cDNA fingerprint from the hepatopancreatic glands of pond snails (*Lymnaea stagnalis*) exposed to benzo[a]pyrene

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ABSTRACT

Identification of differentially expressed genes that could be potentially used as biomarkers of PAH exposure of common invertebrate animal (like freshwater snail) would be a valuable resource for investigators interested in toxicology and biomonitoring of aquatic environments. Therefore, the aim of this research was to investigate effects of waterborne benzo[a]pyrene (B[a]P) exposure on mRNA expression in the pond snail's (*Lymnaea stagnalis*) hepatopancreatic gland. Toward this end, mature individuals of pond snail (*L. stagnalis*) were treated with $50\mu M$ B[a]P solution in a short 36h static exposure test. Differential Display PCR (DD-PCR) was used to generate a unique cDNA fingerprint of genes that were differentially expressed in the tissues of exposed and unexposed snails. To assess the putative identity of the isolated cDNA amplicons (ESTs), BLAST queries were performed to find similarities in their nucleotide sequence. Real-Time qPCR analysis was used to verify the DD-PCR expression profile. Finally, an additional independent exposure study, including higher dose of B[a]P ($100\mu M$), was conducted to validate the expression of selected ESTs. BLAST revealed that only 3 out of 9 isolated ESTs had meaningful information on their putative nucleotide sequence identity. The highest similarity was scored for EST-A1, identified as the transcript of UAP-like protein, found to be up-regulated after B[a]P exposure. The original expression pattern that was observed in DD-PCR step was coherent with results of the qPCR verification for 3 out of 5 analyzed ESTs. However, changes in the ESTs expression were modest and the treatment with B[a]P resulted in significant down-regulation for only 1 unidentified fragment (EST-G42, almost 2-fold; $p < 0.05$) when compared to untreated snails. Although no significant changes were observed for EST-A1 and EST-G42 in the validation study, their expression pattern was consistent with that obtained from DD-PCR. Surprisingly, EST-C5 remained in contrast to the DD-PCR part, but it showed significant down-regulation in group of snails exposed to $100\mu M$ B[a]P (3.5-fold; $p < 0.05$). The obtained results show that diverse genes may be involved in the molecular response of the pond snail's hepatopancreas to treatment with B[a]P. However, further research is needed to confirm the utility of the discovered EST as PAH biomarkers in biomonitoring practices with *L. stagnalis* as bioindicator species.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs; also known as polyarenes) are a group of organic compounds commonly found in the environment. Most PAHs have anthropogenic origins, as they are formed in the process of incomplete combustion, e.g. consumption of fossil fuels (coal, oil, gas) and other conventional energy sources (wood) or waste incineration. PAHs may also arise from non-anthropogenic sources, such as volcano eruptions, forest fires, or degradation of organic matter in the soil, however, the natural sources do not contribute significantly PAH emission

(Maliszewska-Kordybach 1999; Thiele and Brummer 2002). Although the great deal of PAHs are emitted into the air, they eventually end up in the surface waters, i.e. lakes and rivers which serve as a final sink for many environmental pollutants. High concentrations of PAHs are usually found in sediments (e.g. Baumard et al. 1997), especially around strongly industrialized regions. Aquatic organisms, including zooplankton, snails, mussels, or fish have the potential to accumulate PAHs (e.g. Nesto et al. 2007), therefore, it is reasonable to think that this group of animals is particularly vulnerable to these compounds.

In the environment, PAHs almost always exist as a complex mixture of different chemical species but routine analyses are usually limited to the 16 most common compounds from the US Environmental Protection Agency priority list (USEPA 1982). Guidance values for PAHs limits in drinking water often refer to the content of benzo[a]pyrene (B[a]P), which is usually one of the major components of PAHs mixtures. Due to the numerous toxicological investigations concerning its effects, B[a]P is considered to be a model PAH compound with previously demonstrated mutagenic and carcinogenic activity (Miller and Ramos 2001).

Although fish, particularly in early developmental stages (Bailey et al. 1996; Prasch et al. 2003; Teraoka et al. 2003), are known to be aquatic species sensitive to PAHs, information concerning the adverse effects of B[a]P and other PAHs on aquatic invertebrates is limited. More knowledge about gene expression, regulation and function of organisms challenged by B[a]P would be of value to investigators and others interested in toxicology and biomonitoring of aquatic environments.

One of the best studied aquatic invertebrate species, used in research fields including behavioural ecology, evolutionary biology, parasitology, aquatic ecotoxicology, and neurophysiology, is the freshwater pulmonate gastropod *Lymnaea stagnalis* (Besnard et al. 2013; Byzitter et al. 2012). For example, several papers have documented its high sensitivity to heavy metals like Co, Cu, Pb, Ni (Leonard and Wood 2013; Munley et al. 2013; Ng et al. 2011). According to Gust et al. (2013), mRNA expression of pond snail genes involved in the immune response can be used to assess toxic effects and seems to be a very promising marker. Bouétard et al. (2013) indicated transcriptomic resources developed in *L. stagnalis* as a suitable biomarker set for elucidating underlying toxicity of chemicals able to induce oxidative stress and other molecular alterations such as endocrine disruption. Additionally, this has been identified as one of the most relevant mollusc species for assessing the reprotoxic effects of chemicals (Giusti et al. 2013). The above studies, in conjunction with the fact that it is common to aquatic habitats throughout Europe, prompted us to use *L. stagnalis* as a test animal.

In recent years a number of molecular approaches have been used to study the adverse effects of various contaminants on aquatic organisms, and modern applications of molecular biology (e.g. microarrays or DNA chips) have opened new possibilities in the field of biomarker discovery. Nonetheless, because differential display PCR (DD-PCR) still offers a robust alternative for performing transcriptome analysis at a low cost (e.g. Brzuzan et al. 2007; Woźny et al. 2012), it became our method of choice.

The aim of this research was to investigate the effects of waterborne B[a]P exposure on mRNA expression in the hepatopancreatic gland of pond snail. In this study, mature individuals of pond snail (*L. stagnalis*) were treated with 50µM B[a]P solution in a short 36h static exposure test. DD-PCR was then used to generate a unique cDNA fingerprint of genes that were differentially expressed in the tissue of exposed and unexposed snails. Real-Time qPCR analysis was used to verify the expression profiles of genes identified with DD-PCR. In order to validate the EST that was elected in the first part of the study, an additional, independent experiment was conducted, involving 36h of snails' exposure to 50 and 100µM B[a]P.

MATERIALS AND METHODS

Snails collection and exposure

Mature pond snail individuals, *Lymnaea stagnalis* (L.), of similar size (total shell length of 4.36±0.27cm and weight of 5.26±0.42g), were collected from the Starodworskie Lake in Olsztyn (Poland) and acclimated for 7 days at room temperature in a 50L aerated glass tank. For snail maintenance and further exposure, we used an aqueous solution of Instant Ocean salts (60mg·L^{-1} of distilled water; the E3 solution) which is commonly used as an environment for freshwater fish species (Westerfield 2000). After the acclimation period, the snails were transferred into plastic beakers (volume of 100mL) and divided into two experimental groups (n=4 each): i) treated to benzo [a]pyrene (50µM of B[a]P in E3 solution +0.2% dimethyl sulfoxide, DMSO; Sigma-Aldrich), and ii) untreated (control) group (E3 solution +0.2% DMSO). After 36h of the exposure (static test without renewal of test solutions), the snails' hepatopancreases were dissected (n=4 from each group), immersed in RNA*later*® preserving solution (Sigma-Aldrich) and stored at -20°C.

mRNA isolation and cDNA synthesis with RH primers

RNA*later*® preserved tissues (n=4 in each group; approximately 25mg per sample) were used to extract mRNA, using PolyATract® System 1000 (Promega) according to the attached protocol, and measured for quantity and purity with UV/Vis Biophotometer (Eppendorf). The isolated mRNA samples (A_{260}/A_{280} ratio above 1.85) were then used for reverse transcription (RT)

using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific). The cDNA reaction for each sample contained $0.15\mu g$ of mRNA and $0.2\mu g$ of random hexamer primers (RH), and the reaction was performed according to the manufacturer's recommendations. At the end of the RT reaction, all cDNA samples were additionally treated with 2 U of RNAse H (Promega) for 30min at 37°C followed by 5min at 65°C (Corton 1998), and after that immediately used for PCR amplification.

Differential-Display PCR

The PCR amplification was carried out in accordance with the original method outlined by Sokolov and Prockop (1994), described by Corton (1998), with modifications. In this procedure, freshly synthesized cDNA samples were used for PCR reaction with defined but arbitrary decamer oligonucleotides; the BS primers (BS-52: 5'-CAA GCG AGG T-3'; BS-54: 5'-AAC GCG CAA C-3'; BS-55: 5'-GTG GAA GCG T3'; BS57: 5'-GAA AGC AGC T-3'; BS-58: 5'-CAG TGA GCG T-3') combined in 10 primer pairs (52+54, 52+55, 52+57, 52+58, 54+55, 54+57, 54+58, 55+57, 55+58, 57+58). Each PCR sample consisted of $10\mu L$ of 2X DreamTaq™ Green PCR Master Mix (Thermo Scientific), $0.9\mu M$ of each BS primer, $2\mu L$ of cDNA as a template, and PCR-grade water up to a final volume of $20\mu L$. The reaction was carried out on MasterCycler Gradient 5331 thermocycler (Eppendorf) with the following program: 95°C for 2 min of initial denaturation, then 45 cycles of: 95°C for 30s, 34°C for 1min, 72°C for 1min, and 72°C for 5min of final elongation. To generate the cDNA fingerprint, PCR products were electrophoresed in 6% polyacrylamide gel. Electrophoresis was carried out at 120V for 2h in 1 x TBE buffer using a D-code system (Bio-Rad Laboratories Inc.; Hercules). The migration patterns were visualized by staining with SYBR®Gold (1:10 000; Invitrogen) for 20 minutes, followed by UV transillumination in Gel Logic 200 system (Kodak). After gel visualization, the differentially expressed cDNA amplicons (Expressed Sequence Tags; ESTs) were excised from the gel, melted in sterile water and used for reamplification with the same set of primers and the same PCR conditions. Reamplified PCR products were cleaned using Cleanup kit (A&A Biotechnology) and stored at -20°C for the cloning procedure.

Cloning and sequencing of ESTs

The obtained ESTs were ligated into the pTZ57R/T vector and cloned using InsTAclone™ PCR Cloning Kit (Thermo Scientific) according to the accompanying manual. The cloned plasmids were extracted using Plasmid Mini isolation kit (A&A Biotechnology) and sequenced under contract at Genomed (http://www.genomed.pl). BLAST queries (Altschul et al. 1997) were then performed to compare the obtained ESTs sequences to sequences already deposited in GenBank® databases (http://www.ncbi.nlm.nih.gov). The results of sequence identification (BLAST matches) were considered significant at an expected value of $E < 10^{-5}$.

RNA isolation and cDNA synthesis with oligo d(T)$_{18}$ primers

To verify the expression of the isolated ESTs, total RNA was extracted from the RNA*later*® preserved tissues (20mg per sample; n=4 from each group) using Total RNA Mini isolation kit (A&A Biotechnology) followed by DNase I treatment (Thermo Scientific). Total RNA with a A260/A280 ratio above 1.85 was converted to cDNA using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific), according to the manufacturer's instructions. The reaction mixture for cDNA synthesis with each sample contained $1\mu g$ of total RNA and $0.5\mu g$ of oligo d(T)$_{18}$ primers. After synthesis, the strand cDNAs were stored at -20°C until they were used.

Real-Time PCR

Based on the obtained nucleotide sequences of the ESTs, primers for Real-Time PCR assay were designed using the Primer-BLAST tool at the NCBI-NIH website (http://www.ncbi.nlm.nih.gov) and purchased from Genomed. Due to the short length and unfavorable thermodynamic characteristics of a few EST sequences, primers were designed for only 5 of the 9 ESTs. Details of the Real-Time PCR primers and their amplicons are presented in Table 1 and Appendix 1.

Real-Time qPCR was carried out on an ABI 7500 Real-Time PCR System thermocycler (Applied Biosystems) in singleplex mode; all samples were estimated in duplicate. Each PCR well included $10\mu L$ of $2\times$FastStart SYBR Green Master ROX mix (Roche Diagnostic), $0.5\mu M$ of each (forward and reverse) primer (Table 1), $1\mu L$ of cDNA (synthesized from total RNA) as a template, and PCR-grade H_2O for a final volume of $20\mu L$. The reaction was carried out in standard thermal conditions: 95°C for 10min, then 45 cycles of 95°C for 15s and 60°C for 1min. To exclude the possibility of cross-contamination, the reaction plate contained negative water controls (NTCs). After each run a melting curve analysis followed by agarose gel electrophoresis was executed to confirm the quality of the PCR products.

To estimate PCR efficiency, cloned plasmids with EST inserts were linearized with *Bam*HI (Thermo Scientific) and used to prepare a series of six 10-fold dilutions with a known concentration of plasmid DNA as a standard. The prepared dilutions served as templates in Real-Time qPCR assays of each of the tested primer pairs. To plot standard curves, Cq values obtained for each dilution series were plotted against the standard concentration. The corresponding efficiencies for each primer pair (Table 1) were calculated according to the equation: Eff. $= 10^{[-1/\text{slope}]}$ (Pfaffl 2001), and used for qPCR data analysis.

Table 1. Details of the Real-Time PCR primers used in the study for target ESTs.

Primer		Sequence [5' → 3']	Amplicon length [bp]	Melting peak [°C]	PCR efficiency [%]	R²
EST-A1	F:	tgccaggagcagtgaagaac	219	80.8	92.4	0.996
	R:	tgctgagccaacttctccag				
EST-B4	F:	cagcgaggtgtgaagaggaa	154	79.4	98.5	0.999
	R:	tctttcagggaagcgtcagc				
EST-C5	F:	tacatggttgtgaggccgac	185	78.0	98.4	0.998
	R:	ggtcccacatcagaggcaac				
EST-G42	F:	ggatgcaaggacgtcgagaa	213	82.0	97.1	0.999
	R:	caccgattccttccggttca				
EST-I2	F:	atgccagagagttggcgaat	239	81.5	99.4	0.999
	R:	ctgtctcgcagaaacgctct				

R² – coefficient of determination.

Data analysis

Data obtained from the Real-Time PCR assay was used to compute the non-normalized ratio of EST expression in the B[*a*]P-treated group relative to the control group of snails (expression ratio, R). The calculations were performed with REST©2009 software (Pfaffl et al. 2002), including the calculated PCR efficiencies. Due to the limited information on pond snail mRNA sequences, no reference genes were used to normalize the EST expression data. However, all samples were standardized by using the same amount of template in cDNA synthesis (total RNA) and further qPCR (cDNA). Differences between groups (control versus treated) were assessed for statistical significance using a randomization test calculated with REST©2009 software (Pfaffl et al. 2002) with 5000 iterations performed for each EST.

EST validation study

In order to validate the ESTs selected (DD-PCR) and verify (qPCR) in the first part of the study, an additional experiment was conducted, involving the same exposure period (36h of static test) and two doses of B[*a*]P (50 and 100*µ*M). Briefly, *L. stagnalis* individuals (total shell length of 5.21±0.26cm and weight of 9.85±1.74g) were collected from Starodworskie Lake. Acclimation and exposure were performed following the protocol described in the *snails collection and exposure* section, except for inclusion of an additional group of snails exposed to 100*µ*M of B[*a*]P. RNA*later*® preserved tissues of snails from the validation study were used to extract total RNA, synthesize cDNA with oligo d(T)$_{18}$, and analyze EST expression level, according to the same procedures described above.

RESULTS

DD-PCR yield and identification of ESTs

The use of 10 BS primer pairs combinations in DD-PCR step resulted in the generation of 4 to 12 distinguishable bands in the gel, and the size of the DNA fragments varied from approximately 100 bp to more than 1000 bp. A total of 13 cDNA amplicons (ESTs) were found to be differentially expressed among samples from the control and the B[*a*]P-treated snails. However, only 9 of the isolated ESTs were successfully cloned and used in further *in silico* analysis. After BLAST analysis, 6 out of the remaining 9 ESTs revealed high similarity (E>10^{-5}) to known mRNA sequences deposited in GenBank database. However, only 3 BLAST hits (for EST-A1, EST-F11, and EST-F23) had full information on the deposited mRNA sequences; the highest similarity scored for EST-A1, which was identified as the transcript of UDP-N-acetylhexosamine pyrophosphorylase-like protein (Table 2). For the rest of the sequences (EST-G42, EST-I1, and EST-I2), BLAST queries showed no significant similarity to any deposited nucleotide sequence (E>10^{-5}).

Verification of EST expression by Real-Time PCR

Time PCR products for each primer pair revealed single bands at expected lengths (Table 1). PCR efficiencies for the tested primers were high, ranging from 92 to 99%; the coefficient of determination values for each standard curve was above 0.99 (Table 1).

Table 2. Characterization of differentially expressed sequence tags (ESTs) in the pond snail's hepatopancreas after 36h of static exposure to 50μM aqueous solution of benzo[a]pyrene.

EST	EST length [bp]	Expression pattern (DD-PCR)	Expression verification (qPCR)[a]	Putative identity	E value	Query coverage [%]	GenBank ID (species)
EST-A1	859	↑	1.27 (S.E. 0.74-2.03)	UDP-N-acetylhexosamine pyrophosphorylase-like protein 1-like	$5e^{-106}$	98	XM_003439611.1 (Oreochromis niloticus)
EST-B4	342	↓	1.19 (S.E. 0.70-2.08)	gene product (Dana\GF23678)	$1e^{-21}$	95	XM_001965116.1 (Drosophila ananassae)
EST-C5	289	↑	1.31 (S.E. 0.66-2.19)	hypothetical protein	$5e^{-6}$	91	XM_002115557.1 (Trichoplax adhaerens)
EST-E1	362	↑	Not available	predicted protein (NEMVEDRAFT_v1g210745)	$2e^{-20}$	90	XM_001630066.1 (Nematostella vectensis)
EST-F11	298	↓	Not available	protein SFI1 homolog	$4e^{-10}$	57	XR_141436.1 (Mus musculus)
EST-F23	276	↓	Not available	protein SFI1 homolog	$2e^{-8}$	53	XR_141436.1 (Mus musculus)
EST-G42	1233	↓	0.62* (S.E. 0.44-0.87)	Unidentified			
EST-I1	145	↓	Not available	Unidentified			
EST-I2	527	↑	0.91 (S.E. 0.50-1.64)	Unidentified			

Up (↑) or down (↓) regulation of EST expression after treatment with benzo[a]pyrene;
a – fold-change of non-normalized expression ratio (and a range of standard error);
* – significant difference between the control and treated groups of animals (randomization test; $p<0.05$)

Table 2 shows non-normalized expression ratio of selected ESTs in the hepatopancreas of pond snails after exposure to B[a]P. For all of the tested ESTs, the observed expression changes were modest (the modulation of mRNA level was not greater than 2-fold). However, the treatment with B[a]P resulted in significant down-regulation of EST-G42 (R=0.62; S.E. 0.44-0.87; $p=0.046$) in comparison to the untreated (control) group of snails (R=1.00; Figure 1). Interestingly, the original expression pattern that was observed in gels of DD-PCR step was coherent with 3 out of 5 analyzed tags, i.e. EST-A1, EST-C5, and EST-G42 (Table 2).

Validation of EST expression in independent exposure study

The expression pattern of EST selected for validation study (Figure 1) was similar to that obtained from the verification of DD-PCR results with qPCR (Table 2), except of EST-C5. Surprisingly, expression of EST-C5 was suppressed in both treated groups with significant down-regulation observed after exposure to 100μM of B[a]P (R=0.28; S.E. 0.09-0.65; $p=0.028$). Although changes in expression ratio of EST-A1 and EST-G42 were insignificant (Figure 1), their expression pattern in the group of snails exposed to 50μM of B[a]P ($R_{A1} =1.66$;

$R_{G42}=0.48$) remained consistent with that obtained by DD-PCR and qPCR from the first part of the study (Table 2).

DISCUSSION

Gene expression studies on pond snails exposed to various environmental contaminants may improve our knowledge about their effects in the field and highlight a role of the species as a bioindicator in biomonitoring practices. Nonetheless, studies on molluscs, especially snails, are still rare (Bandow and Weltje 2012) and only a few papers have been proven particularly valuable for studying environmental influences on gene expression and disease in a vast number of different invertebrate species (Bouétard et al. 2013; Giusti et al. 2013). Consequently, in this study we used the Differential Display (DD-PCR) method to generate a unique cDNA fingerprint from the hepatopancreatic gland of pond snails exposed to model environmental contaminant, benzo[a]pyrene. Differentially expressed cDNA amplicons (ESTs) that were isolated from treated and untreated snails were isolated and their nucleotide sequences were subjected to in silico identification. Then, Real-Time PCR assay was applied to verify the original expression pattern of isolated EST
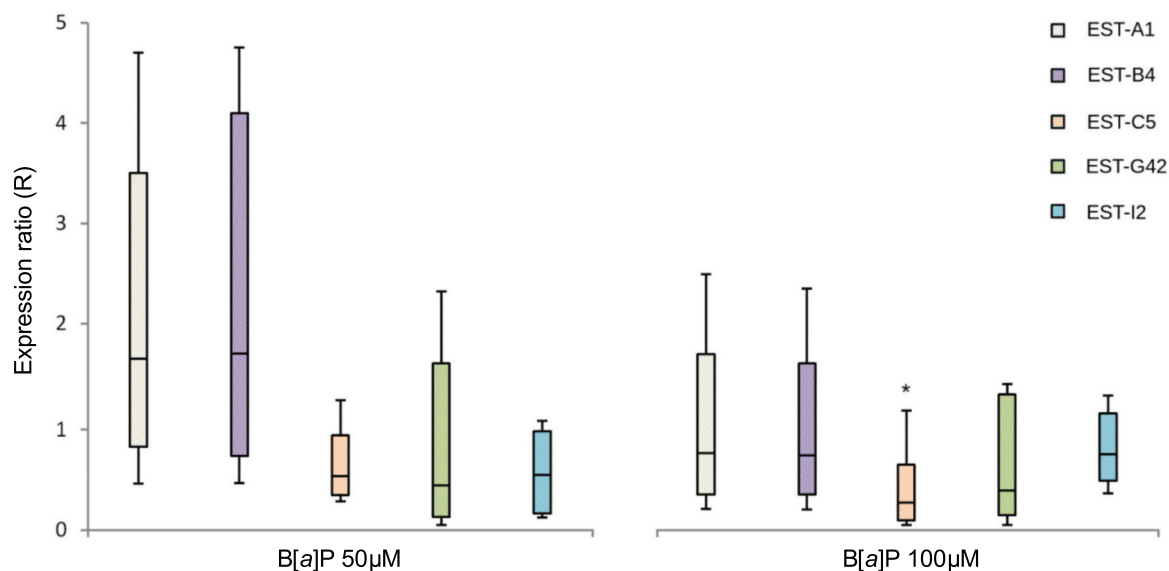
Figure 1. Validation of the EST selected from the first part of the study (see Table 1 for details). Non-normalized expression ratio (R) of selected expressed sequence tags (ESTs) in hepatopancreatic gland of pond snails after 36h of static exposure to 50 and 100μM aqueous solution of benzo[*a*]pyrene, relative to untreated group of snails (control; R=1.0). The box area encompasses standard error, the line in the middle represents the sample's ratio and the whiskers represent 95% confidence interval. The asterisk indicates a significant difference between the control and treated groups of animals (randomization test; * *p*<0.05). All values were calculated using REST©2009 software.

observed in DD-PCR. An additional independent exposure study, including a higher dose of B[*a*]P (100μM), was conducted to validate the expression of ESTs elected by DD-PCR and confirmed with qPCR in the first part.

The highest similarity was scored for EST-A1, identified as the transcript of UDP-N-acetylhexosamine pyrophosphorylase-like protein. UDP-N-acetylhexosamine pyrophosphorylase (UAP; EC 2.7.7.23) is an enzyme engaged in nucleotide sugar metabolism, and the protein is widely distributed in nature: from bacteria to animal tissues (Wang-Gillam et al. 1998). In humans, the protein is encoded by the *UAP1* gene, the functional enzyme of which catalyzes the synthesis of both UDP-N-acetylglucosamine (UDP-GlcNAc) and UDP-N-acetylgalactosamine (UDP-GalNAc), important metabolites (Wang-Gillam et al. 1998). In insects, the *UAP1* has been shown to be required for chitin production, a major structural component of the exoskeleton (Arakane et al. 2011) but chitin is also present in mollusc shells (Peters 1972). Only limited information exists on the possible engagement of the *UAP* in cellular response of invertebrates to exposure to chemical compounds. For example, treatment of the red flour beetle (*Tribolium castaneum*) larvae to diflubenzuron (an insecticide known for inhibiting the production of chitin) resulted in up-regulation of *UAP* protein level, and the observed modulation was suggested to be possibly related to the insecticide's main mechanism of action (Merzendorfer et al. 2012). If mollusc *UAP* serves a similar function to the insect protein (i.e. it is involved in the chitin

synthesis), then the differential expression pattern of this gene observed in our study (Table 2) caused by B[*a*]P exposure may have consequences on mollusc anatomy, i.e. impairing the process of shell formation. Indeed, the existing data at least partially seems to correspond with the hypothesis. For example, Choy et al. (2007) reported on the significant shell growth reduction of the larval Pacific oyster (*Crassostrea gigas*) from broodstock exposed to waterborne B[*a*]P.

Importantly, the EST-A1 original expression regulation found in DD-PCR step (the up-regulation after exposure to 50μM of B[*a*]P) was verified by Real-Time qPCR (Table 2). In additional validation study (Figure 1), the general expression pattern of EST-A1 in the hepatopancreatic gland of snails exposed to the same dose of B[*a*]P was consistent with that obtained in the first part of the study (Table 2). However, the observed expression changes were modest and statistically insignificant in both cases (Table 2; Figure 1; p>0.05). Among the possible explanations for the modest effect could be the small number of experimental individuals taken for the study, together with the large inter-individual variation in mRNA expression, or an inappropriate (short) exposure period. This suggests that different experimental conditions (e.g. long-term renewal exposure) should be included in future studies involving larger experimental groups of animals (i.e. n>4). Consequently, further research is needed to assess the function of snail *UAP* gene and to fully elucidate its role in the cellular response to PAHs exposure.

EST-F11 and EST-F23 were identified as SFI1 protein homolog (Table 2) which is a large product, localized mainly in the cell's centrosome of higher eukaryotes but is also found in yeast (Martinez-Sanz et al. 2006). The SFI1 is an essential protein involved in cell cycle progression and assembly of the mitotic spindle (Ma et al. 1999; Martinez-Sanz et al. 2006), however, information concerning invertebrate animals is very limited. Due to problems with our cloning procedure and PCR primers design, the original (DD-PCR) expression of EST-F11 and EST-F23 could not be verified by the Real-Time PCR. Therefore, to avoid excessive speculation, the discussion concerning a possible role of SFI1 homolog in the cellular response to exposure to B[a]P should not be overemphasized.

For the rest of the obtained EST sequences (EST-G42, EST-I1, and EST-I2), BLAST queries showed no significant similarity to any deposited nucleotide sequence ($E>10^{-5}$). The Basic Local Alignment Search Tool (BLAST) is arguably the most widely used program in bioinformatics, because it allows sequence comparison on currently huge sequence databases. However, the rate of success in *in silico* identification of nucleotide sequences depends on a few factors: i) sequence deposits of the test species (and other closely related animals), ii) length of sequences submitted for the BLAST queries. It seems that in the case of the three unidentified EST sequences, at least one of the factors was the reason for the poor result of BLAST search.

Until recently, the lack of large-scale genomic or transcriptomic information has been a serious drawback in the molecular research involving *L. stagnalis*. Yet, the two studies, conducted by Feng et al. (2009) and Sadamoto et al. (2012), provided novel information by successful sequencing of the pond snail's transcriptome from the central nervous system, and enlarging the public databases of mollusc species. Nonetheless, the current genetic information of the snail has not yet been fully explored (e.g. for other tissues), thereby limiting our search for EST putative identity (Table 2).

As for EST-G42, the qPCR assay not only confirmed the original expression pattern from DD-PCR but also showed significant down regulation in hepatopancreas of snails exposed to B[a]P (p<0.05; Figure 1). Although EST-G42 expression changes in the validation study were not significant among treatment groups (Figure 1), the expression pattern remained coherent with this obtained in the first part of the study

(Table 2). In addition, the validation also revealed significant down-regulation of EST-C5 and the results suggest that the response may be dose-dependent (Figure 1). Despite the change in EST-C5 under-expression being in contradiction to the first part of the study, we believe that together with EST-G42 they may serve as promising candidate-biomarkers of B[a]P exposure for *L. stagnalis*. However, in case of the EST sequences with unknown identity it is difficult to discuss their possible role in the molecular mechanism of B[a]P action and assess their utility in biomonitoring practices. However, future studies involving methods allowing discovery of full-length cDNA sequences, like 3'- and 5'-RACE (Rapid Amplification of cDNA Ends) would be a convenient step (and less expensive to Next Generation Sequencing methods) to elucidate the possible role of EST-C5 and EST-G42 in the response to B[a]P challenge.

Taken together, the results of this study show that genes of diverse function may be involved in the molecular response of the pond snail's hepatopancreas to short-term treatment with B[a]P, extending existing knowledge of the PAHs mode of action. While the observed differential expression pattern of the isolated ESTs do not guarantee any pathophysiological or pathoanatomical roles for them, further research with different experimental conditions (e.g. long-term renewal exposure test) and methods (e.g. RACE) may improve our understanding concerning possible consequences of environmental contamination with PAHs and strengthen the role of *L. stagnalis* in environmental biomonitoring.

ACKNOWLEDGEMENTS

Appendix 1. FASTA nucleotide sequences of ESTs obtained by DD-PCR with highlighted Real-Time PCR amplicons and bracketed primer-specific sites.

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>EST-A1
1      TCAGCGAGGTGTGAAGAGGAAGGTCAAGGTCACCTGCTCCAGTTCTGGGA      50
51     TCAGCTGAATGAGGAGCAGCAGAAGTCTCTTGTAGCTGASATCGAACACA      100
101    TCAACCTGAAGGAAGCCAATGAGTATTTCCAAGAAGCTGACGCTTCCCTG      150
151    AAAGAAACCAATGAAAAATAGATGAACACTTGATGCCCTTGGAGCCTGC       200
201    AGTGTGCGGGAGTGTTGCCAGGAGCAGTGAAGAACTCTTACAAAAATATT     250
251    TGAAAACAGGTTTAACTGCCATAGGTAACAACGAAGTGGCAGTGCTACTG     300
301    CTGGCTGGAGGTCAGGGCACAAGGTTGGGGGTCCCTTACCCTAAAGGGAT     350
351    GTACAATGTTGGGCTTCCTTCTGGGAAGACCCTGTACCAGATACAAGCTG     400
401    AGAGGCTACTGAAGCTGGAGAAGTTGGCTCAGCAGGAGACTGGCAAATCA     450
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451    TGCAATGTGCCATGGTACATTATGACAAGTGAACACACAAAAGAAGCAAC    500
501    AGATACCTTTTTCCAGAAAAACAACCATTTTGGCCTTAGTAAAGAAAATG    550
551    TCATATTGTTTGAGCAGAGTTTGCTCCCCTGTTTTGACTTCAGTGGCAAA    600
601    ATTATTTTAGAATCTCAATCTAAGGTGGCACTGGCTCCAGCTGGCGATGG    650
651    AGGCTTGTACCAAGCCCTTAGAAATAATGGTGTCCTTGATGACCTTACGT    700
701    TACGAGGAGTGAAATATGTTCATGTCTACTGTGTGGACAACATATTGGTT    750
751    AAAATGGCCGACCCCGTGTTCATTGGTTTCTGCCTGAGCAAAGATGTGGA    800
801    GTGTGGGGCAAAGGTTGTGGAAAAGACCATGCCCAAGGAAGCTGTGGGTG    850
851    TTGCGCGTA                                          859
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>EST-B4
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1      TCAGCGAGGTGTGAAGAGGAAGGTCAAGGTCACCTGCTCCAGTTCTGGGA    50
51     TCAGCTGAATGAGGAGCAGCAGAAGTCTCTTGTAGCTGAGATCGAACACA    100
101    TCAACCTGAAGGAAGCCAATGAGTATTTCCAAGAAGCTGACGCTTCCCTG    150
151    AAAGAAAACCAATGAAAAAATAGATGAACACTTGATGACCTTGGAGCCTGC    200
201    AGTGTGCGGGAGTGTTGCCAGGAGCAGTGAAGAACTCTTACAAAAATATT    250
251    TGAAAACAGGTTTAACTGCCATAGGTAACAACGAAGTGGCAGTGCTACTG    300
301    CTGGCTGGAGGTCAGGGCACAAGGTCGGGGGACGCTTCCACA          342
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>EST-C5
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1      TGGAGCAGCTACATCTGGGACAGAGTCAACACCCACCTCAAAAGTGGTGTC    50
51     CAAGCCGGCCAATGACCACACTTTGGGCACACAAGGAATAGGATGGTACA    100
101    TGGTTGTGAGGCCGACTGATGTGGGATACGCAAGGGCGTCCATTCTTGAA    150
151    TCTCCTGCTTTTGGTGCCACGGAAGAAGGTTGTAGTATTGAGTTTTATTA    200
201    TTTTAATGATGGCAGCGGTTCCATCAAGCTTTACCTGTATCCTGATGGTG    250
251    TTACCAACTATGTTGCCTCTGATGTGGGACCTCGCTTGA            289
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>EST-G42
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1      GAGCGTGCAGAGTTGTGGTTCATAGCGGGACGTTGACGTACCAGATGAAG    50
51     ATCCTGGAGCGGTCAAGTCACACTGTCTACCAGAATTTGAGGCAGCTGAT    100
101    AACTGTGAAGTGGAGGTCAAGGTTACAGCAGGATGCGTCTGCTTGGTCTG    150
151    AAAGTTCACATAATTGATTACACTTGAAACGTGCACCGAGTACGCTACAG    200
201    ATGATGTTATACACGTGGATTGTTGTGTCGCCTGAAACTGGGTCAAAGGT    250
251    GACCTCGTCGAGACGTCACAAGACGTAGGCGTGTGCTGGCTCGGGCAGGG    300
301    CTGCACGGAGATCGCTTCATTTACACCGACAGCCGCCGCTGTCAAGAACC    350
351    GAGCCGTCCCTTGTGAAGCCAGTCTTTCCGGTGAAGGTTGGCACGGGCCA    400
401    CCGATGGGAGTTAATTTCACTGACGCGTCCATGTTTTCCCGTAGCGCGTC    450
451    TCTCGGGATGTCTGTCGGTGTTTCCAGATCTAAGGATGGCGCCGTTTGAC    500
501    TCTGTGATCTGTTCTCTTCTACAGGCAGGTCCTCATCGGTCAGCGACAGA    550
551    ACCCCCGTGTCCCATGGATGCAAGGACGTCGAGAACGGCTCCCACGGACT    600
601    TTGAGAGTCCGAACTGGACGCCACAAGATGATCCTCGATATCGTCAGGCA    650
651    TGCTGATTCCGGAGCTAATCGTCCGCGCAAGAGAAGGATTTAAAATAAAC    700
701    TGTTCGCCGGTCGTCGCTAGGGACGCTGGTGGACCGGAAGCACTAACGCT    750
751    CGGCAGTTTGAACCGGAAGGAATCGGTGCTGGGAGCGTTGAAGAAGGCGT    800
801    CGTCCGTCTGGTGGGTGGTGACGGCCCCAGTGTTTGTGCTCGAGACTGGG    850
851    GGCATTCGCGATCGTCTGCTGATGGAAATCGAAACTCTTTACCGTTGTTT    900
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901   CCACCTCGACTGTCGCGAAGTCGATGAAATCATCGCTAAAACTACAAACA      950
951   TTCTGAGAGCATCTTAACGGCTCTGGCACACTGAATGAACTCACGGGTGT      1000
1001  TCCTTGCAGCACGTTCGTCGTTTCGGGTCGCTCGGCARACGAMAATGAAT      1050
1051  CTAAAACGTCCAATGTGCCCAAGTTAAAGTTTTATTGGGCATGATCAAGT      1100
1101  CGAAGCTCAGTGAGTATGAAATAGGTTTAAGTCACTGCTGACTGTTGCTT      1150
1151  CAAACGGTTCGAGGGTTCCTGGAGTAAGCATGCGATGCATTGTCTTTGTT      1200
1201  GAGCTGCATGCTGTAGTGTGGGATGGTGCGCGT                      1233
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>EST-I2
1     TCAGTGAGCGTGTCAGGGAGTAAGCGTGTCGGAGCCACGTTGGGGTGTTG      50
51    CTATACGGCATGCCAGAGAGTTGGCGAATCAGAGAACATTGCGTATGAAA      100
101   GAGTAGGCGGACCGGAGAGACAGCGCGTCCAAGACGAAGTAAGTTGGCGT      150
151   GTCAGAGAAGGGGCGTGTCAGAGTGAGGCGTGTCAGTGAGTAGGTGTGAT      200
201   AGAATATGGGCGTGTCAGGTATTTGACGTGTCAGAGAGATAGTGTTTCAT      250
251   AGACGGAGTTAGTTGGTGTGTCACARAAAGAGCGTTTCTGCGAGACAGCG      300
301   TGTCAGAACAGGGGTTTGCCAGAGCAGGGGCGTTTCARAGAGAGGGCGTG      350
351   TCAGAGAGATGGCGCGTCAGAGAATGGGAGTATCAGAGATGGCGTGTAGA      400
401   ATGTATGCATTAACATTTATTGACAGTACTTTGATATCTTTTTTTTTCCA      450
451   CTGGCAATTACTCTACTGCATTTTATGCATGGAACATTCTTTTTTTACTA      500
501   GGGAGACTTGGTTGTTACGCTTCCACA                            527
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