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► **To cite this version:**

Bowen Li, Chonggang Wang, Kai Ye, Ang Yu, Yixin Chen, et al.. Differential gene expression in the brain of in response to exposure to polychlorinated biphenyls (PCBs). *Marine Environmental Research*, 2008, 66 (5), pp.548. 10.1016/j.marenvres.2008.09.001 . hal-00563053

HAL Id: hal-00563053

<https://hal.science/hal-00563053>

Submitted on 4 Feb 2011

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Accepted Manuscript

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PII: S0141-1136(08)00205-5
DOI: [10.1016/j.marenvres.2008.09.001](https://doi.org/10.1016/j.marenvres.2008.09.001)
Reference: MERE 3287

To appear in: *Marine Environmental Research*

Received Date: 30 April 2008
Revised Date: 30 July 2008
Accepted Date: 5 September 2008



Please cite this article as: Li, B., Wang, C., Ye, K., Yu, A., Chen, Y., Zuo, Z., Differential gene expression in the brain of *Sebastiscus marmoratus* in response to exposure to polychlorinated biphenyls (PCBs), *Marine Environmental Research* (2008), doi: [10.1016/j.marenvres.2008.09.001](https://doi.org/10.1016/j.marenvres.2008.09.001)

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1 **Differential gene expression in the brain of *Sebastiscus marmoratus* in**
2 **response to exposure to polychlorinated biphenyls (PCBs)**

3
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15 **Abstract**

16 Effects of exposure to polychlorinated biphenyls (PCBs) on *Sebastiscus marmoratus* were
17 investigated using a suppression subtractive hybridization method. A total of 108 gene
18 sequences were identified as having the potential for being differentially expressed, and 45
19 could be identified with homologous database sequences. Functions with which they were
20 associated included long-term potentiation and neurotransmitter release, neuroendocrine,
21 mitosis and cell proliferation, energy-related metabolism, general metabolism, signal
22 protein, hemopoiesis system, immune system, and structure. The expression of 17 of these
23 genes was analyzed in the brain using real time fluorescent quantitative PCR. The present
24 study provided a basis for studying the response of fish to PCB exposure and allowed the
25 characterization of new potential neurotoxicol biomarkers of PCB contamination in
26 seawater.

27

28 **Keywords:** *polychlorinated biphenyls, neurotoxicity, Sebastiscus marmoratus, suppression*
29 *subtractive hybridization library, real time PCR*

30 1. Introduction

31 Polychlorinated biphenyls (PCBs) are a class of industrial compounds consisting of
32 paired phenyl rings with various degrees of chlorination (Tilson and Kodavanti, 1997).
33 Although their production was banned in the 1970s, this was after more than a billion
34 kilograms were produced, and so they remain ubiquitous, persistent environmental
35 contaminants that are routinely found in samples of human and animal tissues (Fisher,
36 1999). The central nervous system is one of the target organs for PCBs (Altmann et al.,
37 2001), and PCBs are known to be neurotoxic, inducing neurochemical and behavioral
38 disruptions in humans and experimental animals (Brouwer et al., 1995). Cognitive/motor
39 deficits were found among children who had accidental perinatal exposure to PCBs in
40 cooking oil (Rogan and Gladen, 1992).

41 It has been proved that disruption of the hypothalamus-pituitary-interrenal axis by
42 PCBs involves brain glucocorticoid receptor down-regulation (Aluru et al., 2004). The
43 effects of PCBs on brain development may be attributable, at least in part, to their ability to
44 reduce circulating levels of thyroid hormone. However, the developmental effects of PCB
45 exposure are not simply a function of PCB-induced hypothyroidism (Zoeller et al., 2000).
46 A high dose of PCBs appears to result in neuronal loss, as reflected by a decrease in
47 glucocorticoid receptor (GR), heat shock protein 90 (hsp90), and heat shock protein 70
48 (hsp70) expression, leading to the disruption of the negative feedback regulation of cortisol
49 during stress (Aluru et al., 2004). In recent years, learning deficits and endocrine disrupting
50 actions have been observed whereby the non-coplanar congener exhibits neurotoxicities via
51 different mechanisms with coplanar PCB (Zhou et al., 2004).

52 However, the neurotoxic mechanism of PCBs is complex and actually still unclear.
53 The aim of this study was to identify the transcripts which were up- or down-regulated by
54 PCBs in the brain of the marine fish *Sebastiscus marmoratus* using suppression PCR
55 subtractive hybridization (SSH). This is a highly effective genome-wide approach that
56 enriches for differentially expressed mRNA transcripts by generating subtracted cDNA
57 libraries (Alonso and Leong, 2002; Diatchenko et al., 1996; Straub et al., 2004).
58 Differential expression of some of the genes identified by SSH was confirmed by real time
59 quantitative PCR. This method is very sensitive for the detection and quantification of gene
60 expression levels, in particular for low abundance mRNA (Feng et al., 2007).

61

62 **2. Materials and methods**

63 **2.1. Chemicals**

64 Aroclor 1254, a commercial PCB mixture was purchased from Sigma-Aldrich
65 (Sigma-Aldrich Co., USA), and a PCR-Select cDNA Subtraction Kit was obtained from BD
66 Biosciences (BD Biosciences, UK). All other chemicals were of analytical grade and were
67 obtained from commercial sources.

68 **2.2. Treatments and sample collection**

69 All animal experiments were conducted according to the research protocols approved
70 by Xiamen University Institutional Animal Care and Use Committee. Female *Sebastiscus*
71 *marmoratus* weighing 23-27 g, were captured from the coastal area of Xiamen city, Fujian
72 Province, China. Before the exposure experiment, the fish were acclimated in tanks (25 fish
73 in each tank) containing 50 L of aerated sand-filtered seawater, with natural photoperiod

74 for 7 days. Fish were dosed via single intraperitoneal injection with PCBs (3 mg/kg body
75 weight in corn oil) or with only corn oil as a control. After 3 days, fish were randomly
76 sampled from each group. The isolated brains were frozen in liquid N₂ and immediately
77 stored at -80 °C until analysis.

78 **2.3. Suppression subtractive hybridization**

79 For the SSH procedure (PCR-Select cDNA Subtraction Kit, BD Biosciences, UK)
80 mRNA was isolated from fish brains (RNAiso Reagent and *OligotexTM-dT30<Super>*
81 mRNA Purification Kit, TaKaRa Biotechnology (Dalian), PR. China) and two libraries
82 constructed in a pMD18-T vector (TaKaRa Biotechnology (Dalian), PR. China), enriched
83 for genes which had been up-regulated (forward subtracted) or down-regulated (reverse
84 subtracted) in response to PCBs. For each subtracted cDNA library, 150 random white
85 colonies were selected and PCR amplifications were performed with nested PCR (primer
86 sequences: forward 5'-TCG AGC GGC CGC CCG GGC AGG T-3', reverse 5'-AGC GTG
87 GTC GCG GCC GAG GT-3'). Candidate positive clones from the subtracted cDNA library
88 were selected for sequencing. The sequence data were analyzed using the Vec Screen
89 program (NCBI) for vector masking, and all adaptor sequences were removed. Each
90 sequence of the isolated clones was used for homology searches in GenBank by BLASTN
91 (NCBI)

92 **2.4. Real time fluorescent quantitative PCR**

93 The real time fluorescent quantitative PCR analysis used total RNA isolated from
94 single-brain pools (n=6). SYBR green I was used as the fluorescent dye for real time
95 quantitative PCR on a Rotorgene 3000 (ABI, USA). The housekeeping gene of beta-actin

96 was used as the endogenous assay control (primer sequences: forward 5'-AGG GAA ATC
97 GTG CGT G-3', reverse 5'-ATG ATG CTG TTG TAG GTG GT-3'). PCR reactions were
98 performed in triplicate for each cDNA sample.

99 The Relative Expression Software Tool - Multiple Condition Solver (REST-MCS ©-
100 version 2) was used to calculate the relative expression of target genes mRNA in real time
101 fluorescent quantitative PCR using Pair Wise Fixed Reallocation Randomization Test©
102 (Pfaffl et al., 2002). The mathematical model used is based on the mean crossing point
103 deviation between sample and control group of target gene, normalized by the mean
104 crossing point deviation of reference genes. Specific amplification efficiencies are included
105 in the correction of the quantification ratio. Significant differences between groups were
106 determined by ANOVA and secondary testing was done by using Tukey's LSD.

107

108 **3. Results and discussion**

109 PCR screening of colonies for recombinants was done with insert-specific primers in
110 parallel with the generation of archived, 96-well format, bacterial stock cultures. Colonies
111 containing inserts were identified using agarose gel electrophoresis of the PCR products.
112 Eighty clones of forward subtraction and 28 of reverse subtraction were selected and
113 sequenced. A total of 108 expressed gene sequences were identified by BLAST searches.
114 Seventy-nine transcripts were obtained and analyzed by BLASTN (nucleic acid) and
115 TBLASTX (translated protein query search) and a selection are shown in Table 1. The
116 homology search revealed that 45 clones contained substantial sequence homologies
117 ($E\text{-value} < 1e^{-6}$) with known gene fragments or full-length cDNAs (most belong to

118 *Tetraodon nigroviridis*) and 34 possibly novel genes, showing no significant homologies
119 with any known sequences in the GenBank.

120 The clones could be arranged into a number of distinct functional groups
121 representative of protective mechanisms associated with responses to PCB exposure,
122 including genes involved in PCB metabolism, such as *cytochrome b* and *cytochrome c*
123 (Goksoyr and Forlin, 1992); some correlated with neural functions, such as *snap25a*
124 (Roberts et al., 1998), *rtn1* (De Craene et al., 2006); and of mechanisms of energy
125 metabolism and cellular regulation, while a number were cryptic (classifications are given
126 in Table 1).

127 **Positions of Table 1**

128 The real time quantitative PCR primers for 20 candidate ESTs were designed, and 17
129 were used for quantitative PCR (Table 2). The differential expression of 17 ESTs included
130 10 which encoded known proteins likely to be involved in PCB neurotoxicity, 5 which
131 belong to function-unknown cDNA/mRNA, and 2 were potentially novel gene transcripts.
132 The results showed the presence of differentially expressed genes in the brains of the
133 PCB-treated fish compared with the control group (Figure 1).

134 **Position of Table 2**

135 **Position of Figure 1**

136 Long-term potentiation (LTP) is a persistent synaptic enhancement induced by
137 high-frequency stimulation of afferents and has been suggested to be an important
138 component of the cellular basis of certain forms of memory and learning (Collingridge,
139 1987). LTP has been introduced in neurotoxicological studies on PCBs, and PCB-induced

140 LTP deficits reveal that there are multiple factors have to be considered besides the
141 N-methyl-D-aspartate (NMDA) receptor (Altmann et al., 2001; Gilbert et al., 2000). In the
142 present study, EST 13 and EST 14 contained substantial sequence homologies with genes
143 of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor β subunit
144 and calmodulin respectively, which correspond to LTP (Sandkuhler, 2007). It has been
145 reported that LTP deficits responding to PCBs can only be partly related to the reduction of
146 binding sites for the NMDA receptor (Altmann et al., 2001). Our result suggested that the
147 AMPA receptor was also induced by PCB-treatment. The down-regulation of these genes
148 could induce LTP deficits, which may be one likely mechanism for neurochemical and
149 behavioral disruptions responding to PCBs. EST 9 is homologous with the gene of 25 kDa
150 synaptosomal-associated protein (SNAP-25), which is a presynaptic protein involved
151 in neurotransmitter exocytosis at the nerve terminal (Roberts et al., 1998). At the
152 neuronal synapse, the central role of SNAP-25 is evident from the impairment of
153 neuronal secretion by botulinum neurotoxin A/E, a toxin that proteolytically
154 inactivates SNAP-25 (Hayashi et al., 1994). Moreover, loss of SNAP-25 expression
155 in mice results in embryonic lethality late in gestation with evoked
156 neurotransmitter release absent at the neuromuscular junction and central
157 synapses (Washbourne et al., 2002). This study showed that the expression of
158 SNAP-25 was decreased after PCB-treatment, which is likely to have induced the
159 disruption of synaptic plasticity and LTP, resulting in the inordinateness of central
160 neural system functions. EST 1 is homologous to a gene of Tob (the transducer of
161 ErbB-2), which is well known as a negative regulator in peripheral cells (Matsuda et al.,

162 1996). The elevated expression level of Tob responding to PCB-treatment suggested that
163 Tob might be another potential core gene involved in the neurotoxicity of PCBs.

164

165 **4. Conclusions**

166 In conclusion, our results identified a number of ESTs suitable for further
167 development as environmental biomarkers, most of which had novel sequences and
168 although some remained cryptic, those putatively identified were informative regarding the
169 potential mechanisms of PCB neurotoxicity. However, further investigation is required to
170 understand the effects of PCB-induced neurotoxicity in marine fish.

171

172 **Acknowledgements**

173 This work was supported by the National Natural Science Foundation (40606027) of
174 China and by Fund (MEL0606) of the State Key Laboratory of Marine Environmental
175 Science, Xiamen University, China. Professor John Hodgkiss is thanked for his assistance
176 with English.

177

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235 Table 1 Characterisation of ESTs isolated from polychlorinated biphenyls-treated
236 *Sebastiscus marmoratus*.

237

238 Table 2 Primers used for real time quantitative PCR.

239

240 Figure 1. Figure 1. Gene expression profile of genes that were affected by PCBs: The
241 values were gene absolute regulation calculated by REST-MCS ©- version 2 from
242 duplicate PCR determinations of three independent experiments for each set of conditions
243 tested (PCB-treated and the control). * & ** indicate expression levels significantly
244 different from the control ($p < 0.05$ & $p < 0.01$ respectively) by Tukey's LSD.

245

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246 Table 1
 247 Characterisation of ESTs isolated from polychlorinated biphenyls-treated *Sebastiscus marmoratus*
 248

Homologous gene & species	EST #	Insert (bp)	E-value	Change	GenBank Access No.
<u>LTP and neurotransmitter release related</u>					
<i>Danio rerio</i> SNAP 25a	9	271	7e ⁻⁴⁷	↓	<u>FF403915</u>
<i>Carassius auratus</i> kainate receptor (AMPA)beta subunit	13	384	4e ⁻⁶³	↓	<u>FF403841</u>
<i>O. latipes</i> calmodulin	14	541	2e ⁻¹⁶²	↓	<u>FF403842</u>
<i>Scophthalmus maximus</i> clone sba441 ependymin	20	486	2e ⁻³¹	↓	<u>FF403849</u>
<u>Neuroendocrine</u>					
<i>Gasterosteus aculeatus</i> RTN1	8	321	7e ⁻¹¹²	↓	<u>FF403914</u>
<i>Paralichthys olivaceus</i> carboxypeptidase H	22	587	2e ⁻¹³²	↓	<u>FF403851</u>
<u>Mitosis and Cell proliferation related</u>					
<i>Gillichthys mirabilis</i> transducer of ERBB-2(Tob)	1	305	4e ⁻³⁰	↑	<u>FF403837</u>
<i>Danio rerio</i> dynein cytoplasmic 1 heavy chain 1	6	470	5e ⁻²⁵	↓	<u>FF403892</u>
<i>Oreochromis mossambicus</i> stathmin-like mRNA	23	745	0.0	↓	<u>FF403852</u>
<u>Energy-related metabolism</u>					
<i>Scombridae gen. sp.</i> cytochrome c oxidase subunit VI b precursor	4	268	5e ⁻⁶⁰	↑	<u>FF403870</u>
<i>Sebastes maliger</i> cytochrome b	5	326	1e ⁻⁹¹	↑	<u>FF403881</u>
<i>Rhabdosargus sarba</i> sodium potassium ATPase α subunit	15	754	0.0	↓	<u>FF403843</u>
<i>Helicolenus hilgendorfi</i> ATPase 6	26	165	2e ⁻⁵²	↑	<u>FF403855</u>
<i>Kyphosus vaigiensis</i> NADH dehydrogenase subunit 2	31	272	7e ⁻³⁹	↑	<u>FF403861</u>
<u>General metabolism</u>					
<i>Danio rerio</i> proteasome subunit Y	32	246	5e ⁻³²	↓	<u>FF403862</u>
<i>Sphyræna argentea</i> lactate dehydrogenase-A	52	487	3e ⁻⁴⁷	↑	<u>FF403884</u>
<i>Danio rerio</i> glutamic-oxaloacetic transaminase 1, soluble	56	439	8e ⁻⁹²	↓	<u>FF403888</u>
<u>Signal proteins</u>					
<i>Danio rerio</i> zinc finger protein zic1	10	379	4e ⁻¹⁰⁷	↓	<u>FF403838</u>
<i>Sparus aurata</i> V-Fos transformation effector-like protein	11	468	0.0	↓	<u>FF403839</u>
<i>Danio rerio</i> suppression of tumorigenicity 13 (colon carcinoma) (Hsp70 interacting protein)	21	687	5e ⁻¹²²	↓	<u>FF403850</u>
<i>Siniperca chuatsi</i> clone C311 eukaryotic translation elongation factor -1 gamma	34	270	5e ⁻⁶³	↑	<u>FF403864</u>
<i>Danio rerio</i> zinc finger protein zic1	36	197	1e ⁻⁴¹	↓	<u>FF403866</u>
<i>Homo sapiens</i> zinc finger protein 238 (ZNF238), transcript variant 1	74	310	1e ⁻¹⁸	↓	<u>FF403908</u>

(continued on next page)

249 Table 1 (continued)

Homologous gene & species	EST #	Insert (bp)	E-value	Change	GenBank Access No.
<u>Immune system</u>					
<i>Epinephelus akaara</i> clone 004 MHC class I alpha antigen	25	353	5e ⁻¹¹	↑	<u>FF403854</u>
<u>Hemopoiesis system</u>					
<i>Oryzias latipes</i> embryonic alpha-type globin	12	371	3e ⁻⁵⁹	↓	<u>FF403840</u>
<u>Ribosomal proteins</u>					
<i>Platichthys flesus</i> 60S ribosomal protein L28	24	308	1e ⁻¹²⁵	↑	<u>FF403853</u>
<i>Paralichthys olivaceus</i> ribosomal protein L17	28	456	1e ⁻¹⁴⁵	↑	<u>FF403857</u>
<i>Siniperca chuatsi</i> clone C426 60S ribosomal protein L35	29	180	6e ⁻⁷¹	↑	<u>FF403858</u>
<i>Pagrus major</i> 60S ribosomal protein L24	30	346	6e ⁻⁸⁸	↓	<u>FF403860</u>
<i>Solea senegalensis</i> ribosomal protein L35a	51	394	3e ⁻¹²²	↑	<u>FF403883</u>
<i>Siniperca chuatsi</i> clone C123 ribosomal protein L7	57	561	0.0	↓	<u>FF403889</u>
<i>Pagrus major</i> ribosomal protein L37	65	259	2e ⁻⁷⁶	↑	<u>FF403898</u>
<u>Identified genes but function unknown</u>					
<i>Tetraodon nigroviridis</i> full-length cDNA	7	428	1e ⁻⁶¹	↓	<u>FF403903</u>
<i>Tetraodon nigroviridis</i> full-length cDNA	16	342	3e ⁻²⁵	↓	<u>FF403844</u>
<i>Gasterosteus aculeatus</i> clone CNB134-E09 mRNA	17	467	1e ⁻⁵²	↓	<u>FF403845</u>
<i>Tetraodon nigroviridis</i> full-length cDNA	18	262	6e ⁻²¹	↓	<u>FF403846</u>
<i>Tetraodon nigroviridis</i> full-length cDNA	19	300	2e ⁻¹⁶	↓	<u>FF403847</u>
<i>Tetraodon nigroviridis</i> full-length cDNA	27	410	4e ⁻¹²⁷	↑	<u>FF403856</u>
<i>Gasterosteus aculeatus</i> clone CFW195-D 03 mRNA	33	220	1e ⁻⁷	↑	<u>FF403863</u>
<i>Tetraodon nigroviridis</i> full-length cDNA	38	474	2e ⁻¹⁰	↑	<u>FF403868</u>
<i>Tetraodon nigroviridis</i> full-length cDNA	49	111	3e ⁻¹⁰	↑	<u>FF403880</u>
<i>Gasterosteus aculeatus</i> clone CGX01-B09 mRNA	54	514	3e ⁻⁶⁰	↓	<u>FF403886</u>
<i>Gasterosteus aculeatus</i> clone CNB24-E09 mRNA	62	335	7e ⁻⁰⁹	↑	<u>FF403895</u>
<i>Tetraodon nigroviridis</i> full-length cDNA	73	453	3e ⁻¹⁵	↑	<u>FF403907</u>
<i>Gasterosteus aculeatus</i> clone CNB134-E09 mRNA	78	264	5e ⁻²⁹	↓	<u>FF403912</u>
<u>Unidentified genes</u>					
No homologous region	2	423	—	↑	<u>FF403848</u>
No homologous region	3	453	—	↑	<u>FF403859</u>
No homologous region	35	392	—	↑	<u>FF403865</u>
No homologous region	37	191	—	↑	<u>FF403867</u>
No homologous region	39	328	—	↑	<u>FF403869</u>
No homologous region	40	548	—	↑	<u>FF403871</u>
No homologous region	41	424	—	↓	<u>FF403872</u>
No homologous region	42	496	—	↓	<u>FF403873</u>
No homologous region	43	627	—	↓	<u>FF403874</u>

(continued on next page)

250 Table 1 (*continued*)

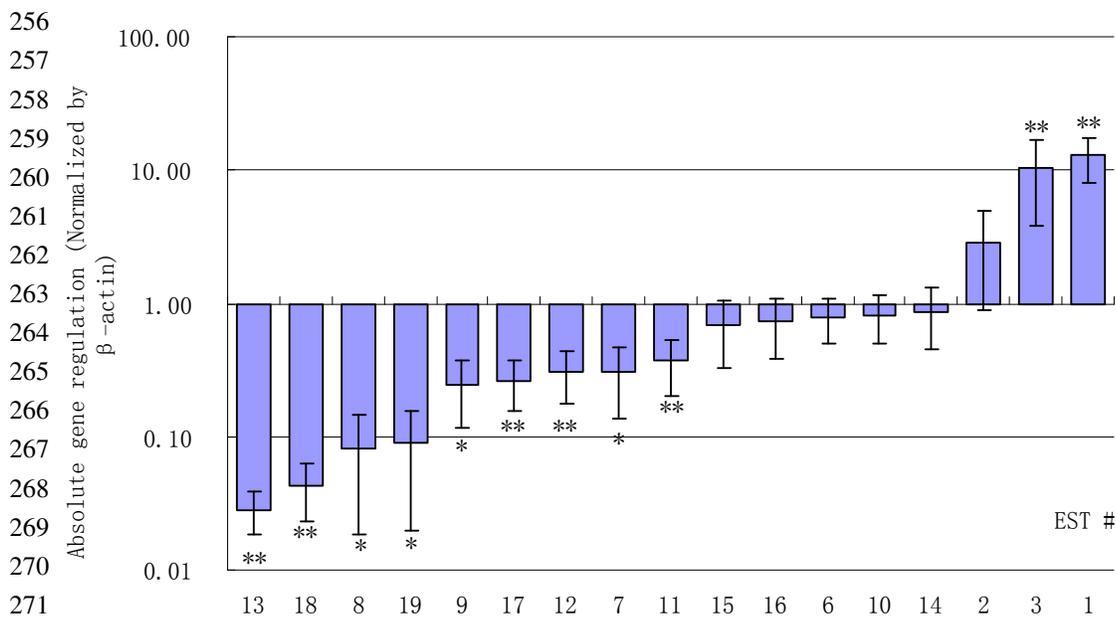
Homologous gene & species	EST #	Insert (bp)	<i>E-</i> <i>value</i>	Change	GenBank Access No.
No homologous region	44	267	—	↓	<u>FF403875</u>
No homologous region	45	595	—	↓	<u>FF403876</u>
No homologous region	46	756	—	↓	<u>FF403877</u>
No homologous region	47	767	—	↓	<u>FF403878</u>
No homologous region	48	611	—	↓	<u>FF403879</u>
No homologous region	50	179	—	↑	<u>FF403882</u>
No homologous region	53	1143	—	↑	<u>FF403885</u>
No homologous region	55	654	—	↓	<u>FF403887</u>
No homologous region	58	234	—	↓	<u>FF403890</u>
No homologous region	59	139	—	↓	<u>FF403891</u>
No homologous region	60	315	—	↓	<u>FF403893</u>
No homologous region	61	510	—	↓	<u>FF403894</u>
No homologous region	63	409	—	↑	<u>FF403896</u>
No homologous region	64	246	—	↑	<u>FF403897</u>
No homologous region	66	332	—	↑	<u>FF403899</u>
No homologous region	67	931	—	↓	<u>FF403900</u>
No homologous region	68	612	—	↓	<u>FF403901</u>
No homologous region	69	268	—	↓	<u>FF403902</u>
No homologous region	70	240	—	↓	<u>FF403904</u>
No homologous region	71	495	—	↓	<u>FF403905</u>
No homologous region	72	519	—	↓	<u>FF403906</u>
No homologous region	75	402	—	↓	<u>FF403909</u>
No homologous region	76	342	—	↓	<u>FF403910</u>
No homologous region	77	555	—	↓	<u>FF403911</u>
No homologous region	79	338	—	↓	<u>FF403913</u>

251

252 Table 2
 253 Primers used for real time quantitative PCR
 254

EST #	GenBank Access No.	Forward Primer(5'→3')	Reverse Primer(5'→3')	Tm(°C)
1	<u>FF403837</u>	ACTATAATCCTTGAAAGTCCAGT	AACTTCAGTCTGAGCAACATG	53
2	<u>FF403848</u>	ACCTGAGGGACAGAAACG	TCCCAGAGCCACAACAC	53
3	<u>FF403859</u>	GAAATATCACAAGCGTGGC	GCTCATCTGAATCCCTTTTC	53
6	<u>FF403892</u>	ATAATCACCTACATTCAATTGCT	TACCATCGGTCAACATTCTC	53
7	<u>FF403903</u>	AGAAAGAATACCCGGACAG	GCGATGACGTGGTCACA	53
8	<u>FF403914</u>	ATACAAAAGCAAAAACCCTAC	GCGAAATGCCACAGA	53
9	<u>FF403915</u>	GCAAAATGTCCACCACTTC	GCACACCAAATCGCCA	53
10	<u>FF403838</u>	GTGCTACTCAAACCTGGACCT-	AATATTTGCGATGAATGGAC	53
11	<u>FF403839</u>	GAAACCCACGCAGAACAG	AGCTCATCACCGAGGATG	53
12	<u>FF403840</u>	ATCTTGAAGTTGGCAGGG	ACTGGAAGGACTTGAACCC	53
13	<u>FF403841</u>	ACGGGCTTCTTTGATTTC	GAATCTGTTCCGGCTCACTC	53
14	<u>FF403842</u>	CACAGACAGCGAGGAGGA	CGCCGTCATCATTTGTAC	53
15	<u>FF403843</u>	AAGAAGTCCATCGCCTACA	GGTTCCTGGGCTGTCTC	53
16	<u>FF403844</u>	TTTTGGGGGATTTTATGC	CACAGAAGATACCTTGGACAG	53
17	<u>FF403845</u>	CCTCATCATTCGTAGTCAGC	CACTTGGATAGGGGTTGG	53
18	<u>FF403846</u>	GAGAAGAGGGCTTTTAGAGAG	TTGATAGCAGTTGTAATTGTCTG	53
19	<u>FF403847</u>	TTGAATCACTTCTCCTCTG	GCCAAGGCTATACCTGCT	53

255



273 Figure 1. Gene expression profile of genes that were affected by PCBs.

274 The values were gene absolute regulation calculated by REST-MCS ©- version 2 from
275 duplicate PCR determinations of three independent experiments for each set of conditions
276 tested (PCB-treated and the control). * & ** indicate expression levels significantly
277 different from the control ($p < 0.05$ & $p < 0.01$ respectively) by Tukey's LSD.

278