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Use of the enhanced frog embryo teratogenesis assay-*Xenopus* (FETAX) to determine chemically-induced phenotypic effects

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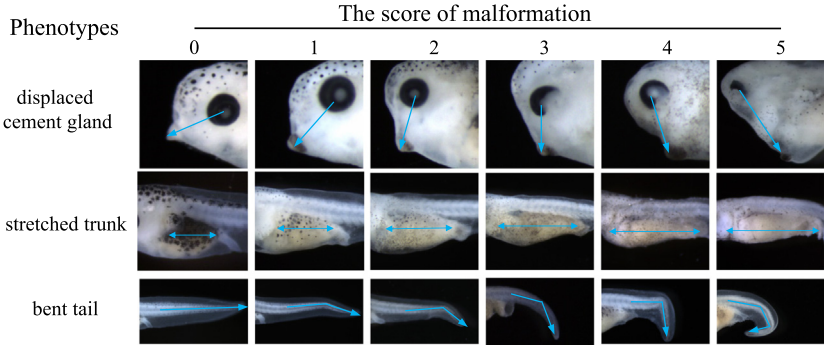
HIGHLIGHTS

- The FETAX is an established method for testing developmental toxicity.
- Test chemicals induced multiple malformations in *Xenopus tropicalis* embryos.
- We proposed a phenotypic method with 20 phenotypes and a 0–5 scoring system.
- The phenotypic profiles were characteristic of different test chemicals.
- The phenotypic method increased the sensitivity and quantitative measurement.

GRAPHICAL ABSTRACT



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ABSTRACT

The frog embryo teratogenesis assay-*Xenopus* (FETAX) is an established method for the evaluation of the developmental toxicities of chemicals. To develop an enhanced FETAX that is appropriate for common environmental contaminants, we exposed *Xenopus tropicalis* embryos to eight compounds, including tributyltin, triphenyltin, $CdCl_2$, pyraclostrobin, picoxystrobin, coumoxystrobin, *all-trans*-retinoic acid and *9-cis*-retinoic acid. Multiple malformations were induced in embryos particularly following exposure to tributyltin, triphenyltin and pyraclostrobin at environmentally relevant concentrations. Based on the range of observed malformations, we proposed a phenotypic assessment method with 20 phenotypes and a 0–5 scoring system. This derived index exhibited concentration-dependent relationships for all of the chemicals tested. Furthermore, the phenotype profiles were characteristic of the different tested chemicals. Our results indicate that malformation phenotypes can be quantitatively integrated with the primary endpoints in conventional FETAX assessments to allow for increased sensitivity and measurement of quantitative effects and to provide indicative mechanistic information for each tested chemical.

1. Introduction

The FETAX is a 96-h whole-embryo test that utilizes the embryos of the frog *Xenopus laevis* and was originally developed by Dumont et al. (1983). In 1991, the American Society of Testing and Materials developed test guidelines for the FETAX, which were subsequently revised and republished (ASTM, 1998). Validation studies have shown that the FETAX is valuable for determining the relative developmental toxicity hazards of chemical agents and complex mixtures (Bantle et al., 1990; Dawson et al., 1989; Fort et al., 1989; Morgan et al., 1996). This test can be used as an alternative prescreening test system for conventional

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mammalian organisms in developmental toxicity testing (Bantle et al., 1999a; Fort et al., 1998; Leconte and Mouche, 2013).

The FETAX assay has consequently been adopted in several ecotoxicology applications to determine the toxicities of common environmental contaminants such as metals (Bosisio et al., 2009; Fort et al., 2006), organic compounds (Bacchetta et al., 2008; Chae et al., 2014; Gutleb et al., 2007), and nanomaterials (Nations et al., 2011). Additionally, this assay can be used to determine the ecotoxicities of environmental samples (Chenon et al., 2003; de Lapuente et al., 2014). The primary endpoints measured in the FETAX include mortality, malformation, and growth. Adding further chemical-specific, quantifiable, and easily identifiable endpoints can further enhance the utility of the FETAX as an environmental monitoring tool.

Phenomics is regarded as the next challenge after genomics and proteomics (Houle et al., 2010). Phenotypes of malformation are particularly important features in the teratogenic assessment of chemicals and have been used in the FETAX (Fort and Paul, 2002; Yu et al., 2011). Bantle et al. (1999b) published guidelines for the identification of malformations of *X. laevis* embryos. Similarly, Kao and Elinson (1988) used the dorsal and axis index (DAI) to evaluate the degree of malformation induced in *X. laevis* embryos by retinoic acids. Likewise, Fort and Paul (2002) developed a characteristic abnormality approach to characterize the responses of *X. laevis* embryos to a teratogenic agent. This scoring approach provides a more reliable method of assessing responses and increases the predictability and versatility of the FETAX (Fort and Paul, 2002). In a similar manner, our previous work involved the development of the index of axis deficiency (IAD) to evaluate the degree of axis malformation and the index of fin deficiencies (IFD) to evaluate fin development in *X.* (*Silurana*) *tropicalis* embryos (Shi et al., 2012; Yu et al., 2011). Recently, a morphological scoring system for ranking tissue-specific malformations in a zebrafish (*Danio rerio*) teratogenicity assay was also proposed (Brannen et al., 2010; Panzica-Kelly et al., 2010). Therefore, endpoints that utilized malformation phenotypes could equally be exploited to develop an enhanced FETAX for use in chemical impact assessments.

The West African clawed frog *X. tropicalis* is an emerging model animal for developmental biology. This frog is closely related to *X. laevis* and shares virtually all of the advantages of *X. laevis* as an embryological system. Additionally, *X. tropicalis* is smaller, has a much shorter generation time, and produces more eggs. *X. tropicalis* can be used effectively as a test organism in the FETAX model (Fort et al., 2004). To date, *X. tropicalis* has been successfully used as a first-line in vivo model for chemical screening and for the testing of reverse engineering approaches (Schmitt et al., 2014; Wheeler and Liu, 2012).

In the present study, we exposed *X. tropicalis* embryos to eight environmentally relevant compounds including tributyltin, triphenyltin, $CdCl_2$, pyraclostrobin, picoxystrobin, coumoxystrobin, *all-trans*-retinoic acid and *9-cis*-retinoic acid. These test materials cover types of contaminants that are regarded as potential factors that lead to declines in amphibian populations (Alsop et al., 2004; Higley et al., 2013). Tributyltin (TBT) and triphenyltin (TPT) have been widely used as biocides in antifouling paints and are well-known endocrine-disrupting chemicals. Such chemicals are already known to induce unique malformations in *X. tropicalis* embryos at environmentally relevant concentrations (Guo et al., 2010; Yu et al., 2011). $CdCl_2$ is a common heavy metal with teratogenic properties in *X. laevis* embryos (Sunderman et al., 1991). Pyraclostrobin, picoxystrobin and coumoxystrobin are common strobilurin fungicides (Balba, 2007). *All-trans*-retinoic acid and *9-cis*-retinoic acid are retinol drugs and known teratogens (Yu et al., 2011). Both of these acids are typically used to validate new developmental toxicity methods (Brannen et al., 2010).

Based on the phenotypes observed and published in the literature, we proposed a phenotypic method that involves 20 phenotypes and a 0–5 scoring system with the aim of developing an enhanced FETAX with integrated phenomics that was applicable to environmentally relevant chemicals at concentrations found in the environment.

2. Materials and methods

2.1. Chemicals

Eight chemicals were selected for testing (Table 1). Coumoxystrobin was purchased from Luyuan Agricultural Materials Co., Ltd. (Liaoning, China). Dimethyl sulfoxide (DMSO) and 3-amino-benzoic acid ethyl ester (MS-222) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The remaining chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Husbandry of *X. tropicalis* and exposure experiments

X. tropicalis adults were obtained from Nasco (Fort Atkinson, WI, USA). The husbandry of the frogs adhered to the method of Yu et al. (2011). The 48-h exposure experiments were conducted following FETAX (ASTM, 1998). In brief, with the exception of $CdCl_2$, the test chemical solutions were dissolved in DMSO (<0.05%) and prepared immediately prior to exposure. Ringer solution and DMSO controls were included. The exposure levels of each chemical were determined based on the levels that have been reported in the literature, and the nominal concentrations were used in the present study (Table 1). Four replicates were performed for each control and treatment group. Twenty embryos with jelly coats at stage 10 were randomly transferred into acid-washed glass Petri dishes (9-cm diameters) with FETAX medium, DMSO control solution or the test chemical solution. The dishes were incubated at 26 ± 0.5 °C for 24 h in the dark to avoid the photodecomposition of some of the chemicals. Any dead embryos were removed, and the media renewed at 24-h intervals. After 48 h of exposure, 4 replicate dishes were sampled from each control and treatment, and the surviving embryos were immediately anesthetized with 100 mg/L MS-222 and preserved in 70% ethanol for morphological observations.

2.3. Morphological observations and phenotypic determination

The embryos were observed under a Carl Zeiss Discovery V8 Stereo microscope (MicroImaging GmbH, Göttingen, Germany), and the images were taken with an AxioCam digital camera. The available literatures involving FETAX was collated, and the various descriptions of

Table 1
Chemicals used in the experimental exposure.

Compounds	CAS	Purity	Concentrations in the study	Maximum environmental concentrations	References
Tributyltin	1461-22-9	≥95%	50, 100, 200 ng/L	425.3 ng Sn/L	Jiang et al. (2001)
Triphenyltin	639-58-7	≥95%	1, 3, 5, 6, 8 μg Sn/L	6.0 μg/L	Jones-Lepp et al. (2004)
$CdCl_2$	10049-05-5	>95%	0.1, 0.5, 1, 2, 3 mg/L	0.27 μg/L Cd^{2+}	Hamed and Emara (2006)
Pyraclostrobin	175013-18-0	>98%	0.1, 0.5, 1, 2.5, 5 μg/L	150 μg/L	Bartlett et al. (2002)
Picoxystrobin	117428-22-5	>98%	5, 10, 25, 35, 45 μg/L	238 ng/L	Reilly et al. (2012)
Coumoxystrobin	ND[a]	20%	0.5, 1.5, 1.75, 2, 2.5 μg/L	ND	ND
All-trans-retinoic acid	302-79-4	≥97%	1, 5, 10 μg/L	47.6 ng/L	Inoue et al. (2010)
9-Cis-retinoic acid	5300-03-8	≥97%	0.25, 0.5, 1, 2.5, 5 μg/L	ND	ND

[a] ND, no data.

the malformation phenotypes used in the previously published work were analyzed.

2.4. Statistical analyses

The data were analyzed using SPSS17.0 software. Each dish of 20 embryos was considered as a replicate, and there were 4 replicate dishes per group (n = 4). Student's t-tests were applied for comparisons of two groups (e.g., the Ringer and DMSO controls). The mean differences between the treatment and control groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's HSD test (homogeneous variances) or Tamhane–Dunnett test (heterogeneous variances) and multiple comparisons.

3. Results

3.1. Chemically-induced phenotypes

Multiple malformations were induced by the test compounds, particularly TBT, TPT and pyraclostrobin, at environmentally relevant concentrations of 50 ng/L–8 μg/L (Fig. 1). The most common chemically-induced phenotypes included abnormal eyes, enlarged proctodaeum, bent axis, narrow fins, and skin hypopigmentation (Fig. 1). In parallel, various descriptions of additional phenotypes that have been published in the FETAX-related literature were compiled (Supplementary 1).

3.2. Phenotype-based morphological scoring system

A phenotypic scoring system comprising twenty selected phenotypes with six grades for each phenotype (according to their severity of malformation) was piloted (Fig. 2). The sizes of eyes and changes in skin pigmentation included two opposite sub-phenotypes. All of the phenotypes of each embryo were evaluated, and an exact score in the range of 0–5 was assigned to each phenotype. In the numerical scoring system, normal anatomic structures were assigned a score of 0. Scores of 1–5 signified abnormalities of increasing severity (1–2 = mild abnormality, 3 = moderate abnormality, and 4–5 = severe abnormality).

3.3. Novel endpoints derived from the phenotypic scoring system

The score of malformation (SOM) and the profile of phenotypes were taken as two examples of novel endpoints based on the results of phenotypic classification. The SOM was calculated as the average value of the total scores for the twenty phenotypes (Fig. 3). The SOM exhibited concentration-dependent relationships for each of the tested chemicals (Fig. 3). For comparison, the traditional percentage of malformation index was also provided, for which the maximum value used was 100% (Fig. 3).

The phenotypic profiles of each chemical were observed using the newly developed scoring system (Fig. 4), which also exhibited dose dependence (Fig. 4).

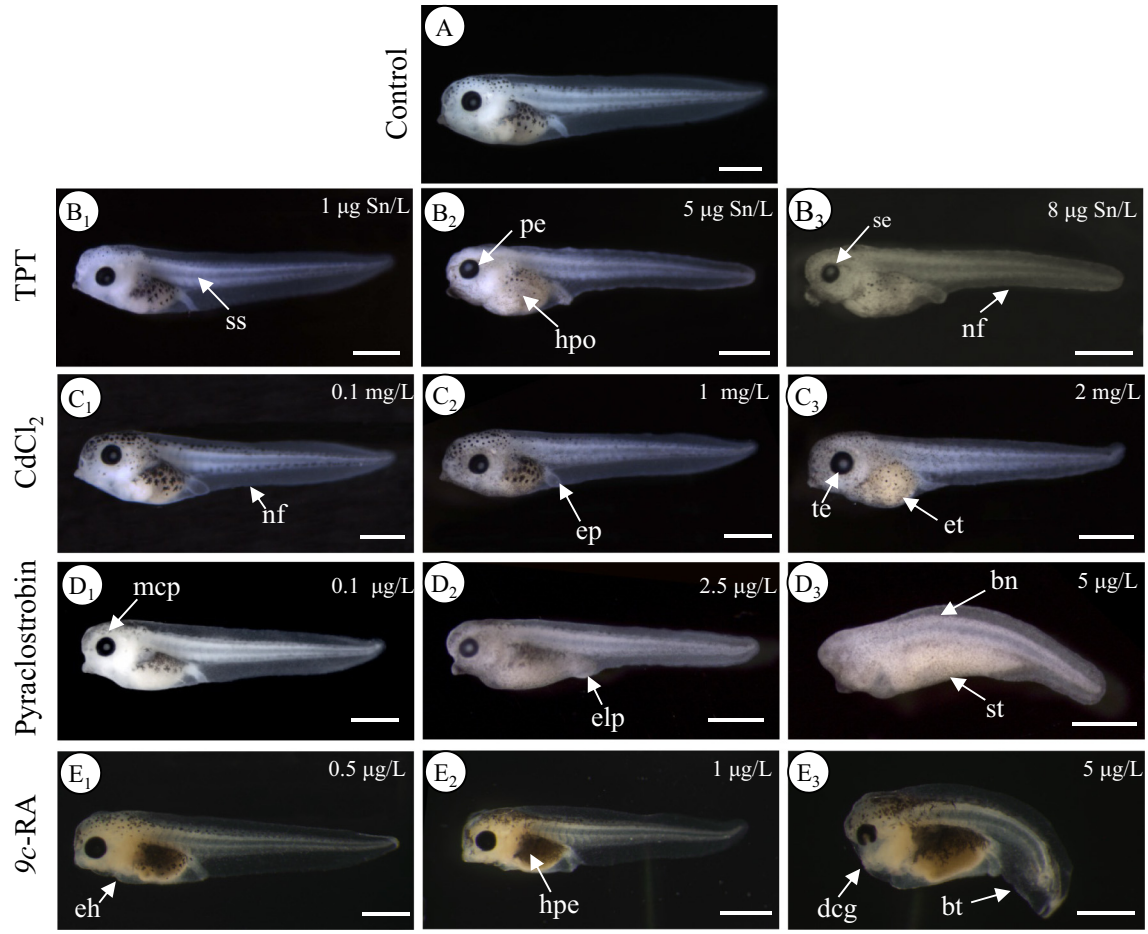

Fig. 1. Malformations of *X. tropicalis* embryos induced by the test chemicals. Abbreviations: bn, bent notochord; bt, bent tail; cg, cement gland; dcg, displaced cement gland; eh, edema in heart; ep, edema in proctodaeum; elp, enlarged proctodaeum; et, enlarged trunk; f, fin; hpe, hyperpigmentation; hpo, hypopigmentation; mcp, microcephaly; nf, narrow fin; p, proctodaeum; pe, protruding eye; se, small eye; ss, somite segmentation; st, stretched trunk; te, turbid lense of eyes. Scale bar = 0.5 mm.

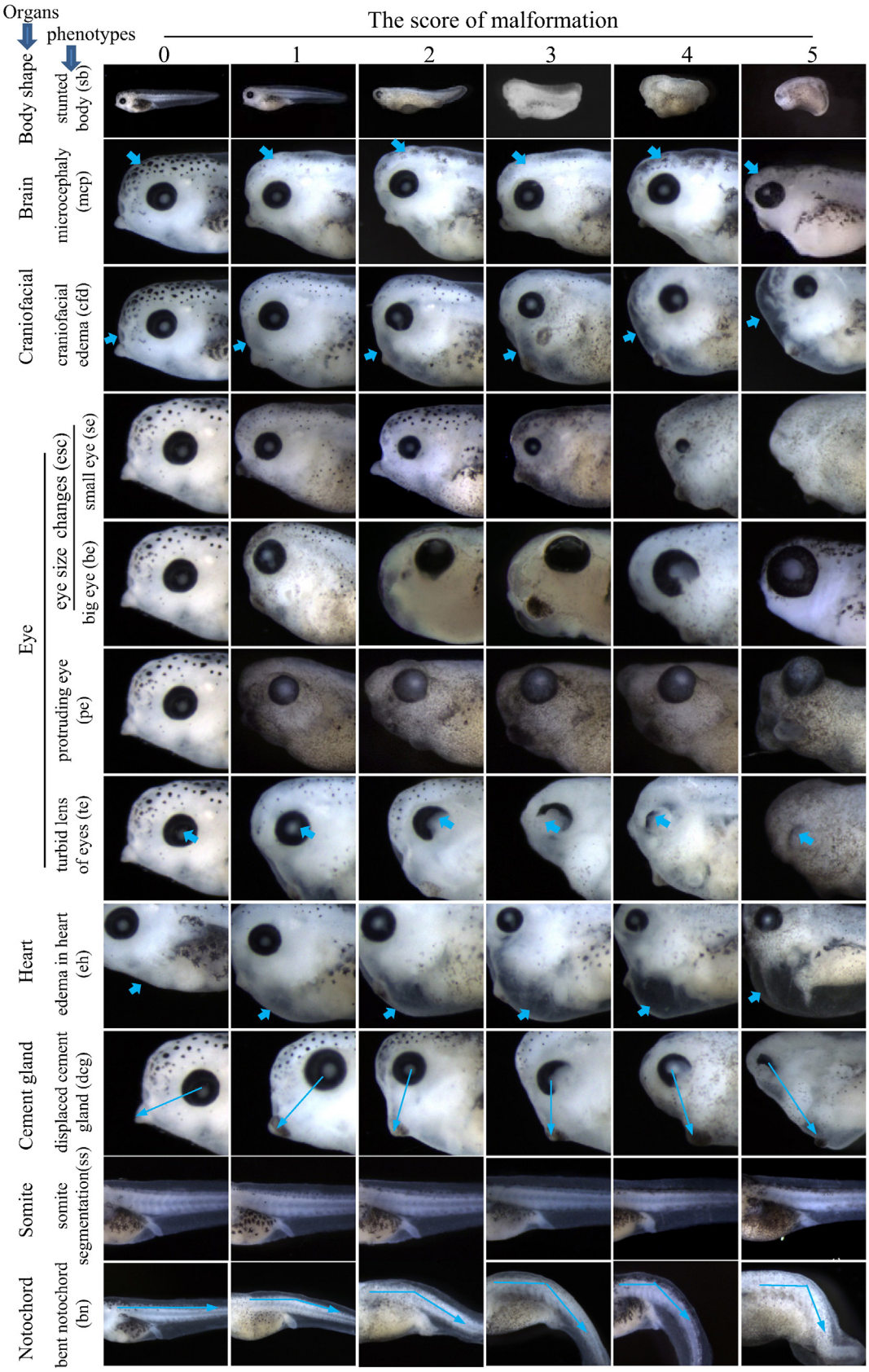

Fig. 2. Phenotype classification and scoring system for *X. tropicalis* embryos. This system contains twenty phenotypes with six grades (0–5) for each phenotype based on the degree of malformation. The sizes of the eyes and changes in the skin pigmentation include two opposite sub-phenotypes.

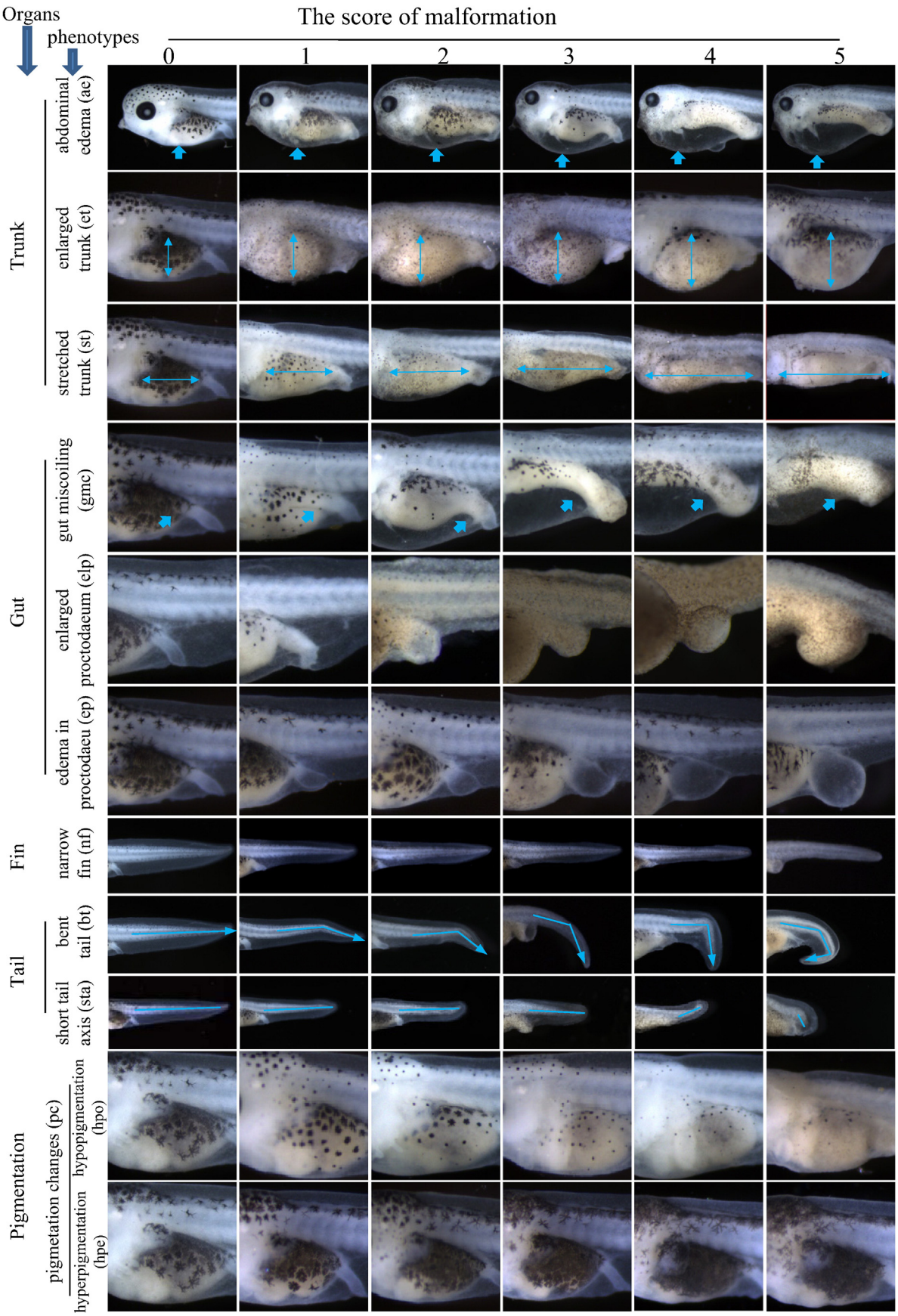

Fig. 2 (*continued*).

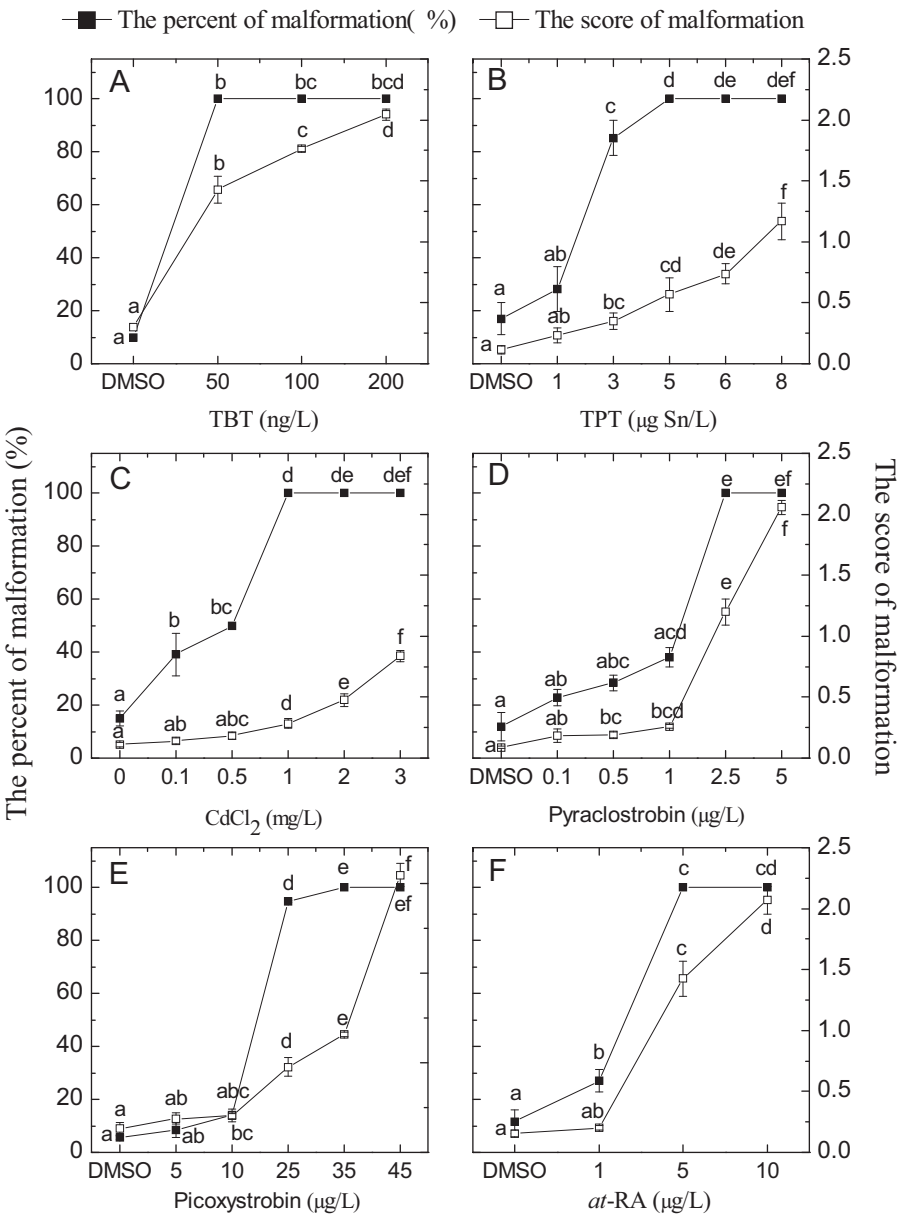

Fig. 3. Comparisons of the percent of malformation and the score of malformation induced by selected compounds in *X. tropicalis* embryos after 48 h of exposure. Each value represents the mean ± the SD of four replicates. ANOVA and multiple comparisons were used to calculate the significant differences. The letters above the bars indicate significant differences ($P < 0.05$). If two arbitrary concentrations of each test chemical have the same letter, they were not significantly different.

4. Discussion

In the present study, we piloted a phenotype-based method to enhance the assessment of the teratogenic effects of common environmental contaminant chemicals. The FETAX has been widely used as an assay of developmental toxicology (Chenon et al., 2003; El-Merhibi et al., 2004; Fort et al., 2006). No extra equipment is needed for the use of the phenotype-based method, and existing data can be reanalyzed and examined with the enhanced FETAX method.

Herein, we applied the enhanced method to study the effects of selected-common and environmentally relevant chemicals. The results indicated that $CdCl_2$ induced gut malformation, ocular anomalies, bent notochords, misshapen dorsal fins, facial dysplasia, cardiac deformities, and dermal blisters in *X. tropicalis*. These results are consistent with the work of Sunderman et al. (1991). Similarly, the phenotypes (i.e., microcephaly, loss of external eyes and bent axis) observed are consistent with those found in *X. laevis* embryos following exposure to *all-trans* retinoic acid (Degitz et al., 2000).

The present work builds on previous studies in that some indices already assess overall morphological changes or single deficiencies in *Xenopus* embryos (Kao and Elinson, 1988; Shi et al., 2012; Yu et al., 2011); however, these indices are only used for limited teratogenic factors. Deficiencies in all of the main structures of *Xenopus* embryos have been included in the present 20-phenotype array, and these phenotypes are consistent with the most common phenotypes described in the literature (Fig. 1).

Compared to single phenotypes or indices, the multiple phenotype assessment system arguably better reflects the complex effects of chemicals (Kao and Elinson, 1988; Shi et al., 2012; Yu et al., 2011). In theory, the use of greater numbers of phenotypes should produce better results. Further studies are needed to validate the present system by utilizing a greater number of test compounds and environmental samples. Specifically, additional phenotypes and sub-phenotypes will be collected, compared and selected from the exposure experiments. The selected phenotypes and sub-phenotypes should be easily distinguished and exhibit significant concentration-effects. Therefore, the present system

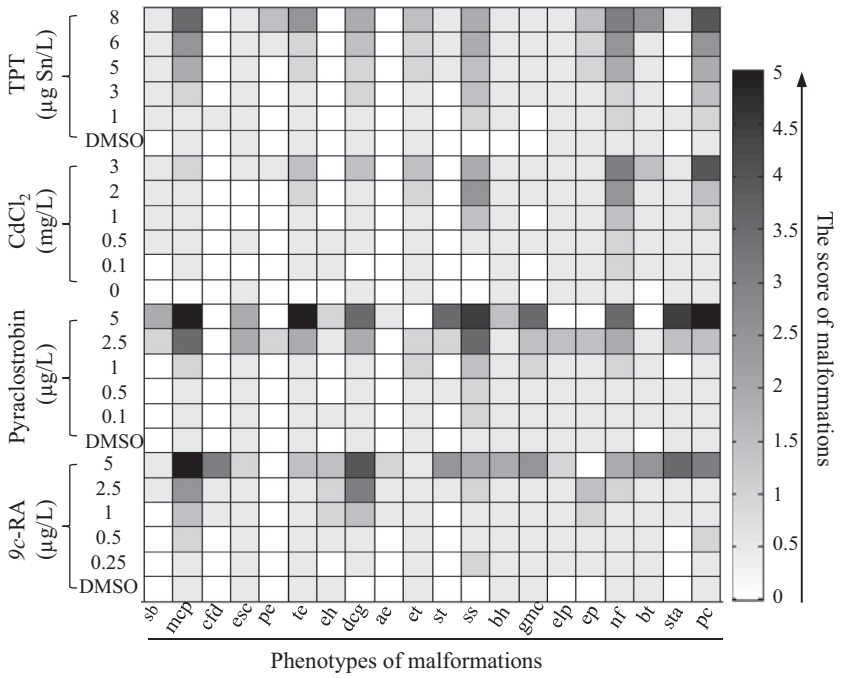

Fig. 4. The profile of the phenotypes of malformation induced in *X. tropicalis* embryos by the selected compounds. Abbreviations: ae, abdominal edema; bn, bent notochord; bt, bent tail; cfd, craniofacial edema; dcg, displaced cement gland; eh, edema in heart; ep, edema in proctodaeum; elp, enlarged proctodaeum; et, enlarged trunk; esc, eye size changes; gmc, gut miscoiling; mcp, microcephaly; nf, narrow fin; pc, pigmentation changes; pe, protruding eye; sta, short tail axis; ss, somite segmentation; st, stretched trunk; sb, stunted body; te, turbid lens of eyes.

can be updated with additional sensitive phenotypes or sub-phenotypes and the removal of some improper phenotypes in the future.

Two examples were used to validate the application of this phenotypic method. The percent of malformation has often been used to assess the degree of teratogenicity of the test compounds at specific concentrations (Bonfanti et al., 2004; Bosisio et al., 2009; Degitz et al., 2000; Zhu et al., 2013). However, this index loses its power in distinguish the differences in the teratogenic effects of compounds at high concentrations (Bonfanti et al., 2004). In contrast, using the enhanced method, the malformation scores remained valid even when the percent of malformation reached 100%; thus, the enhanced method represents a valid endpoint.

There is a further advantage to the enhanced method in that entire phenotypic profiles can be obtained rather than descriptions of limited profiles that have typically been reported in recent studies (Nations et al., 2011; San Segundo et al., 2013). Several approaches have been suggested for improving the performance characteristics of the FETAX compared to those of mammalian teratogenicity assays (Fort and Paul, 2002; Leconte and Mouche, 2013). One significant improvement is to base the EC50 on characteristic malformations only rather than on all malformations (Fort and Paul, 2002). Characteristic malformations refer to those that increase in frequency and possibly severity with increasing concentrations of the test substance. The characteristic malformations can be numerically distinguished from the whole phenotypic profiles obtained from the phenotypic method piloted herein.

Phenotypes may also reflect the underlying mechanisms of the effects of each chemical and reflect the endpoints of altered gene expression (Paules, 2003). For example, the absence of ventral fins and the presence of posterior anus phenotypes were apparent in *X. tropicalis* embryos following exposure to mixtures of TPT and a retinol drug (bexarotene or LGD1069) in our previous study (Shi et al., 2012). In parallel, molecular studies have shown that the loss of gene expression in *Bmp7* and *Tsg* in *X. laevis* embryos also results in reduced ventral fins and even posteriorized anuses (Zakin et al., 2005). As such, phenotype profiles may also provide indications of potential target genes for further mechanistic studies.

In summary, we developed a method of classifying and scoring the phenotypes of malformations in *X. tropicalis* embryos based on chemical exposures at environmentally relevant doses. This method was successfully integrated with the primary endpoints used in the FETAX, which allowed for a sensitive and quantitative assessment of the effects and indications of the mechanistic underpinnings of the tested compounds.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2014.11.086.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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