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Characterization of the *Fundulus heteroclitus* embryo transcriptional response and development of a gene expression-based fingerprint of exposure for the alternative flame retardant, TBPH (bis (2-ethylhexyl)-tetrabromophthalate)

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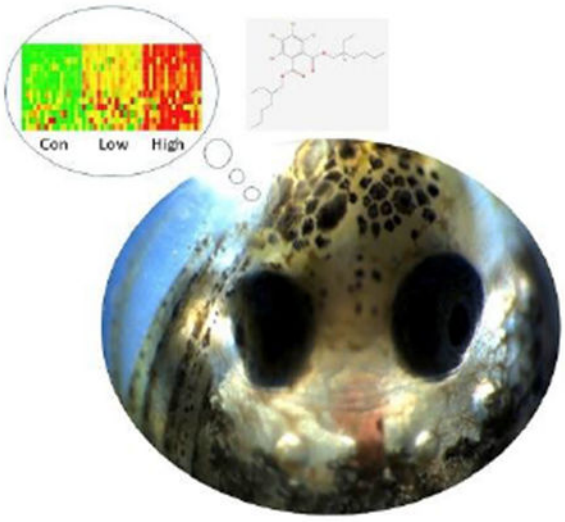
Abstract

Although alternative Flame Retardant (FR) chemicals are expected to be safer than the legacy FRs they replace, their risks to human health and the environment are often poorly characterized. This study used a small volume, fish embryo system to reveal potential mechanisms of action and diagnostic exposure patterns for TBPH (bis (2-ethylhexyl)-tetrabromophthalate), a component of several widely-used commercial products. Two different concentration of TBPH were applied to sensitive early life stages of an ecologically important test species, *Fundulus heteroclitus* (Atlantic killifish), with a well-annotated genome. Exposed fish embryos were sampled for transcriptomics or chemical analysis of parent compound and primary metabolite or observed for development and survival through larval stage. Global transcript profiling using RNA-seq was conducted (n = 16 per treatment) to provide a non-targeted and statistically robust approach to characterize TBPH gene expression patterns. Transcriptomic analysis revealed a dose-response in the expression of genes associated with a surprisingly limited number of biological pathways, but included the aryl hydrocarbon receptor signal transduction pathway, which is known to respond to several toxicologically-important chemical classes. A transcriptional fingerprint using Random Forests was developed that was able to perfectly discriminate exposed vs. non-exposed individuals in test sets. These results suggest that TBPH has a relatively low potential for developmental toxicity(at least in fishes), despite concerns related to its structural similarities to endocrine disrupting chemicals and that the early life stage *Fundulus* system may provide a convenient test system for exposure characterization. More broadly, this study advances the usefulness of a biological testing and analysis system utilizing non-targeted transcriptomics profiling and early developmental endpoints that complements current screening methods to characterize chemicals of ecological and human health concern.

Graphical Abstract



Keywords

TBPH; Transcriptomics; Killifish; AHR; RNA-seq

Introduction

Flame retardants are commonly added to household products and electronics in order to meet flammability standards. Polybrominated diphenyl ethers (PBDEs) were among the most commonly used flame retardants; however, these chemicals have been phased out over the last 15 years due to rising concern over their persistence in the environment, potential for bioaccumulation, of possible negative health impacts. The removal of the PBDEs has prompted the use of novel brominated flame retardants (NBFRs).

Bis (2-ethylhexyl)-tetrabromophthalate (TBPH) is part of several commercial formulations (Firemaster 550, BZ-54) of brominated flame retardants intended to replace the PBDEs. As the use of PBDEs has declined, there has been a corresponding increase in the frequency of detection and concentration of the commercial products containing TBPH (Dodson et al., 2012). TBPH is listed as a high production volume chemical with a 2015 national aggregate production volume between 500 and 5000 tons (https://chemview.epa.gov/chemview?tf=0&ch=26040-51-7&ma=4-11-1981377&tds=0&tdl=10&tas1=1&tas2=asc&tas3=undefined&tss=&modal=template&modalId=109016&modalSrc=4&modalDetailId=&modalCdr=109016). The inclusion of flame retardants into household products in the U.S. is largely driven by California Technical Bulletin TB117 (Stapleton et al., 2012): however, TBPH has been identified in house dust world-wide, indicating potentially widespread human exposure (Ali et al., 2012; Ali et al., 2011; Dodson et al., 2012; Schreder and La Guardia, 2014; Stapleton et al., 2008). Infants and young children may be at particular risk for exposure given an increased frequency of hand-to-mouth contact coupled with the presence of these chemicals in house dust, baby products and children's hand wipes (Stapleton et al., 2011; Stapleton et al., 2014). Air samplingevidence, as well as the identification of TBPH in remote areas, suggest that TBPH has the potential for long-range transport and is being

globally distributed (Ma et al., 2012). These flame retardants are also frequently identified in environmental media, including air (Ma et al., 2012), solid waste leachates and wastewater treatmentsludge (La Guardia et al., 2010). TBPH is poorly absorbed in mammals (Knudsen et al., 2017) and in fish (Nacci et al., 2018). Furthermore, in mammals, TBPH is minimally metabolized and eliminated fairly rapidly (75% after 24 h, 98% after 72 h in rats) through the feces (Knudsen et al., 2017). Yet TBPH has been commonly found in both human serum (11-164 ng/g lw) and breast milk (0.8-6.6 ng/g lw) (Zhou et al., 2014), in aquatic mammals (<0.04-342 ng/g lw) (Lam et al., 2009), and in fish (nd – 15.7 ng/g lw) (Houde et al., 2014) suggesting that persistent contact even with low levels of TBPH is sufficient to maintain chronic biological exposure.

Widespread exposures and TBPH's structural relationship with diethylhexyl phthalate (DEHP) have prompted concerns about the potential for adverse endocrine activity by TBPH. Specifically, DEHP is a suspected endocrine disrupting compound with anti-androgenic (Lee and Koo, 2007) and thyroid effects (Liu et al., 2015). In addition, there is limited evidence *in vivo* across taxa of TBPH affecting endocrine-controlled processes, i.e., growth (in rodents) (Patisaul et al., 2013) and reproduction (in fish) (Saunders et al., 2015). *In vitro* data, often using mammalian systems such as ToxCast screening (https://www.epa.gov/chemical-research/toxicity-forecasting), suggest a much greater range of possible targets and effects for TBPH. For example, TBPH and its primary metabolite mono-(2-ethyhexyl) tetrabromophthalate (TBMEHP) affects steroid hormone levels inconsistently when tested in various systems (Mankidy et al., 2014) (Saunders et al., 2013) (Fic et al., 2014) (Krivoshiev et al., 2016). Evidence supporting TBPH-induced thyroid activity is equally mixed (Egloff et al., 2011; Klopcic et al., 2016; Patisaul et al., 2013). Other putative TBPH or TBMEHP targets and activities identified include the glucocorticoid receptor (Klopcic et al., 2016), lipid accumulation (Tung et al., 2017) adipocyte differentiation (Springer et al., 2012). Taken together, TBPH has been shown in various experimental systems to have the potential to act through diverse mechanisms of action (MOAs).

The first goal of the current study was to provide novel information on potential and realized effects of TBPH by evaluating changes in development and expression levels of the transcriptome of exposed *Fundulus heteroclitus* (Atlantic killifish, or referred to here as killifish) embryos. The killifish was selected for study because it is well-studied as a lab and field ecological model (e.g. (Burnett et al., 2007),), and has a well-characterized genome, which greatly simplifies analysis and interpretation. This study also provides information complementary to results of TBPH exposures conducted using adult killifish (Nacci et al., 2018). The use of whole embryos was paired with RNA-seq to reveal transcriptome- and organism-level patterns without *a priori* focus on a particular tissue or specific hypothesis of potential effects. The second goal of this study was to demonstrate the process of identifying a transcriptomics-based fingerprint that could be used to identify exposures to compounds with MOAs to TBPH in real-world samples.

2. Materials and methods

2.1. Killifish embryo exposures

Embryo-Larval Assay (ELA) procedures for killifish have been described in detail elsewhere (Nacci et al., 2005; 2010), and were conducted for this study with minor modifications as described here. Adult killifish (~100-200 fish) were collected using baited traps at an uncontaminated estuarine site in Barnstable, MA ('Scorton Creek'; (Nacci et al., 2010)). Fish were returned to US EPA ORD aquarium facilities (Narragansett, RI), and maintained in ~300-~600 L flowing sea water tanks for > six months or up to two years, and then used as breeding stock. These stocks provided hundreds of embryos that were collected following unstimulated or manually induced spawning events, held at 23 °C overnight, then screened to remove dead or abnormal embryos. Typically, batches of normally developed embryos from a single tank of breeding stock were used in a single ELA.

Each ELA used embryos exposed individually from one to seven days post-fertilization (dpf) in ten mL of amended sea water. To mitigate the transfer of exposure solution, all embryos from a single treatment were transferred to a single vial containing 10 mL clean sea water before further processing. Then, one subset of embryos from each treatment (ten) was distributed to ten individual tubes for flash freezing by immersion in liquid nitrogen and archived at −80 °C until genomic or chemical analysis.

Another subset of embryos from each treatment (20) was allowed to develop and observed for biological effects until seven days post hatching (dph). be Each of these embryos was was transferred to a well of a 12-well disposable plate (Thermo Fisher Scientific, Rockville, MD, USA) containing uncontaminated sea water-dampened 20 mm Restek Cellulose filters (made for ASE 200 extraction cells, Restek, Bellefonte, PA, USA), and allowed to develop at 23 °C. At 14 dpf, plates were rocked gently and sea water added to each well to initiate hatching. Individual larvae were maintained in single wells containing 3 mL sea water, incubated at 23 °C, fed 48-h hatched *Artemia* ad lib daily, and renewed with sea water on alternate days until seven dph (~21 days post-fertilization) when the ELA was terminated. Throughout the ELA, individuals were assessed daily for hatching and survival, and were phenotyped microscopically at 10 dpf, when abnormalities of developmental stage and features were noted (e.g. (Whitehead et al., 2010),).

2.2. TBPH solutions

LO and HI dose stock solutions of TBPH were made in acetone and diluted 1000 fold into sea water to nominal exposure concentrations of 9.865 μg TBPH/mL and 98.65 μg TBPH/mL respectively. Acetone used as carrier and basis for stocks was first eluted through an activated charcoal column to remove any trace organic contamination. Prior studies have shown that ELA success does not differ between acetone carrier (0.1%) and unamended control embryos (data not shown). TBPH (AK111508, neat, >95% purity) was purchased from Ark Pharm, Inc (Libertyville, IL). These stocks had been used in a prior study where adult killifish were exposed to TBPH via diet, which demonstrated that TBPH is poorly bioaccumulated from diet (Nacci et al., 2018). However, given the low water solubility and

high log Kow of TBPH (Hanson et al., 2019) water-born exposures were expected to produce high concentrations of TBPH in lipophilic embryos (EPA, 2015).

2.3. Analysis of TBPH and TBMEHP

Individual embryo weighing approximately 6 mg (Supplemental Materials: Table S1) were analyzed for TBPH and TBMEHP using the isotopic dilution method of Bradley et al. (2013). The method of Bradley et al. is for the analysis of foods including fish and eggs for phthalic acid, phthalate mono-esters, and phthalate diesters. The method is described in detail in the Supplemental Materials. Briefly, the embryos after homogenization were spiked with surrogates and then, extracted using 1 mL aliquot of acetonitrile:dichloromethane +5% Acetic Acid (1:1 v/v) mixture. After centrifugation, the supernatant was transferred to glass test tube, and this process was repeated two additional times. The combined extract was evaporated to dryness, reconstituted in 300 μL of acetonitrile, sonicated, and then, frozen. The frozen extracts were centrifuged and supernatant transferred to GC vials. Internal standards were added to the vials and extracts analyzed using Thermo GC-Q Exactive GC-HRAM-MS system for TBPH and Agilent 6410 series LC-QQQ system for TBMEHP.

2.4. RNA isolation

Total RNA was isolated from embryos using the MagMAX™-96 Total RNA Isolation kit (ThermoFisher, Waltham, MA) following the manufacturer's protocol supplied with the kit, except as noted below. Homogenization of embryos was performed in 1.5 ml microcentrifuge tubes using a Bullet Blender© Storm 24 homogenizer (Next Advance, Troy, NY) with a 3.2 mm stainless steel bead added to each sample. Each embryo was homogenized in 100 μl of MagMax Lysis/Binding solution supplemented with 0.7 μl β-mercaptoethanol. Contaminating DNA was removed from samples using the supplied Turbo DNase. Both the duration of DNase treatment and incubation temperature were increased, to 20 min and 37 °C, to increase the effectiveness of digestion. RNA was eluted from the magnetic beads using 25 μl of Elution Buffer. During the elution step, samples were incubated at 37 °C.

RNA was quantified on a Synergy HTX Multi-Mode Reader using a Take3 micro-volume plate (BioTek, Winooski, VT). RNA quality was analyzed on a 2100 Bionalyzer using the RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA). RNA Integrity Number (RIN) values averaged 9.8 for all samples. Samples were diluted with nuclease-free water to a concentration of 50 ng/μl.

Libraries were prepared (n = 16 per treatment) using the TruSeq Stranded mRNA Library Prep Kit for Neoprep (Illumina, San Diego, CA) according to the manufacturer's protocol. One hundred ng RNA was used as input into the automated NeoPrep System (Illumina, San Diego, CA). The NeoPrep is able to prepare libraries for up to 16 samples. Samples were blocked by treatment for a multiplex level of 16 samples. This level of multiplexing provides a theoretical sequencing depth of 18.75 M sequences per sample. Following library preparation, individual samples were quantified using the Qubit fluorometer (Thermofisher), normalized to 10 nM and pooled.

RNA-sequencing: Library pools were QC'd and quantified using a combination of Qubit dsDNA HS, either Caliper LabChipGX HS DNA or Agilent Bioanalyzer High Sensitivity DNA and the Kapa Illumina Library Quantification qPCR assays. Each pool was loaded onto one lane of an Illumina HiSeq 4000 flow cell and sequencing performed in a 1×50bp single read format using HiSeq 4000 SBS reagents. Base calling was done by Illumina Real Time Analysis (RTA) v2.7.7 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.1.

2.5. Data analysis

2.5.1. RNA-seq data analysis—RNA-seq data analysis were carried out in two steps. The first step was to identify differentially expressed genes between the control and TBPH treatment groups. The analysis also examined the biological mechanisms and pathways, i.e., GO terms and Pathway enrichment analysis, to identify potential TBPH MOA. The second step was to develop classifiers using gene expression profiles identified in the first step.

2.5.2. Identification of differently expressed transcripts—Raw sequencing data were first subjected to quality control check, using the FastQC (Brown et al., 2017), for read quality, GC content, presence of adaptors, overrepresented *k*-mers and duplicated reads derived from sequencing issues, PCR bias, or contaminations. Using BWA (Li and Durbin, 2009), reads then were mapped to the Atlantic killifish's reference transcript sequences (version 3.02, https://www.ncbi.nlm.nih.gov/genome/?term=Fundulus+heteroclitus) from the NCBI genome annotation database (NCBI Annotation Release 101). Quantification of individual transcripts and identification of differentially expressed genes were then carried out by our internal RNA-seq analysis pipeline based on the improved version of EpiCenter (Huang et al., 2011). Read counts in each biological replicate were normalized so that the average number of total mapped reads was same all replicates across all groups. To reduce false positive hits from background noise, transcripts with average normalized read counts <100 in both groups in comparison were filtered out prior to differential expression analysis. A 5% false discovery rate (FDR) was used as the cutoff of statistically significant genes for further analysis.

2.5.3. Gene enrichment and pathway analysis—The list of significant genes at the 5% FDR cutoff were used for the gene enrichment and pathway analysis with the popular online tool DAVID (Huang da et al., 2009). The same set of genes were projected to corresponding human homolog genes to identify possible affected pathways in the human species using another online tool Reactome (Fabregat et al., 2016).

2.5.4. Classification and predication—We used several methods to avoid overfitting of the model due to the disparity between the dimensionality of the data (tens of thousands) and the replicate number (n = 16 per treatment). The first was to ensure selection of highly relevant features and to reduce data dimensionality by filtering out non-relevant features and noise. We removed lowly expressed transcripts in all groups, i.e. the highest average group count per sample <100, and we filtered out all non-significant transcripts using a fairly stringent FDR cutoff (FDR ≤ 5%). This dramatic reduction of non-relevant features not only reduced the overfitting risk but also increased computational efficiency.

Second, we chose classification methods robust to the overfitting problem. We avoided any overly complex models prone to overfitting by using models that can explicitly penalize the increase of the number of parameters. We tested three different classification methods to find the best predication model for the classification of control and treatment groups (TBPH_LOW and TBPH_HIGH). The three classification methods are: 1) Penalized Logistic Regression (PLR), an efficient regression method designed to overcome the overfitting issue by penalizing increasing model complexity (Schimek, 2017; Sun and Wang, 2012), 2) Random Forests (RF), an ensemble classification method based on decision tree, but without the overfitting tendency of the decision tree method by taking majority decision from a forest of multitude decision trees (Chen and Ishwaran, 2012; Diaz-Uriarte and Alvarez de Andres, 2006), 3) Partial Least Squares Discriminant Analysis(PLS-DA), which bears some similarity to principal components analysis, and is particularly suited to these cases when predictors have more variables than observations as in this dataset (Boulesteix and Strimmer, 2007; Ding et al., 2014; Huang et al., 2013). Although all three methods were tested, detailed discussion below is limited to the highest performing method, RF.

Third, we applied 10-fold cross-validation to further mitigate the overfitting problem. For each method, we randomly split the original training data into 10 equal sized subsamples, and then used 9 subsamples for training and the remaining for validating a model. This process was repeated 10 times and the average of results was reported as the performance of the model. We did the classification and prediction analysis with three sets of training and testing datasets of different partitions of all samples: 12 for training and 4 for testing, 10 for training and 6 for testing, and 8 samples each.

Instead of using the read counts normalized by the mean of total read count across samples, we used RNA-seq data normalized by the RPKM method (Reads Per Kilobase of exon model per Million reads) (Mortazavi et al., 2008) for classification and prediction analysis. This is because total number of sequenced reads or read depth coverage can vary greatly across different RNA-seq experiments. The RPKM normalization method removes the library-size effects on model training of classifiers, and thus alleviates the problem of performance change due to the library-size difference between the training samples and future samples to be predicted.

3. Results

3.1. Chemical analysis of TBPH and TBMEHP in embryos

A significant amount of TBPH was found in embryo tissue at both the low (574 μg/g dwt, standard deviation = 255) and high exposures (3497 μg/g dwt, standard deviation = 2047; Supplementary Table 1). The TBPH metabolite, TBMEHP, was present in concentrations orders of magnitude less than TBPH (TBPH LO 0.0770 μg/g dwt, standard deviation = 0.0308; TBPH HI 0.380 μg/g dwt, standard deviation = 0.170) and accounts for approximately 0.017% and 0.014% of the measured tissue TBPH, suggesting minimal metabolism.

3.2. Non-toxic embryo exposures

An ELA conducted using replicate embryos as those processed and analyzed for chemical analysis and transcriptomics showed that high aqueous TBPH exposures were non-toxic to killifish embryos. Specifically, there were no gross morphological abnormalities noted among treatments, and survival to 7 dph were similar for controls (100% survival), TBPH_LOW (nominal aqueous exposure concentration, 9865 μg TBPH/L; 88% survival) and TBPH_HIGH (nominal exposure concentration, 98650 μg TBPH/L; 94% survival).

3.3. Limited scale but highly significant dose-dependent responses

Initial RNA-seq data analysis demonstrated that the read counts per sample and the corresponding mappable proportions are consistent across treatment groups. On average, each sample has 22 million reads with 78.2% reads mapped to the killifish transcriptome (see Suppl. Table 1). Although only a few transcripts were differentially expressed between the control and TBPH_LOW (6) or TBPH_HIGH groups (13), at the 5% FDR cutoff. All differentially expressed genes identified in the TBPH_LOW group were also identified in the TBPH_HIGH group (see Table 1, Table 2). For all these transcripts, there was only moderate within-group variation in expression in both the control and TBPH_LOW groups, but was more substantial in the TBPH_HIGH group (Suppl. Figure 1). All differentially expressed genes (DEGs) in the comparison between control and TBPH_LOW were CYP P450 related transcripts with the majority being either a transcript variant or pseudogene of CYP P450 1B1. Although this was largely also the case for the TBPH_HIGH group, there were several other transcripts represented with more moderate change of expression levels. A clear dose response was observed in all significant transcripts (see Fig. 1 and Suppl. Figure 2). In particular, CYP450 1A1 (LOC105930482) and 1B1 (LOC105916580) genes were up-regulated 3.34 and 7.70 fold respectively in the TBPH_LOW group and 6.98 and 21.26 fold in the TBPH_HIGH group. Taken together with the high number of replicates employed, this strongly underscores the validity of these results.

3.4. Sample classification and prediction

A second goal of this study was to develop a classifier capable of accurately identifying exposures to TBPH. Three different classification algorithms for binary classification between the control and TBPH_LOW and TBPH_HIGH respectively, as well as between the control and TBPH_LOW and TBPH_HIGH combined were evaluated. The final model was evaluated using a training set consisting of 10 samples, while the remaining 6 were held as a test set. This split ratio was selected as it yielded the overall best prediction probabilities (see Suppl. Table 2 for example). Further, it maximized training while reducing the risk of model overfitting. The RF models performed the best overall with perfect prediction accuracy across three differently partitioned sets of training and testing datasets. In classifications between the combined TBPH_LOW and HIGH and the control group, both the PLR and PLS models also performed well, albeit worse than RF model. Poor performance of the PLS model likely resulted from the low number of DEGs observed. Generally, a higher probability of class assignment was observed for the RF models compared to the other models (Table 3, Suppl. Table 3, Suppl. Table 4). As shown in Table 3, the final three classifiers based on the RF models generally more confidently assign a holdout sample to its

correct group with a high probability, suggesting high reliability of these classifiers for predicting future samples. This also indicates that the gene expression profiles embedded in these classifiers are of true signature differences between the control and TBPH samples. Given such striking differences in gene expression profiles between different groups as shown in Fig. 2, perfect prediction should not be a surprise.

4. Discussion

4.1. TBPH effects and MOAs

TBPH is widely used as a flame retardant in common household products and has been detected in human and animal tissue samples (Liu et al., 2016), household and workplace dust samples, air samples and environmental media and thus presents a significant exposure risk both to humans and the environment. Many studies of TBPH and its metabolite, TBMEHP, have utilized a variety of *in vivo* and *in vitro* approaches and model systems, and identified a wide variety of putative effects (Gramec Skledar et al., 2016; Klopcic et al., 2016; Saunders et al., 2015; Xiang et al., 2017). However, taken as a whole, results among studies were generally weak, inconclusive or contradictory, suggesting observed effects may be secondary, or may be highly dependent on the test system employed. In this study, laboratory exposures of killifish embryos produced tissue concentrations ranging from 574 to 3497 μg TBPH/g dwt and 0.1-0.4 μg TBMEHP/g dwt. Yet, despite these high concentrations there were no overt signs of toxic effects in TBPH-exposed embryos when raised through to larval stages (seven dph). These results are consistent with a companion study where minimal adverse biological effects were observed in adult killifish ranging up to ~20 μg TBPH/g dwt following laboratory dietary exposures (Nacci et al., 2018). To put these exposure concentrations into context, the concentrations of TBPH in wild biota worldwide range up to ~1 μg/g, although data are limited and concentrations are likely to rise (reviewed in (Nacci et al., 2018)). Together, these laboratory exposures, which exceeded by 1-3 orders of magnitude currently measured tissue concentrations of TBPH, demonstrated that TBPH produced little overt toxicity in the killifish life stages tested.

Despite the lack of clearly identified biological or toxicological effects from TBPH exposures, few studies have examined gene expression responses as an alternative experimental line of evidence to further characterize TBPH effects. Those studies that have utilized gene expression generally target genes associated with suspected MOA, such as endocrine activity (Mankidy et al., 2014; Saunders et al., 2015), PPAR-α and γ activation (Springer et al., 2012), lipogenesis (Springer et al., 2012) and PXR activation (Gramec Skledar et al., 2016) using targeted approaches such as QPCR. To the best of our knowledge, no studies have conducted global transcript profiling either *in vitro* or in a vertebrate model system. Thus, global transcript profiling, requiring no *a priori* toxicity information, was applied as a non-targeted approach to identify potential MOAs for TBPH in killifish embryos.

Despite the high levels of TBPH achieved in the current study, few genes were identified as differentially expressed in exposed killifish embryos. Based on gene identity there was no indication of direct effects on the endocrine system or any obvious links to other MOAs previously identified. Consistently, no significant morphological effects or mortality were

observed in the treated groups. All genes identified in the TBPH-LOW were also present in the TBPH-HIGH exposure group, suggesting a dose response and further supporting that observed differential expression was biologically meaningful. Based on gene set enrichment analysis (Table 3), as well as other resources, two main processes are evident: aryl hydrocarbon receptor (AHR) pathway activation, and induction of cytochrome P450s (CYP P450), some of which are activated via the AHR.

Many of the genes identified as differentially expressed in both the TBPH_LOW and TBPH_HIGH treatment groups are known to be either regulated through the AHR pathway or have been previously shown to be differentially regulated following treatment with the most potent AHR agonist, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Of the 13 genes differentially expressed in the TBPH_HIGH treatment, ten have previously shown to associated with the AHR or with TCDD exposure. CYP P450 1A1 is well-known to be induced by AHR, and it, as well as the aryl hydrocarbon receptor repressor (AHRR), are considered “AHR core” genes, which are commonly expressed across wide variety of experimental systems (Watson et al., 2017). Moreover, the AHRR promoter is inducible by TCDD and can be activated by either AHR1 or 2. CYP1A1, 1B1 and AHRR were shown to be strongly upregulated in zebrafish embryos exposed to mITP, component of FM-550 with dioxin-like effects; whereas, up-regulation was not observed for CYP1B1 or AHRR in AHR2-null embryos under the same exposure conditions, suggesting they are downstream of AHR2 activation (Haggard et al., 2017). Xiong et al. (2008) also observed upregulation of CYP1A1 and 1B1 transcripts in zebrafish embryo following TCDD exposure. Most relevant to the test system used here, laboratory and field studies have identified several “AHR core” genes associated with sensitivity to dioxin-like compounds in killifish (Nacci et al., 2016; Proestou et al., 2014; Reid et al., 2016; Reitzel et al., 2014; Whitehead et al., 2012)

While the majority of genes identified as differentially expressed had relatively moderate changes in expression relative to the control, CYP450 1A1 and 1B1 related genes, demonstrated much more drastic treatment effects. These genes were highly differentially expressed in both the TBPH-LOW and HIGH treatment groups and exhibited a dose-dependent response. The CYPs are well known to play critical roles in the metabolism of drugs and environmental toxicants. Cytochrome P450 1A1 is generally shown to be responsive for xenobiotics metabolism while P450 1B1 is associated with sterol metabolism in human drug tests (Guengerich, 2006, 2008; Guengerich et al., 2005). And while their potential role in TBPH metabolism is unclear, little metabolism of TBPH has been observed in this and other tested systems (often mammalian) (Knudsen et al., 2017; Roberts et al., 2012). No differential expression of CYP genes was observed in the only other TBPH study identified that targeted CYP expression (CYP1A4, CYP1A5, CYP2H1, CYP3A37 and UGT1A9) (Egloff et al., 2011). However, this study used chicken embryonic hepatocyte cultures, a drastically different test system than the one employed here.

Within TBPH treatment groups, individual responses varied greatly, with a three-fold difference in expression level of P450 1A1 within the TBPH_HIGH treatment group. Since the adverse effects or toxicity of TBPH on an exposed organism could be affected by the genetic variability in these P450 enzymes, as shown in human studies (Lynch and Price, 2007), this may have implications for assessing the risk posed by TBPH on human and

environmental health. Negative health effects may also be indirect, increased cytochrome P450 enzymes also could reduce effectiveness of many medical drugs or result in other side effects due to interactions of drugs and cytochrome enzymes in humans. It has been shown that variants of P450 enzyme genes can greatly affect drug or metabolite levels in both blood and urine, leading to therapeutic failure, toxicity or even death when the patient receives the commonly recommended dosage of a drug (Nebert and Dalton, 2006; Nebert et al., 2013). TBPH is commonly found in house dust, exposures are highly likely to occur as mixtures with other household product-derived chemicals. Thus, CYP induction by TBPH may alter sensitivities to other chemicals dependent on CYP pathways for metabolism.

Despite the considerable overlap between differentially expressed genes identified in TBPH exposures and those with TCDD and AHR activation, as well as evidence that the debrominated analog, DEHP, can activate AHR (Kruger et al., 2008), there is no prior experimental evidence that TBPH may act as an AHR agonist, although activation of the AHR and the resulting formation of DNA adducts was suggested as a possible mechanism for TBPH genotoxic effects (Bearr et al., 2010). In fact, evidence to the contrary exists. TBPH did not induce developmental heart malformations in zebrafish embryos, a well-established effect of TCDD exposure early in development (McGee et al., 2013). Moreover, using H4IIE reporter gene constructs driven by dioxin response elements, TBPH demonstrated no TCDD-like potencies or cytotoxic effects (Saunders et al., 2013).

In the killifish embryo system used in this study, the over representation of genes directly or indirectly associated with the AHR pathway, especially given the stringency of differential expression analysis and the significant overlap of gene lists between treatment conditions, strongly suggests that TBPH activates the AHR either directly or indirectly. However, given the high exposure and internal TBPH concentrations, TBPH would be considered a very weak AHR agonist. To provide context, CYP1A (or its proxy, ethoxy resorufin-*o*-deethylase, EROD, activity) is induced in killifish embryos by the dioxin-like PCB congener, 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) (Nacci et al., 2010; Whitehead et al., 2012; Whitehead et al., 2010) at estimated embryos concentrations (Nacci et al., 1999) of ≤20 ng PCB126/g dwt. Therefore, TBPH is ~10^4 times less potent as an activator of the AHR pathway in killifish embryos than PCB126, and by extension (Van den Berg et al., 1998) about ~10^7 times less potent than TCDD. At such low potency, it cannot be ruled out that trace impurities in the TBPH stock (>95% purity) as tested may have contributed to this signal.

TBPH was non-toxic to killifish embryos and transcriptomic analysis detected few disturbed pathways, although mammalian studies suggest many potential MOAs. This disparity suggests that killifish may be less sensitive to TBPH than some other fish species, and/or fish species may be less sensitive to TBPH than mammals. That fish, in general, and killifish, more specifically, are known to be relatively sensitive to AHR agonists (Van Veld, 2008; Whitehead et al., 2012) is consistent with the detection of TBPH as a very weak AHR agonist in this study. While transcriptomic analyses provide a comprehensive assessment of potential MOAs, embryo transcriptomics certainly does not reflect comprehensively all pathways important in the regulation of specific organ functions and life events. Thus, our

study in context with others suggests that a suite of screening systems and methods is valuable to evaluate comprehensively chemical risks.

4.2. Classification

Gene expression fingerprints for TBPH exposure were able to differentiate TBPH treated-embryo from untreated. Given that evaluation of the fingerprint was done using a holdout set, this underscores the veracity of the gene expression results. The small number of genes that comprise the fingerprint and their almost complete overlap with AHR responsive genes, suggests that the fingerprint may not be specific to TBPH, but rather is generalized to AHR activation. The power of gene expression fingerprints, as well as other bioassays, lies in their ability to provide information about putative biological activity of unknown samples, whether this be identifying MOA of uncharacterized chemicals or of highly complex mixtures often found in environmental samples. Though there are many efforts to do this through the use of *in vitro* testing, this has yielded mixed results, as these systems often require the manipulation of environmental samples and cannot currently account for metabolism (Thomas et al., 2012). Whereas, in the current system, these limitations are minimized, while concurrently permitting the observation of whole organism biological and toxicological effects. Further, the small size of killifish embryos permits safe and efficient exposures, with potential for testing in a high or medium throughput system. Thus there are many attributes of the biological system used here that, like that of the zebrafish embryo, provide advantages for chemical screening. Killifish also offer the advantage of using an ecologically-important species, with a well-annotated genome. Our system identified a previously unreported activity for TBPH through the activation of the AHR pathway in killifish embryos. The expression pattern of AHR activation is not to unique to TBPH, and is associated with major classes of organic contaminants, including those that are dioxin-like. However, our development of this test system provides a feasible option that complements the current suite of chemical screening methods useful for a number of regulatory applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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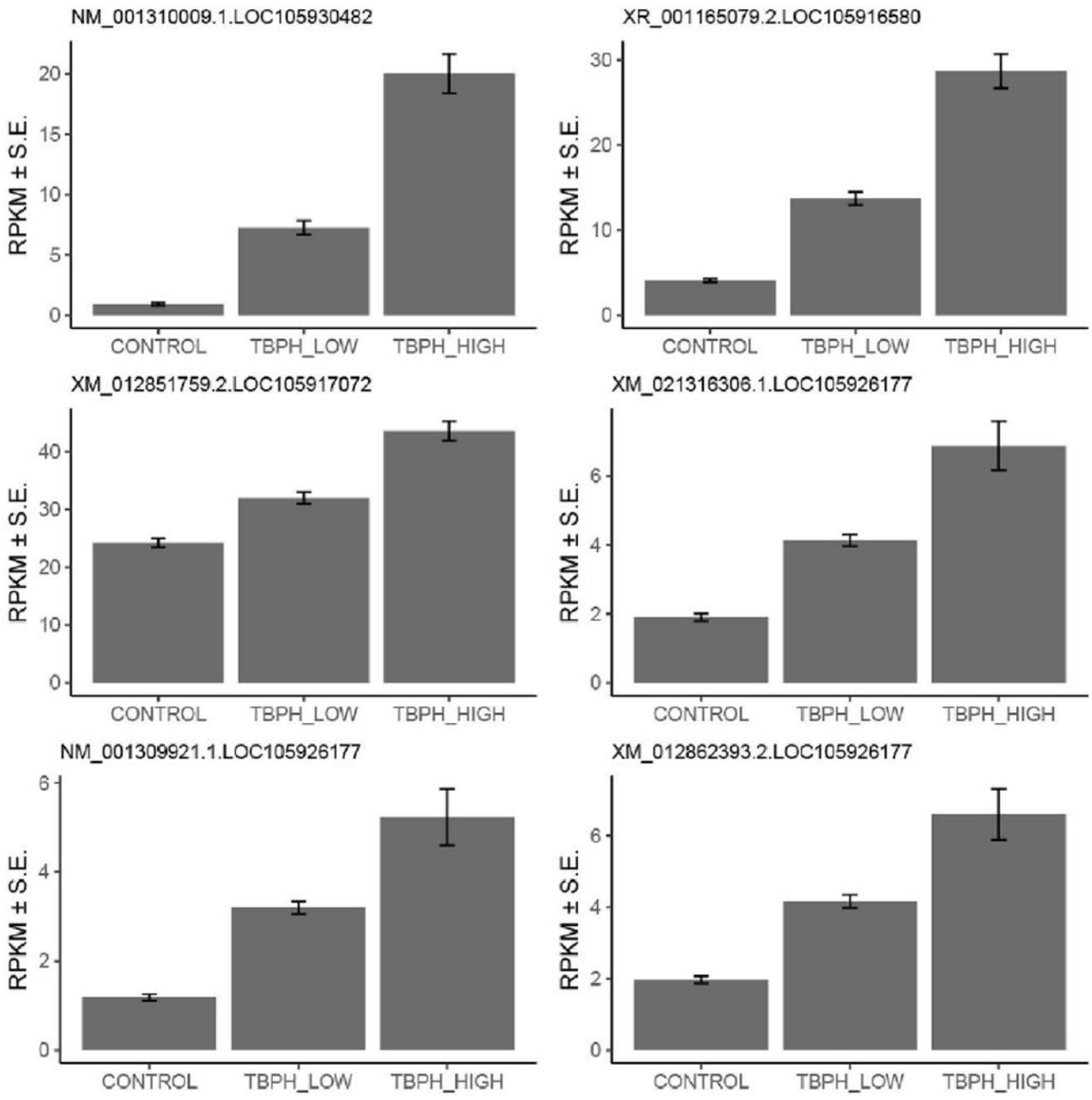

Fig. 1.

Dose-dependent expression of the top 6 significant transcripts among three groups. The six bar-plots show the mean expression level of the top 6 significant transcripts, one for each transcript as indicated by transcript ID at top-left of each plot. In each bar-plot, x-axis shows experimental groups and y-axis is mean expression level in RPKM (Reads Per Kilobase of transcript per Million mapped reads). The height of each box indicates estimated mean expression level and the corresponding error bar shows the 95% confidence interval of the mean estimate (mean ± standard error (S.E)). The mean expression of these six transcripts

were statistically significant in both control-TBPH_LOW and control-TBPH_HIGH comparisons at the 5%.FDR cutoff.

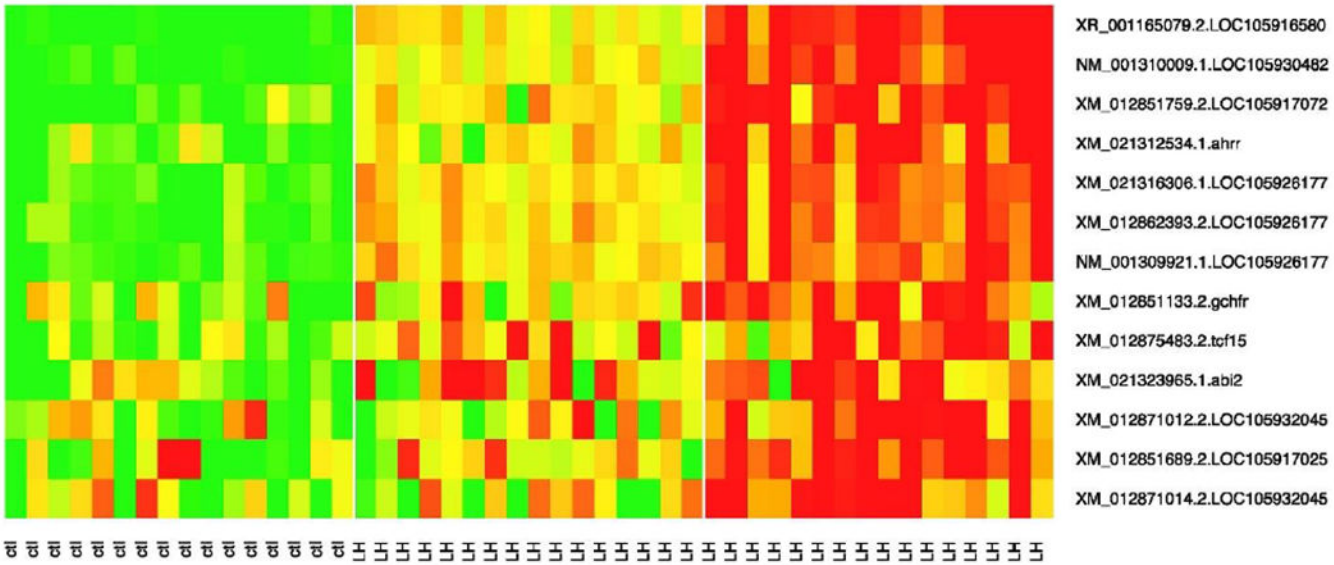

Fig. 2.

Gene expression level heat map of top 13 differentially expressed transcripts. in the heatmap plot, each column stands for an experimental sample, and each row stands for a transcript as annotated on the right size The left pane, consisting of the first 16 samples, is of the control group, the middle pane of the next 16 samples is of TBPH_LOW group, and the right pane of the remaining 16 samples is of TBPH_HIGH group. In the row label, ctl stands for the control group, while LH is for the combined TBPH_LOW and TBPH_HIGH group. The head map colors indicate relative expression level with green representing the lowest expression, and red for the highest expression level. The plot clearly shows dose-dependent response of these genes with increasing expression level from the control, TBPH_LOW, and TBPH_HIGH.

Table 1.

Differentially expressed transcripts between the control and TBPH_LOW groups at the 5% FDR cutoff.

geneID	symbol	type	fold	pvalue	FDR	gene name
NM_001310009.1	LOC105930482	mRNA	7.70	3.05E-16	6.67E-12	cytochrome P450 1A1 (LOC105930482)
NM_001309921.1	LOC105926177	mRNA	2.66	1.32E-13	1.44E-09	cytochrome P450 1B1 (LOC105926177)
XR_001165079.2	LOC105916580	transcript	3.34	9.33E-13	6.80E-09	cytochrome P450 1B1 pseudogene (LOC105916580)
XM_021316306.1	LOC105926177	mRNA	2.16	7.15E-12	3.91E-08	cytochrome P450 1B1 (LOC105926177), transcript variant X2
XM_012862393.2	LOC105926177	mRNA	2.10	1.68E-11	7.35E-08	cytochrome P450 1B1 (LOC105926177), transcript variant X1
XM_012851759.2	LOC105917072	mRNA	1.32	1.13E-06	4.10E-03	cytochrome *b*5 (LOC105917072)

Table 2.

Differentially expressed transcripts between the control and TBPH_HIGH groups at the 5% FDR cutoff.

geneID	symbol	type	fold	pvalue	FDR	gene name
XR_001165079.2	LOC105916580	transcript	6.98	1.33E-66	2.93E-62	cytochrome P450 1B1 pseudogene (LOC105916580)
NM_001310009.1	LOC105930482	mRNA	21.26	3.18E-26	3.51E-22	cytochrome P450 1A1 (LOC105930482)
XM_012851759.2	LOC105917072	mRNA	1.80	1.04E-11	7.62E-08	cytochrome *b*5 (LOC105917072)
XM_021312534.1	ahrr	mRNA	1.79	3.73E-10	2.06E-06	aryl-hydrocarbon receptor repressor (ahrr), transcript variant X1
XM_021316306.1	LOC105926177	mRNA	3.59	9.82E-08	4.33E-04	cytochrome P450 1B1 (LOC105926177), transcript variant X2
XM_012862393.2	LOC105926177	mRNA	3.32	3.74E-07	1.37E-03	cytochrome P450 1B1 (LOC105926177), transcript variant X1
NM_001309921.1	LOC105926177	mRNA	4.34	5.10E-07	1.61E-03	cytochrome P450 1B1 (LOC105926177)
XM_012851133.2	gchfr	mRNA	1.72	3.71E-06	1.02E-02	GTP cyclohydrolase I feedback regulator (gchfr)
XM_012851689.2	LOC105917025	mRNA	1.29	1.02E-05	2.49E-02	aryl hydrocarbon receptor-like (LOC105917025)
XM_012875483.2	tcf15	mRNA	1.36	1.18E-05	2.59E-02	transcription factor 15 (tcf15)
XM_012871012.2	LOC105932045	mRNA	1.21	2.12E-05	4.25E-02	brain-specific angiogenesis inhibitor 1-associated protein 2-like (LOC105932045), transcript variant X1
XM_021323965.1	abi2	mRNA	1.15	2.47E-05	4.53E-02	abl interactor 2 (abi2), transcript variant X14
XM_012871014.2	LOC105932045	mRNA	1.23	2.88E-05	4.87E-02	brain-specific angiogenesis inhibitor 1-associated protein 2-like (LOC105932045), transcript variant X2

Table 3.

The prediction probabilities of holdout testing samples by three random-forest (RF) based binary classifiers. The classifier 1 is for the control and TBPH_LOW groups; the classifier 2 for the control and TBPH_HIGH groups; the classifier 2 for the control and the combined TBPH_LOW and TBPH_HIGH groups. Samples ES1-ES16 are of the control group; ES17-ES32 are of TBPH_LOW, and ES33-ES40 are of TBPH_HIH.

RF Classifier 1			RF Classifier 2			RF Classifier 3		
sample	Control	TBPH_LOW	sample	Control	TBPH_HIGH	sample	Control	TBPH
ES2	100.0%	0.0%	ES2	93.4%	6.6%	ES2	91.2%	8.8%
ES5	100.0%	0.0%	ES5	84.0%	16.0%	ES5	83.0%	17.0%
ES6	100.0%	0.0%	ES6	98.0%	2.0%	ES6	97.6%	2.4%
ES9	100.0%	0.0%	ES9	90.6%	9.4%	ES9	92.4%	7.6%
ES12	100.0%	0.0%	ES12	93.4%	6.6%	ES12	88.0%	12.0%
ES13	85.2%	14.8%	ES13	86.8%	13.2%	ES13	84.4%	15.6%
ES19	2.2%	97.8%	ES35	24.4%	75.6%	ES18	13.8%	86.2%
ES22	0.0%	100.0%	ES38	0.0%	100.0%	ES19	14.0%	86.0%
ES23	0.0%	100.0%	ES39	3.8%	96.2%	ES20	12.8%	87.2%
ES25	0.0%	100.0%	ES41	1.2%	98.8%	ES26	8.6%	91.4%
ES27	0.0%	100.0%	ES43	1.4%	98.6%	ES30	11.8%	88.2%
ES32	4.2%	95.8%	ES48	10.4%	89.6%	ES32	18.6%	81.4%
						ES35	4.4%	95.6%
						ES38	0.0%	100.0%
						ES39	0.0%	100.0%
						ES41	0.0%	100.0%
						ES43	0.0%	100.0%
						ES48	3.2%	96.8%

Table 4.

Results from DAVID's function enrichment analysis of significant genes between the control and TBPH_HIGH groups at the 5% FDR cutoff. The gene functional enrichment analysis revealed that the two genes P450 1A1 and 1B1 affected several enriched functional categories including aromatase activity, microsome, and iron ion binding.

Category	Term	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni
GOTERM_MF	GO:0070330~aromatase activity	0.001549	LOC105930482, LOC105926177	2	4	2582	645.5	0.0046
INTERPRO	IPR008066:Cytochrome P450, E-class, group I, CYP1	0.002167	LOC105930482, LOC105926177	4	3	4152	692.0	0.0193
UP_KEYWORDS	Microsome	0.003108	LOC105930482, LOC105926177	3	5	3216	428.8	0.0397
INTERPRO	IPR002401:Cytochrome P450, E-class, group I	0.009366	LOC105930482, LOC105926177	4	13	4152	159.7	0.0812
INTERPRO	IPR017972:Cytochrome P450, conserved site	0.010802	LOC105930482, LOC105926177	4	15	4152	138.4	0.0931
GOTERM_MF	GO:0005506~iron ion binding	0.011232	LOC105930482, LOC105926177	2	29	2582	89.0	0.0333
INTERPRO	IPR001128:Cytochrome P450	0.011519	LOC105930482, LOC105926177	4	16	4152	129.8	0.0990
GOTERM_MF	GO:0020037~heme binding	0.011619	LOC105930482, LOC105926177	2	30	2582	86.1	0.0345
UP_KEYWORDS	Monooxygenase	0.014255	LOC105930482, LOC105926177	3	23	3216	93.2	0.1703
UP_KEYWORDS	Heme	0.015489	LOC105930482, LOC105926177	3	25	3216	85.8	0.1837
UP_KEYWORDS	Endoplasmic reticulum	0.015489	LOC105930482, LOC105926177	3	25	3216	85.8	0.1837
GOTERM_CC	GO:0005789~endoplasmic reticulum membrane	0.023406	LOC105930482, LOC105926177	3	27	2294	56.6	0.0686
UP_KEYWORDS	Iron	0.025339	LOC105930482, LOC105926177	3	41	3216	52.3	0.2837
UP_KEYWORDS	Oxidoreductase	0.04914	LOC105930482, LOC105926177	3	80	3216	26.8	0.4806