Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/etap

Two-dimensional proteomic analysis of gonads of air-breathing catfish, *Clarias batrachus* after the exposure of endosulfan and malathion

*C. Laldinsangi, K. Vijayaprasadarao, A. Rajakumar, R. Murugananthkumar, Y. Prathibha, C.C. Sudhakumari, S.K. Mamta, A. Dutta-Gupta, B. Senthilkumaran**

Department of Animal Sciences, School of Life Sciences-Centre for Advanced Studies, University of Hyderabad, P.O. Central University, Hyderabad 500046, India

ARTICLE INFO

Article history:
Received 23 October 2013
Received in revised form
6 March 2014
Accepted 8 March 2014
Available online 29 March 2014

Keywords:
Catfish
Endosulfan
Malathion
MALDI-TOF-MS
Prolactin
Ubiquitin

ABSTRACT

Endocrine disrupting chemicals have raised public concern, since their effects have been found to interfere with the physiological systems of various organisms, especially during critical stage of development and reproduction. Endosulfan and malathion, pesticides widely used for agricultural purposes, have been known to disrupt physiological functions in aquatic organisms. The current work analyzes the effects of endosulfan (2.5 parts per billion [ppb]) and malathion (10 ppb) on the reproductive physiology of catfish (*Clarias batrachus*) by evaluating protein expression profiles after 21 days of exposure. The proteomic profile of testis and ovary after exposure to endosulfan showed downregulation of proteins such as ubiquitin and Esco2, and upregulation in melanocortin-receptor-2 respectively. Malathion exposed ovary showed upregulated prolactin levels. Identification of proteins differentially expressed in gonads due to the exposure to these pesticides may serve as crucial indications to denote their disruptive effects at the level of proteins.

1. Introduction

Research on endocrine disruptors has received significant attention in recent times, owing to the elevated concerns of their potentially harmful effects on the physiological systems of humans and other organisms inhabiting both terrestrial and aquatic ecosystems. Endocrine disrupting chemicals (EDCs) are a heterogeneous group of compounds, mostly from anthropogenic sources, that have the ability to mimic natural hormones in living systems which can interfere with normal physiological and reproductive functions in various ways, and their ability to bind and/or block estrogen and androgen receptors in particular have been intensely studied (Tabb and Blumberg, 2006; Dutta-Gupta, 2013). These EDCs consist of industrial effluents, pesticides, pharmaceutical agents, etc. (Diamanti-Kandarakis et al., 2009). Among these, the effects of endosulfan, an organochloride (Embrandiri et al., 2012) and malathion, an organophosphate pesticide (Prathibha et al.,

* *Corresponding author*. Tel.: +91 40 23134562/4662; fax: +91 40 23010307/23010120.
E-mail addresses: bsksl@uohyd.ernet.in, senthilkumaranb@yahoo.com (B. Senthilkumaran).
http://dx.doi.org/10.1016/j.etap.2014.03.007
1382-6689/

2014) are noteworthy, since their widespread use in agriculture in many parts of the world including India lead to their run-off into water bodies, posing direct threats to the aquatic ecosystem.

Extensive studies on the consequences of endosulfan exposure have shown anomalies such as decline in plasma vitellogenin levels (Chakravorty et al., 1992), impairment of metabolism (Tripathi and Verma, 2004), hyperactivity and behavioral changes (Jonsson and Toledo, 1993), neurotoxicity (Carlson et al., 1998), impediment of spermatogenesis (Dutta et al., 2006) in fish. Endosulfan is known to competitively bind to androgen receptors in humans, impart estrogenic effects in breast cancer cell lines and congenital defects, cancers and mental retardation (Embrandiri et al., 2012). In zebrafish, exposure to endosulfan concentrations as low as 10 ng/L, have been found to decrease hatching rates considerably, gonadosomatic index was greatly reduced, and vitellogenin levels in male were also increased, which implies its adverse effects on the reproductive aspects of fish (Han et al., 2011). Exposure of the catfish, *Heteropneustes fossilis* to this pesticide at sublethal concentrations have also shown a decrease in the levels of total phospholipids during crucial periods of reproduction such as pre-spawning and spawning phases which may also adversely affect fertility and the annual reproductive cycle, as these lipids play key roles in the supply of nutrition and energy (Singh and Singh, 2007). Quantitative real-time PCR analysis of genes related to testicular development along with histological analysis in juvenile catfish, *Clarias batrachus* from our laboratory demonstrated significant downregulation of transcript levels of testis-related transcription factors and steroidogenic enzyme genes *vis-à-vis* impairment of spermatogonial proliferation (Rajakumar et al., 2012). Although this pesticide had been banned from use in the Stockholm Convention 2011 (Rajakumar et al., 2012), many studies have found its persistence in the environment, due to bioaccumulation. Hence, a study on this aspect, at sub-lethal doses comparable to its concentration in the environment is still very much relevant.

Malathion at sublethal doses has been shown to cause genotoxicity (Amer et al., 2002), sperm damage and immobility (Akbarsha et al., 2000) in mice, physiological and behavioral changes (Beauvais et al., 2000), acetylcholinesterase inhibition (Brewer et al., 2001), teratogenic effects during development (Cook et al., 2005), metabolic dysfunction (Venkataramana et al., 2006), and alteration of immune responses (Plumb and Areechon, 1990; Harford et al., 2005) in fish. Thyroid hormone levels were also shown to be affected in protogynous teleost, *Monopterus albus* exposed to malathion which infer the disruption of the endocrine systems leading to possible reproductive impairment in fishes (Singh, 1989), and these results also correlate with the findings in the catfish, *C. batrachus* where malathion exposure led to retardation of growth (Lal et al., 2013), and disruption in expression levels of gonad and brain specific genes (Prathibha et al., 2014).

The assessment of the toxicity of these and other widely used pesticides on fish species has evolved to be a very significant aspect due to their ecological relevance and economic importance. Since prior studies have shown the potential of these pesticides to impair reproductive function in both adult and juvenile fishes (Arcand-Hoy and Benson, 1998; Rajakumar et al., 2012), it is necessary to explicate different angles as to how the effects are exerted, and stratify various benchmarks at the transcriptomic and proteomic levels that indicate critical levels of exposure. As fishes are responsive to even subtle levels of exposure to external contaminants by alterations in expression pattern of genes and proteins, it would be crucial to analyze the changes at the level of proteome, since, it is known to be highly dynamic, and assessment of the proteomic profiles would serve as valuable links that bridge the effects at the molecular and organismal levels (Lemos et al., 2009). Various studies have outlined parameters that have been brought into account for evaluating the consequences of these harmful pesticides, one being the process of reproduction which may be vulnerable to chemicals which have the potency to disrupt the endocrine functions in the hypothalamo-hypophyseal-gonadal axis. Assessment of the phenotypic consequences of such exposures must be supported by the information on the alterations at the functional level like proteins, which may aid in establishing the 'expression signatures' for various classes of stressors, and also identification of their modes of action (Biales et al., 2011).

Our present study aims to analyze the implications of two commonly used pesticides (endosulfan and malathion) on the reproductive physiology of catfish by evaluating the protein expression profiles after exposure at sub-lethal concentrations. Though endosulfan has been banned completely now, indifferent usage of this pesticide in early days in several countries warrant investigation of this sort. Further this study may caution the indiscriminate use of malathion to control agricultural pests. We assess the differential expression of proteins in the gonads, both ovary and testis, after separate exposures in an attempt to highlight the effects of these pesticides on critical reproductive functions at the level of proteins.

2. Materials and methods

2.1. Animals and treatments

Catfish, *C. batrachus*, exhibits an annual reproductive cycle which can be divided in four different phases, preparatory (February–April), pre-spawning (May–June), spawning (July–August), post-spawning/regressed (September–January). Mature spermiating male and gravid female fishes were injected with human chorionic gonadotropin (1500 IU/fish) and *in vitro* fertilization (IVF) was performed to obtain catfish hatchlings. These hatchlings were then reared in indoor glass tanks with filtered fresh water and proper aeration under ambient temperatures (~25 °C). After 2–3 days post-hatch, the hatchlings were fed with live tube worms followed by commercially available fish feed *ad libitum*. Catfish were reared until eight months old and were then used for the study, which was conducted during mid-March. At this stage, the catfish can be comparable to fishes undergoing recrudescence or mid-preparatory phase, where pre-vitellogenic follicles appear predominantly in females, while males have primary to secondary spermatocytes overwhelmingly.

Male and female catfish of similar age were divided into three groups wherein one group was maintained as

control, and the other two groups were treated either with 2.5 parts per billion (2.5 ppb or μg/L) endosulfan (Lot and Batch: E0344, Nagarjuna Agrichem Ltd., Hyderabad, India) or 10 ppb malathion (Hyderabad Chemicals Ltd., Hyderabad, India), for a period of 21 days. Treatment was administered by dissolution of the pesticides into the fish tanks. The endosulfan group comprising 8 month old catfish was maintained in filtered water dissolved with 2.5 ppb of the pesticide. The malathion group comprised of 8 month old female catfish maintained in filtered water in which 10 ppb of the pesticide was dissolved. Each group was maintained with a replicate. The doses used are based on our pilot studies which have been shown to be lower than the LC_{50} values reported for the two pesticides (Tripathi and Verma, 2004; Prathibha et al., 2014). Working stocks for both endosulfan and malathion were maintained and prepared everyday for treatment. All the groups were handled in similar conditions, and all the tanks were replenished with filtered water daily (control) or filtered water containing endosulfan or malathion for 21 days. The fishes were fed with live tube worms *ad libitum* during experimentation. After the treatment period, tissues were collected by sacrificing the fish from all three groups following general animal ethical guidelines. Gonads were dissected out from the fishes, snap frozen with liquid nitrogen, and stored in −80 °C for proteomic analysis. Gonads of three to four fishes were pooled (taken as one biological sample) for each group to get sufficient quantities of protein for isolation. We repeated each 2D analysis at least three times with different pools of gonad samples each time to get consistent results.

2.2. *Two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time of flight mass spectrometric (MALDI-TOF-MS) analysis*

2.2.1. *Sample preparation and tissue extraction*

Protein extraction from the tissues was performed by homogenization in buffer consisting of 6 M Urea, 4% CHAPS, 50 mM DTT and 100 mM PMSF over ice. The homogenized tissue was then centrifuged at 12,000 × *g* for 30 min at 4 °C. The supernatant was pipetted out, precipitated, and used for further 2D gel electrophoresis. Protein concentration was estimated as per the method of Bradford.

2.2.2. *Two-dimensional gel electrophoresis*

The supernatant collected from the gonadal tissue homogenate was subjected to clean-up using the 2-D Clean-up Kit (GE Healthcare Life Sciences, USA) as per the manufacturer's instructions. The cleanup process was followed by dissolution of the separated protein precipitate in rehydration buffer constituting 7 M urea, 2 M thiourea, 4% CHAPS, 2% ampholytes of pH 3–10, 70 mM DTT and 3.5% bromophenol blue. IPG strips (Immobiline Drystrip, 3–10 pH linear 18 cm, GE Healthcare) were rehydrated in 350 μL of the protein solution for ~20 h under low viscosity mineral oil (Merck Genei, Bangaluru, India). The rehydrated IPG strips containing 300 μg protein were then subjected to Isoelectric Focusing in Ettan IPGphor3 gel apparatus (GE Healthcare) at 20 °C for 70,000 Vh-50 V-1 h, 500 V-5 h, 500 V-5 h, 10000 V-8 h, 500 V-10 h (a total of 70,000 Vh). After IEF, the strips were equilibrated in equilibration buffer (75 mM Tris–HCl pH 8.8,

Table 1 – List of identified protein spots from testis of catfish after the exposure of endosulfan with name of spot, expression pattern, molecular weight, pI, protein name, accession number, score and species of ray-finned fish to which the proteins were identified: protein identification was performed by database searches (PMF and MS/MS) using MASCOT program (http://www.matrixscience.com) employing Biotools software (Bruker Daltonics). The similarity search for mass values was done with existing digests and sequence information from NCBInr and Swiss Prot database. According to the MASCOT program, the probability of $P < 0.05$ was considered statistically significant and only significant hits were accepted for protein identification.

Name of spot	Expression pattern	Molecular weight in kDa	pI	Protein name	Accession number	Species identified	Score
NE	Newly expressed	10.515	10.07	LYR motif-containing protein 4	gi|256000753	*Salmo salar*	21
DR1	Downregulated	59.076	10.01	Esco2 (establishment of cohesion 1 homolog 2)	gi|325652170	*Oryzias latipes*	51
DP1	Disappeared	8.446	6.56	Ubiquitin	gi|229532	*Homo sapiens*	103
DR2	Downregulated	22.15	8.05	Gamma-crystallin M1-1	gi|632009	*Petenia splendida*	17

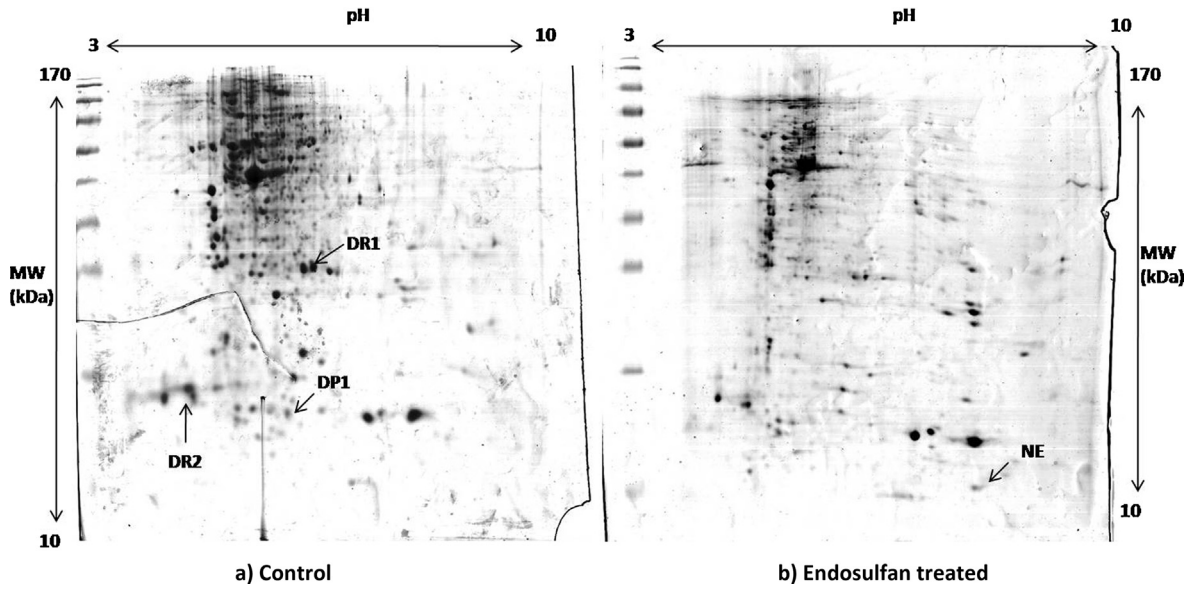

Fig. 1 – Representative two-dimensional electrophoresis gels of catfish testis after endosulfan exposure and control: (a) control and (b) endosulfan treated. Spots indicated by arrows were downregulated (DR1, DR2) and disappeared (DP1) and newly expressed (NE) in the endosulfan exposed fish ($n = 3$ [pooled]). Spots were identified as Esco2 (DR1), LYR motif-containing protein 4 (NE), Ubiquitin (DP1) and gamma-crystallin M1-1 (DR2).

30% glycerol, 2% SDS and a trace of bromophenol blue) containing 10 mg/ml of DTT followed by 20 min in equilibration buffer containing 25 mg/ml of iodoacetamide (an alkylating agent to reduce cysteine residues preventing disulfide bond formation). SDS-PAGE was carried out and the gels were then stained using coomassie brilliant blue R-250.

2.2.3. *Image analysis*

Computer analysis of the gels was carried out using Image Master 2-D Platinum (GE Healthcare) system. Three separate gels were analyzed for each sample and the spots that were found to display significant changes in their expression were chosen for further analysis.

2.2.4. *In-gel digestion, protein identification and database search*

In-gel digestion and MALDI-TOF-MS analysis was conducted with a MALDI-TOF/TOF mass spectrometer (Bruker Autoflex III smartbeam, Bruker Daltonics, Bremen, Germany) according to the method described by Shevchenko et al. (1996) with slight modifications. The protein spots were manually excised from the gels and were destained with 100 μL of 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate (NH_4HCO_3) for 5 times. Thereafter, the gel pieces were treated with 10 mM DTT in 25 mM NH_4HCO_3 and incubated at 56 °C for 1 h. This is followed by treatment with 55 mM iodoacetamide in 25 mM NH_4HCO_3 for 45 min at room temperature (25 ± 2 °C), washed with 25 mM NH_4HCO_3 and ACN, dried in speed vac (Labconco, San Diego, USA) and rehydrated in 20 μL of 25 mM NH_4HCO_3 solution containing 12.5 ng/μL trypsin (sequencing grade, Promega, Wisconsin, USA). The above mixture was incubated on ice for 10 min and kept overnight for digestion at 37 °C. After digestion, it was spun for 10 min and the supernatant was collected in a fresh eppendorf tube. The gel pieces were re-extracted with 50 μL of 1% trifluoroacetic acid (TFA) and ACN (1:1) for 15 min with frequent vortexing. The supernatants were pooled together and dried using speed vac and were reconstituted in 5 μL of 1:1 ACN and 1% TFA. 2 μL of the above sample was mixed with 2 μL of freshly prepared α-cyano-4-hydroxycinnamic acid (CHCA) matrix in 50% ACN and 1% TFA (1:1) and 1 μL was spotted on target plate. Protein identification was performed by database searches (Peptide Mass Fingerprinting and MS/MS) using MASCOT program (http://www.matrixscience.com) employing Biotools software (Bruker Daltonics). The similarity search for mass values was done with existing digests and sequence information from NCBInr and Swiss Prot database. The search parameters were as follows: fixed modification of carbamidomethyl (C), variable modification of oxidation (M), enzyme trypsin, peptide charge of 1^+ and monoisotropic. According to the MASCOT program, the probability of $P < 0.05$ was considered statistically significant and only significant hits were accepted for protein identification.

3. Results

3.1. *Changes in the proteomic profile of catfish gonads after the exposure of endosulfan*

In the gonadal proteomic profile of the endosulfan treated fish, differentially expressed protein spots were identified using 2D gel electrophoresis between control and treated fishes. Differential expression was calculated in fold changes, and only the spots with fold changes greater than 2 were taken for MALDI analysis. Protein spots with lower molecular weight were favored, since most hormones/factors crucial for gonadal development may have low molecular weights, which is a critical criterion in our experiment.

In the testis sample of both treated and control, quantitative image analysis with Image Master 2 D Platinum (GE Healthcare) system comparing these gels (Fig. 1) revealed that out of 76 spots analyzed, 15 spots in treated group had significant changes in their expressions relative to control, with fold changes greater than a factor of 2 (either upregulation or downregulation). Among these 15 protein spots, 6 spots were downregulated (DR) in the treated group as compared to control and protein spots chosen for MALDI analysis were based on their low molecular weights. Spots that newly appeared (NE) or completely disappeared (DP1) after treatment were also chosen due to the drastic changes in their expression pattern. Table 1 shows the list of proteins identified using MALDI-TOF analysis.

In the ovary samples of endosulfan treated group, quantitative image (Fig. 2) analysis showed that out of 50 protein spots analyzed, 5 protein spots had a significant increase in expression (upregulated, UR), with a fold change greater than a factor of 2 as compared to the control samples (Table 2). Out of these 5 upregulated spots, 3 protein spots of low molecular weight having maximum fold increase were selected for MALDI-TOF analysis. One spot which was newly expressed (NE) in the endosulfan treated group was also analyzed.

3.2. *Changes in the proteomic profile of catfish ovary after the exposure of malathion*

Differentially expressed protein spots (Fig. 3) as compared to control fish were identified from ovary samples of malathion treated groups. The details of the various differential spots identified and analyzed from the gels are summarized in Table 3. Out of 123 spots analyzed, 9 spots showed significant increase in expression, i.e., fold change greater than 2. Out of these 9 spots that had significant differential expression, 2 (low molecular weight) spots that were downregulated (DR1 and DR2) and 1 (low molecular weight) upregulated spot (UR) were chosen for MALDI-TOF analysis. Present study could not be extended to testis due to limitation of sample amounts.

4. Discussion

Results from the current work implies that the exposure of catfish to sub-lethal (low) doses of the pesticides, endosulfan and malathion have the potential to elicit responses independently at the proteomic level of developing gonads. Our 2D proteomic analyses coupled with MALDI-TOF revealed differential effects of these pesticides on catfish gonads.

In male fishes treated with endosulfan, the disappearance of protein factors such as ubiquitin may reflect the adverse effects of this pesticide on the reproductive aspects, since this molecule has been known to play a prominent role in the process of spermatogenesis and sperm maturation as it is considered as the “marker for semen quality and fertility” in mammals including humans, mice and bulls (Bebington et al., 2001; Sutovsky et al., 2004; Baska et al., 2008; Lui and Lee, 2008). Studies suggesting the role of ubiquitin in reproduction have implied that its function may be critical to the protein degradation during gametogenesis which requires ubiquitin-dependent proteolysis (Baarends et al., 1999). Thus,

Table 2 – List of identified protein spots from ovary of catfish after the exposure of endosulfan (all other details are as in Table 1).

Name of spot	Expression pattern	Molecular weight in kDa	pI	Protein name	Accession number	Species identified	Score
UR1	Upregulated	55.337	5.46	Disulfide-isomerase A3 precursor	gi|209153384	*Salmo salar*	57
NE	Newly expressed	33.595	8.18	Poly (rC)-binding protein 2	gi|41055221	*Danio rerio*	45
UR2	Upregulated	34.701	8.92	Melanocortin-2 receptor/ACTH receptor	gi|343887257	*Paralichthys olivaceus*	72
UR3	Upregulated	53.290	6.49	Myelin expression factor 2-like	gi|348538711	*Oreochromis niloticus*	54

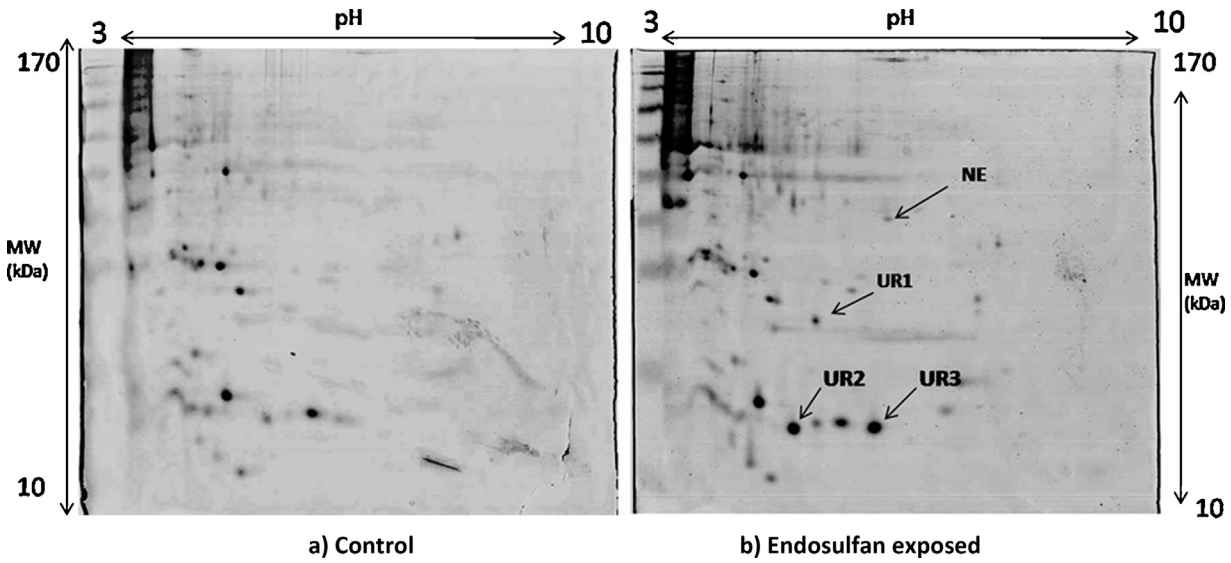

Fig. 2 – Representative two-dimensional electrophoresis gels of catfish ovary after endosulfan exposure and control: (a) control and (b) endosulfan treated. Spots indicated by arrows were upregulated (UR1, UR2, UR3) and newly expressed (NE) in the endosulfan exposed fish ($n = 3$ [pooled]). Spots identified are disulfide-isomerase A3 precursor (UR1), melanocortin-2 receptor/ACTH receptor (UR2), myelin expression factor 2-like (UR3) and poly (rC)-binding protein 2 (NE).

the effect of endosulfan on this protein, leading to its impaired functions in physiological processes may hamper puberty. Though there are no studies in fish implicating ubiquitin in sperm maturation, our earlier finding on endosulfan impairing spermatogonial proliferation (Rajakumar et al., 2012) might support the aforementioned contention. In addition, significant downregulation of the "establishment of cohesion 1 homolog 2" (Esco2) protein, which is a crucial factor for chromosome segregation during mitosis (Bose et al., 2012), may also indirectly link the adverse effects of endosulfan on reproduction, as high levels of cell death and apoptosis have been reported in Esco2 depleted zebrafish models (Mönnich et al.,

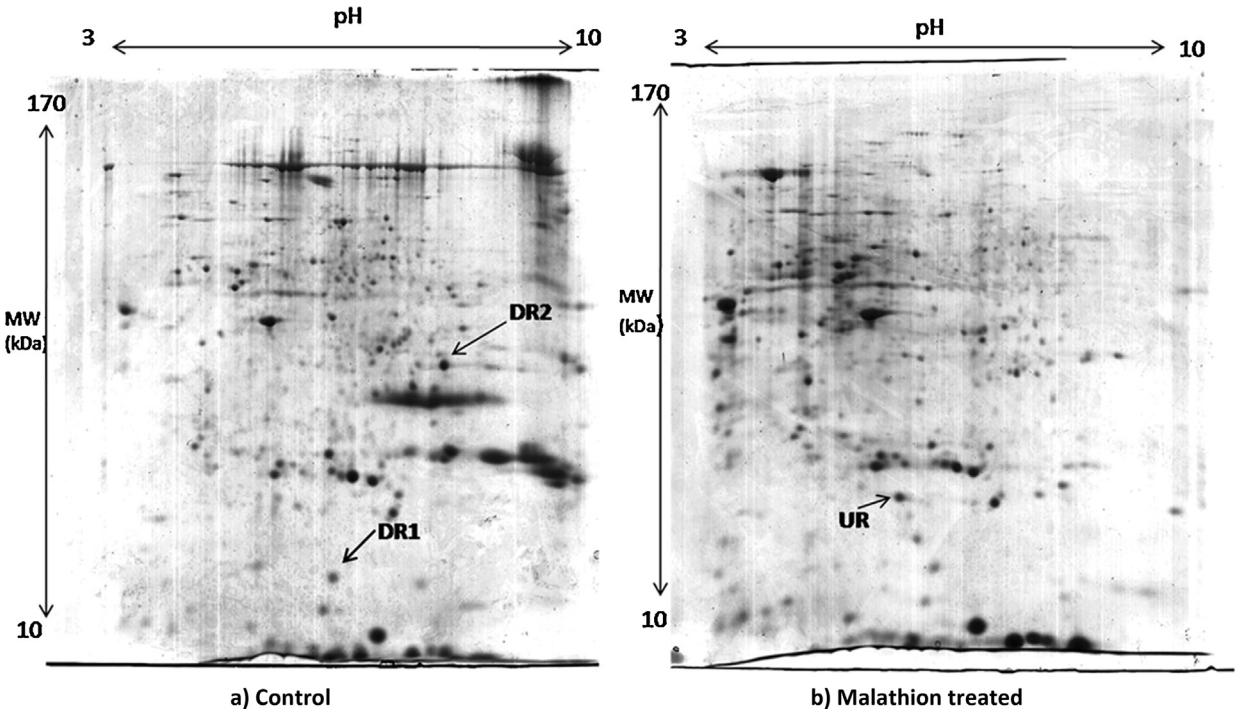

Fig. 3 – Representative two-dimensional electrophoresis gels of catfish ovary after malathion exposure and control: (a) control and (b) malathion treated. Spots indicated by arrows were upregulated (UR) and downregulated (DR1, DR2) in the malathion exposed fish ($n = 3$ [pooled]). Spots were identified as prolactin (UR), progonadoliberin-2 (DR1) and cytochrome P450 1A1 (DR2).

Table 3 – List of identified protein spots from ovary of catfish after the exposure of malathion (all other details are as in Table 1).

Name of spot	Expression pattern	Molecular weight in kDa	pI	Protein name	Accession number	Species identified	Score
UR	Upregulated	23,733	9.24	Prolactin	gi|464464|P34181.1	*Coregonus autumnalis*	16
DR1	Downregulated	9419	9.13	Progonadoliberin-2	gi|157278082	*Oryzias latipes*	17
DR2	Downregulated	59,837	6.58	Cytochrome P450 1A1	gi|3913314	*Opsanus tau*	25

2011) This gene has been localized in both murine meiotic cells (spermatocyte) and embryonic ovary, which suggests its crucial role in meiosis (Evans et al., 2012), and in germ cell renewal (Hogarth et al., 2011) which may imply that disruption of this gene function may possibly hamper reproduction even in non-mammalian vertebrates such as teleosts.

The protein profile of female fish exposed to endosulfan shows an upregulation of melanocortin-2-receptor, which is a crucial factor to receive ligands such as melanocorticoids which is under the influence of adrenocorticotropic hormone (ACTH). But, the molecular mechanisms have not been well established in non-mammalian vertebrates such as teleosts. Tissue distribution pattern of this receptor in rainbow trout showed higher expression in ovarian tissue, which may propose the role of this ligand-receptor complex in the suppression of sex steroid levels mediated via stressors (Aluru and Vijayan, 2008). Since the melanocortin receptors have been proposed to play active roles in ovulation and steroidogenesis of bovine ovary (Amweg et al., 2011), a disturbance by external stressors might have an impact on normal reproductive processes, which is in agreement with findings that establish the negative impact of stress on reproduction in several vertebrates (Alsop et al., 2009).

Catfish exposed to malathion showed upregulation in the level of prolactin in ovary. Prolactin is a versatile peptide hormone that is known to take part in a myriad of biological functions such as metabolism, immunoregulation, behavior, lactation, reproduction etc. (Cooke et al., 2004) Increase in the expression levels of prolactin correlate with findings which demonstrated the elevated plasma levels of this in freshwater teleosts, which may be indicative of a hydromineral effect in response to external stressors (Thangavel et al., 2005). In mice, prolonged elevated levels of prolactin has been known to inhibit GnRH secretion from the hypothalamus, which may in turn inhibit gonadotropins and gonadal steroidogenesis, that may subsequently impair reproductive functions to a great extent (Freeman et al., 2000). Although prolactin is known to be a pituitary hormone, the extrapituitary expression in liver, kidney and gonads are not uncommon (Whittington and Wilson, 2013). The disruption in the expression levels even in those tissues may implicate impairment of sexual development and maturity in fishes. Such an effect of prolactin in fishes remains to be seen, yet the present study may implicate such a phenomenon.

The exposure of catfish to environmentally relevant, sublethal doses of pesticides, endosulfan and malathion caused an alteration in the expression profiles of certain factors which seem to play crucial roles in the normal reproductive physiology of catfish. Further studies are necessary to validate the impact of pesticides that have the potential to disrupt the endocrine systems either directly or indirectly via the impairment of hormone levels, and also to elucidate the identified protein spots as potential biomarkers for monitoring levels of such pesticides in the environment. Nonetheless, our present study provides first evidence for proteomic profile modifications using a teleost fish model after limited period low level exposure of endosulfan and malathion using annually breeding fresh water fish. This study may warrant for curbing indiscriminate use of pesticides to protect agricultural crops.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated to this article can be found, in the online version.

Acknowledgements

This research program was fully supported by a Grant-in-Aid (BT/PR11263/AAQ/03/419/2008) from the Department of Biotechnology (DBT), India to BS. BS is the recipient of DBT-TATA Innovation Fellowship 2014, which is acknowledged. We also acknowledge the instrumentation support of Department of Science and Technology (DST)-PURSE, DST-FIST, DBT-CREBB and University Grants Commission SAP-Center for Advanced Studies programs of the Department of Animal Sciences and School of Life Sciences. CL, AR, RM and YP are thankful to the Council of Scientific and Industrial Research/DBT, India for junior/senior research fellowships. KV is thankful to DBT-CREBB for post-doctoral fellowship. SKM is thankful to the DBT-CREBB and DST-PURSE for post-doctoral fellowships.

REFERENCES

Akbarsha, M.A., Latha, P.N., Murugaian, P., 2000. Retention of cytoplasmic droplet by rat cauda epididymal spermatozoa after treatment with cytotoxic and xenobiotic agents. J. Reprod. Fertil. 120, 385–390.

Alsop, D., Ings, J.S., Vijayan, M.M., 2009. Adrenocorticotropic hormone suppresses gonadotropin-stimulated estradiol release from zebrafish ovarian follicles. PLoS ONE 4, e6463.

Aluru, N., Vijayan, M.M., 2008. Molecular characterization, tissue-specific expression, and regulation of melanocortin 2 receptor in rainbow trout. Endocrinology 149, 4577–4588.

Amer, S.M., Fahmy, M.A., Aly, F.A.E., Farghaly, A.A., 2002. Cytogenetic studies on the effect of feeding mice with stored wheat grains treated with malathion. Mutat. Res. 513, 1–10.

Amweg, A.N., Paredes, A., Salvettia, N.R., Larab, H.E., Ortegaa, H.H., 2011. Expression of melanocortin receptors *mRNA*, and direct effects of ACTH on steroid secretion in the bovine ovary. Theriogenology 75, 628–637.

Arcand-Hoy, L.D., Benson, W.H., 1998. Fish reproduction: an ecologically relevant indicator of endocrine disruption. Environ. Toxicol. Chem. 17, 49–57.

Baarends, W.M., Roest, H.P., Grootegoed, J.A., 1999. The ubiquitin system in gametogenesis. Mol. Cell. Endocrinol. 151, 5–16.

Baska, K.M., Manandhar, G., Feng, D., Agca, Y., Tengowski, M.W., Sutovsky, M., Yi, Y.J., Sutovsky, P., 2008. Mechanism of extracellular ubiquitination in the mammalian epididymis. J. Cell. Physiol. 215, 684–696.

Beauvais, S.L., Jones, S.B., Brewer, S.K., Little, E.E., 2000. Physiological measures of neurotoxicity of diazinon and malathion to larval rainbow trout *Oncorhynchus mykiss* and their correlation with behavioral measures. Environ. Toxicol. Chem. 19, 1875–1880.

Bebington, C., Doherty, F.J., Fleming, S.D., 2001. The possible biological and reproductive functions of ubiquitin. Hum. Reprod. Update 7, 102–111.

Biales, A.D., Bencic, D.C., Flick, R.L., Blocksom, K.A., Lazorchak, J.M., Lattier, D.L., 2011. Proteomic analysis of a model fish species exposed to individual pesticides and a binary mixture. Aquat. Toxicol. 101, 196–206.

Bose, T., Lee, K.K., Lu, S., Xu, B., Harris, B., Slaughter, B., Unruh, J., Garrett, A., McDowell, W., Box, A., Li, H., Peak, A., Ramachandran, S., Seidel, C., Gerton, J.L., 2012. Cohesin proteins promote ribosomal RNA production and protein translation in yeast and human cells. PLoS Genet. 8, e1002749.

Brewer, S.K., Little, E.E., DeLonay, A.J., Beauvais, S.L., Jones, S.B., Ellersieck, M.R., 2001. Behavioral dysfunctions correlate to altered physiology in rainbow trout (*Oncorynchus mykiss*) exposed to cholinesterase-inhibiting chemicals. Arch. Environ. Contam. Toxicol. 40, 70–76.

Carlson, R.W., Bradbury, S.P., Drummond, R.A., Hammermeister, D.E., 1998. Neurological effects on startle response and escape from predation by medaka exposed to organic chemicals. Aquat. Toxicol. 43, 51–68.

Chakravorty, S., Lal, B., Singh, T.P., 1992. Effect of endosulfan (thiodan) on vitellogenesis and its modulation by different hormones in the vitellogenic catfish *Clarias batrachus*. Toxicology 75, 191–198.

Cook, L.W., Paradise, C.J., Lom, B., 2005. The pesticide malathion reduces survival and growth in developing zebrafish. Environ. Toxicol. Chem. 24, 1745–1750.

Cooke, P.S., Holsberger, D.R., Witorsch, R.J., Sylvester, P.W., Meredith, J.M., Treinen, K.A., Chapin, R.E., 2004. Thyroid hormone, glucocorticoids, and prolactin at the nexus of physiology, reproduction, and toxicology. Toxicol. Appl. Pharmacol. 194, 309–335.

Diamanti-Kandarakis, E., Bourguignon, J.P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, R.T., Gore, A.C., 2009. Endocrine-disrupting chemicals: an endocrine society scientific statement. Endocr. Rev. 30, 293–342.

Dutta-Gupta, A., 2013. Teleosts as model for environmental risk assessment. In: Senthilkumaran, B. (Ed.), Sexual Plasticity and Gametogenesis in Fishes. Nova Medical, New York, pp. 49–56.

Dutta, H.M., Misquitta, D., Khan, S., 2006. The effects of endosulfan on the testes of bluegill fish, *Lepomis macrochirus*: a histopathological study. Arch. Environ. Contam. Toxicol. 51, 149–156.

Embrandiri, A., Singh, R.P., Ibrahim, H.M., Khan, A.B., 2012. An epidemiological study on the health effects of endosulfan spraying on cashew plantations in Kasaragod district, Kerala, India. Asian J. Epidemiol. 5, 22–31.

Evans, E.B., Hogarth, C., Evanoff, R.M., Mitchell, D., Small, C., Griswold, M.D., 2012. Localization and regulation of murine Esco2 during male and female meiosis. Biol. Reprod. 87, 1–8.

Freeman, M.E., Kanyicska, B., Lerant, A., Nagy, G., 2000. Prolactin: structure, function, and regulation of secretion. Physiol. Rev. 80, 1523–1631.

Han, Z., Jiao, S., Kong, D., Shan, Z., Zhang, X., 2011. Effects of β-endosulfan on the growth and reproduction of zebrafish (*Danio rerio*). Environ. Toxicol. Chem. 30, 2525–2531.

Harford, A.J., O'Halloran, K., Wright, P.F., 2005. The effects of *in vitro* pesticide exposures on the phagocytic function of four native Australian freshwater fish. Aquat. Toxicol. 75, 330–342.

Hogarth, C.A., Mitchell, D., Evanoff, R., Small, C., Griswold, M., 2011. Identification and expression of potential regulators of the mammalian mitotic-to-meiotic transition. Biol. Reprod. 84, 34–42.

Jonsson, C.M., Toledo, M.C.F., 1993. Acute toxicity of endosulfan to the fish *Hyphessobrycon bifasciatus* and *Brachydanio rerio*. Arch. Environ. Contam. Toxicol. 24, 151–155.

Lal, B., Sarang, M.K., Kumar, P., 2013. Malathion exposure induces the endocrine disruption and growth retardation in the catfish, *Clarias batrachus* (Linn.). Gen. Comp. Endocrinol. 181, 139–145.

Lemos, M.F.L., Soares, A.M.V.M., Correia, A.C., Esteves, A.C., 2009. Proteins in ecotoxicology – how, why, and why not? Proteomics 10, 873–887.

Lui, W.Y., Lee, W.M., 2008. Ubiquitin system in male reproduction and its relevance to contraception. Immunol. Endocr. Metab. Agents. Med. Chem. 8, 14–19.

Mönnich, M., Kuriger, Z., Print, C.G., Horsfield, J.A., 2011. A zebrafish model of Roberts Syndrome reveals that Esco2 depletion interferes with development by disrupting the cell cycle. PLoS ONE 6, e20051.

Plumb, J.A., Areechon, N., 1990. Effect of malathion on the humoral immune response of channel catfish. Dev. Comp. Immunol. 14, 355–358.

Prathibha, Y., Murugananthkumar, R., Rajakumar, A., Laldinsangi, C., Sudhakumari, C.C., Mamta, S.K., Dutta-Gupta, A., Senthilkumaran, B., 2014. Gene expression analysis in gonads and brain of catfish *Clarias batrachus* after the exposure of malathion. Ecotoxicol. Environ. Saf. 102, 210–219.

Rajakumar, A., Singh, R., Chakrabarty, S., Murugananthkumar, R., Laldinsangi, C., Prathibha, Y., Sudhakumari, C.C., Dutta-Gupta, A., Senthilkumaran, B., 2012. Endosulfan and flutamide impair testicular development in the juvenile Asian catfish, *Clarias batrachus*. Aquat. Toxicol. 110–111, 123–132.

Shevchenko, A., Wilm, M., Vorm, O., Mann, M., 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. 68, 850–858.

Singh, P.B., Singh, V., 2007. Endosulfan induced changes in phospholipids in the freshwater female catfish, *Heteropneustes fossilis* (Bloch). J. Environ. Biol. 28, 605–610.

Singh, H., 1989. Interaction of xenobiotics with reproductive endocrine functions in a protogynous teleost, *Monopterus albus*. Mar. Environ. Res. 28, 285–289.

Sutovsky, P., Hauser, R., Sutovsky, M., 2004. Increased levels of sperm ubiquitin correlate with semen quality in men from an andrology laboratory clinic population. Hum. Reprod. 19, 628–638.

Tabb, M.M., Blumberg, B., 2006. New modes of action for endocrine-disrupting chemicals. Mol. Endocrinol. 20, 475–482.

Thangavel, P., Sumathiral, K., Karthikeyan, S., Ramaswamy, M., 2005. Endocrine response of the freshwater teleost

Sarotherodon mossambicus (Peters) to dimecron exposure. Chemosphere 61, 1083–1092.

Tripathi, G., Verma, P., 2004. Endosulfan-mediated biochemical changes in the freshwater fish *Clarias batrachus*. Biomed. Environ. Sci. 17, 47–56.

Venkataramana, G.V., Sandhya Rani, P.N., Murthy, P.S., 2006. Impact of malathion on the biochemical parameters of gobiid fish, *Glossogobius giuris* (Ham). J. Environ. Biol. 27, 119–122.

Whittington, C.M., Wilson, A.B., 2013. The role of prolactin in fish reproduction. Gen. Comp. Endocrinol. 191, 123–136.