone metabolism directly, through induction of CYP1A, or indirectly by down regulation of other P450 activities involved in metabolism of steroid hormones. Whether B(a)P induces a CYP1A-like protein in mysids via an AHR-like receptor protein is unknown. Only recently different AHRs have been cloned out of invertebrates. As many invertebrate animals exhibit significantly different biotransformation systems as compared to vertebrates, and many of them are relatively insensitive to the toxicity of dioxin-like compounds (Hahn, 2002), elucidating the role of an AHR homolog in invertebrates is particularly interesting. Our studies have previously demonstrated that mysids have a biotransformation system that rivals that of many vertebrate animals in complexity (Verslycke et al., 2002). The present study demonstrates that dioxin-like compounds can directly affect steroid metabolism in *N. integer*. As shown in figure 1, a significant increase of phase I hydroxylation derivatives was observed in the 0.43 and 1682.86 $\mu\text{g L}^{-1}$ B(a)P treatments compared to the solventcontrol. For the intermediate concentrations no differences were detected. Whether the observed effects on oxidative metabolism in *N. integer* are caused by an AHR-like regulated induction of xenobiotic-metabolizing enzymes is unknown, but also unlikely as it is generally assumed that the ability of AHR to bind PAHs is a vertebrate innovation (Hahn, 2002). Future studies, however, could look into the identity of the enzymes that are associated with the effects observed in this study, or potentially could look at elucidating the molecular targets for PAHs in mysids and other crustaceans.

A decrease in the elimination of testosterone as reduced or non polar metabolites was observed in the medium by mysids exposed to B(a)P when compared to solventcontrol, and this was significant at 2.39, 28.83 and 339.00 $\mu\text{g L}^{-1}$. The 0.43 and 1682.86 $\mu\text{g B(a)P L}^{-1}$ exposures did not affect the reduced metabolite excretion. As well for the hydroxylated and reduced metabolites as for the phase II metabolites, the highest exposure concentration (1682.86 $\mu\text{g B(a)P L}^{-1}$) was not taken into account for interpretation of the data. The results of the highest concentration did not correlate well with the results of the other exposure concentrations.

Both phase II glucosylation and sulfation were induced when compared to the solventcontrol. For the glucose conjugates, this increase was significant for the concentrations of 2.39 and 339.00 $\mu\text{g L}^{-1}$ treatments, while sulfation was significantly induced at 0.43, 2.39, 28.83 and 339.00 $\mu\text{g L}^{-1}$. No significant effects were seen on phase II conjugation in the highest exposure concentration when compared to the solventcontrol.

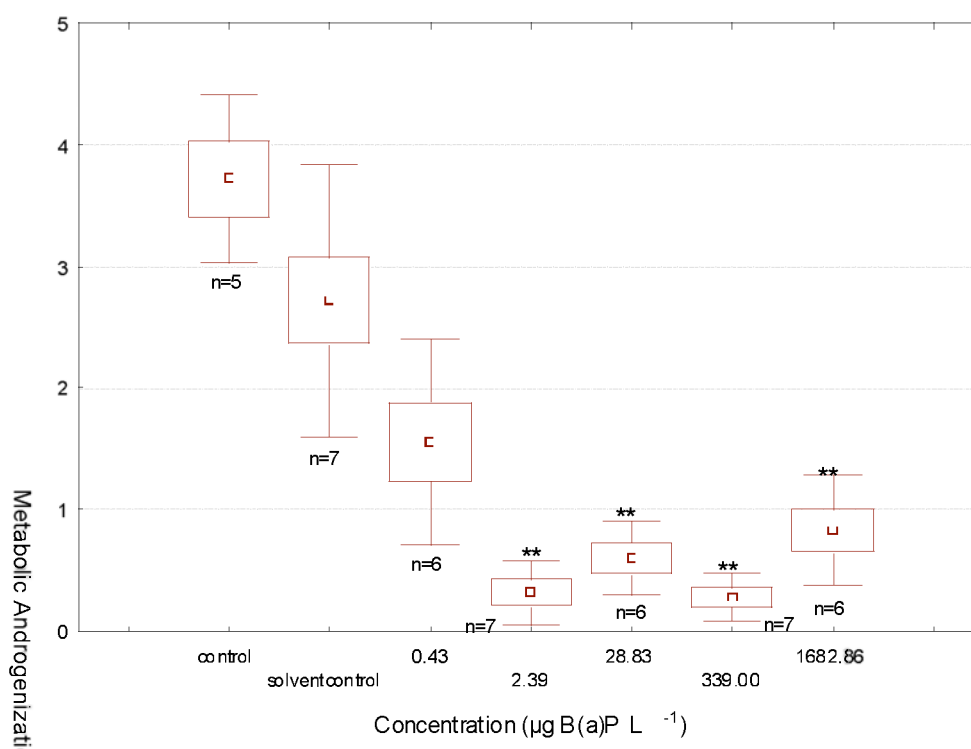


Figure 2: Metabolic androgenization ratio (MAR) of benzo(a)pyrene-exposed *Neomysis integer*, calculated as the ratio of oxido-reduced to glucosylated/sulfated/hydroxylated metabolites of testosterone. Number below the whisker indicates the number of observations.

The overall effect of B(a)P on the metabolic elimination of testosterone can be summarized by the metabolic androgenization ratio (MAR), calculated according to Baldwin et al. (1997) and Verslycke et al. (2003). The MAR is the ratio of the eliminated oxido-reduced derivatives to the eliminated hydroxylated and conjugated derivatives. It integrates the various metabolic processes contributing to both the production of androgenic derivatives and inactivated products of testosterone. The MARs of mysids exposed to 2.39, 28.83, 339.00 and 1682.86 µg B(a)P L⁻¹ were significantly lower than those of unexposed organisms (Fig. 2). This is the combined result of a reduction in reduced metabolites (MAR numerator) and an induction in hydroxylated and conjugated metabolites (MAR denominator). The reduction in reductase activity is also reflected with a decreased presence of the pharmacologically active androgen AED in tissues of *N. integer*, although this was only significant in the 28.83 µg B(a)P L⁻¹ treatment (Fig.3). The increase of AED present in the organism at the lower concentrations (0.43 and 2.39 µg B(a)P L⁻¹) could not be elucidated.

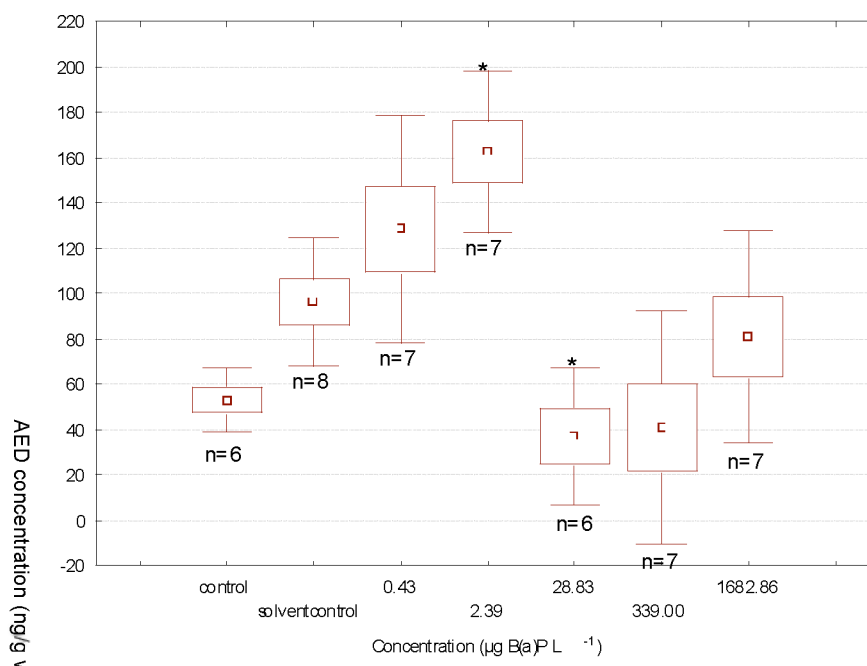


Figure 3: Retention of the androgen androstenedione (AED) by *Neomysis integer* following 96h exposure to B(a)P. Number below the whisker indicates the number of observations.

4. Conclusion

Significant alterations in the testosterone metabolism of *N. integer* were observed following a 96h exposure to the polycyclic aromatic hydrogen benzo(a)pyrene (B(a)P). The overall effect of B(a)P on mysid testosterone metabolism is summarized in a significantly lower metabolic androgenization rate in the four highest exposure concentrations (2.39, 28.83, 339.00 and 1682.86 µg B(a)P L⁻¹). Invertebrates are generally believed to be less sensitive to the effects of dioxin-like compounds because of the lack of an AHR homolog that binds dioxin and related chemicals. Our studies, however, demonstrate significant effects on the enzymatic biotransformation system of *N. integer* after acute exposure to low aquatic concentrations of the polycyclic hydrocarbon B(a)P. Future studies could be directed at identifying the enzymes involved in this response, as well as identifying the molecular targets for B(a)P toxicity in mysids. This study confirms our previous findings that the testosterone metabolism assay of *N. integer* is a sensitive tool to investigate the effects of B(a)P and other chemicals on the biotransformation capacity of mysids. Linking the biochemical effects of dioxin-like chemicals to physiological effects at the organismal level is the subject of ongoing research in many vertebrate models, and should be extended to incorporate invertebrate models that are relevant for regulatory toxicity testing, such as mysids.

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