Chemosphere 120, February 2015, 357–364 DOI: 10.1016/j.chemosphere.2014.07.096

Effects of low dose endosulfan exposure on brain neurotransmitter levels in the African clawed frog *Xenopus laevis*

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Highlights

• *Xenopus laevis* tadpoles were submitted to low environmental concentrations of endosulfan.
• Endosulfan increases levels of brain neurotransmitters.
• The gene coding for the GABA transporter 1 was up-regulated by endosulfan.
• Endosulfan affected both foraging and locomotion.
• Physiological and behavioural approaches are complementary to understand pollutant toxicity.

Abstract

Understanding the impact of pesticides in amphibians is of growing concern to assess the causes of their decline. Among pesticides, endosulfan belongs to one of the potential sources of danger because of its wide use and known effects, particularly neurotoxic, on a variety of organisms. However, the effect of endosulfan was not yet evaluated on amphibians at levels encompassing simultaneously brain neurotransmitters and behavioural endpoints. In this context, tadpoles of the African clawed frog *Xenopus laevis* were submitted to four treatments during 27 d: one control, one ethanol control, and two low environmental concentrations of endosulfan (0.1 and 1 μg L^{-1}). Endosulfan induced a significant increase of brain serotonin level at both concentrations and a significant increase of brain dopamine and GABA levels at the lower exposure but acetylcholinesterase activity was not modified by the treatment. The gene coding for the GABA transporter 1 was up-regulated in endosulfan contaminated tadpoles while the expression of other genes coding for the neurotransmitter receptors or for the enzymes involved in their metabolic pathways was not significantly modified by endosulfan exposure. Endosulfan also affected foraging, and locomotion in links with the results of the physiological assays, but

no effects were seen on growth. These results show that low environmental concentrations of endosulfan can induce adverse responses in *X. laevis* tadpoles. At a broader perspective, this suggests that more research using and linking multiple markers should be used to understand the complex mode of action of pollutants.

Keywords

Endosulfan – Toxicity – Neurotransmitters – Physiology – Behaviour - Amphibians

1. Introduction

According to the Red List of IUCN, the International Union for Conservation of Nature, one third of amphibians is threatened with extinction (Stuart et al., 2004). This worldwide decline of amphibian populations has become a major issue at it is now at its peak and interpreted as the sixth mass extinction (Wake and Vredenburg, 2008). Several causes have been suggested to explain this decline, such as UV radiations (Kiesecker et al., 2001), parasites and pathogens (Christin et al., 2004 and Venesky et al., 2010), predation and competition by invasive species (Broomhall, 2002 and Denoël et al., 2005), and pollution, including industrial and agricultural contaminants (Gillardin et al., 2009a, Gillardin et al., 2009b, Sparling and Fellers, 2009 and Denoël et al., 2013b).

Among the pesticides used in agriculture, the organochlorine insecticide and acaricide endosulfan is still extensively used against pests in tropical countries especially on crops such as tea, coffee, cotton and rice. Endosulfan is often sprayed in agricultural areas nearby wetlands and by run-off, it reaches aquatic systems (Bernabò et al., 2007) where it causes many damages to non-targeted species (Chopra et al., 2010). The pesticide is found in all biological compartments (air, water, sediments, organisms), sometimes far away from the zone of application and even in the food web as endosulfan bioaccumulates and biomagnifies (Weber et al., 2009).

Endosulfan is considered as a highly toxic insecticide and has been recently classified among the Persistent Organic Pollutants (UN, 2011). It affects the nervous system of a large range of organisms, including humans (EPA, 2002). In mammals, endosulfan acts on the central nervous system by altering neurotransmission in the brain (Gant et al., 1987 and Naqvi and Vaishnavi, 1993) and by activating cholinergic, dopaminergic and serotonergic (Agrawal et al., 1983) mechanisms. Endosulfan also induces neurological disorders, hyperactivity, convulsions, paralysis and memory problems (Usha and Harikrishnan, 2004).

Amphibians are particularly affected by endosulfan exposure. As the majority of pesticides are applied in spring, when amphibians reproduce, the eggs and larvae are particularly vulnerable and easily absorb pollutants from the environment (Bernabò et al., 2007). For this reason, there is a need to develop monitoring methods to assess the risks induced by such pollutants. Detrimental effects of endosulfan such as reduced survival (Rohr et al., 2003, Jones et al., 2009 and Shenoy et al., 2009), growth inhibition (Dimitrie, 2010), disturbed behavioural patterns (Denoël et al., 2012 and Denoël et al., 2013a), body deformities (Lavorato et al., 2013), or metamorphosis timing perturbations (Lavorato et al., 2013) have been already reported on

amphibians. Nervous system is the main target of endosulfan (EPA, 2002) but, to the best of our knowledge, no study investigated the impacts of endosulfan on the nervous system of amphibians.

The objective of the present study was to investigate how low concentrations of endosulfan are susceptible to induce neurological disorders in *X. laevis* tadpoles with a focus on neurotransmitters, but also considering behavioural traits. To address this question, tadpoles were exposed to environmental concentrations of endosulfan (0.1 and 1 μg L^{-1}) during 27 d. The responses included physiological (brain neurotransmitter concentrations), molecular (expression of genes coding for neurotransmitter receptors and enzymes involved in their metabolic pathways), life-history (growth) and behavioural (feeding and locomotor activity) variables.

2. Materials and methods

2.1. Animals breeding and housing

Adult African clawed frogs (*Xenopus laevis*) were obtained in 2010 from the National Breeding Center of *Xenopus*, University of Rennes, France. Animals were maintained in dechlorinated water at 22 ± 1 °C with a 12:12 photoperiod schedule. Freshwater was changed every day. To induce reproduction, one adult couple of *X. laevis* was left for a night in FETAX solution (625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4{\cdot}2H_2O$ and 75 mg $SO_4{\cdot}7H_2O$ per L distilled water) for amplexus after injection with 800 IU of Human Chorionic Gonadotropin HCG (Sigma, Germany) in dorsal lymphatic bags. Cleaving embryos of stage 8–13 (Nieuwkoop and Faber, 1994) were placed in FETAX medium until they hatched (48 h post-fertilization, pf). When tadpoles reached stage 46 (5 d pf), they were fed a mixture of spirulin algae (JBL Novo Fect, Belgium).

2.2. Endosulfan exposure

A set of 120 *X. laevis* tadpoles (stage 46) were placed separately and randomly in 120 glass bowls (capacity: 500 mL) filled with 400 mL of FETAX solution during the whole experiment. They were split in four treatments (30 tadpoles for each): control, ethanol control (1.3×10^{-5}%) to 0.1 and 1 μg L^{-1} nominal concentrations of endosulfan diluted into ethanol vehicle (1.3×10^{-5}%). The ethanol control was used because of the necessity to use ethanol with endosulfan. Endosulfan (SIGMA, PS81) was used as a mixture of isomers α/β in a ratio 70/30 and the exposure duration was 27 d (from stage 46 to stage 54). The period of pesticide exposure (premetamorphosis) was chosen as young tadpoles are known to be very sensitive and permeable to pollutants during this stage. From d 0 to d 14, solutions were renewed every 3 d. However, as tadpoles became larger and produced more metabolites, water renewal was done every two days from d 14 to d 20, and daily from d 20 to d 27, in order to keep the animals in adequate experimental conditions. Previous mass spectrometry analyses showed that nominal and real concentrations of endosulfan were similar at low ranges in using a similar design as used in this study (Denoël et al., 2013a). Temperature was maintained constant at a mean ± SE of 22.91 ± 0.04 °C.

2.3. Brain neurotransmitter concentrations

After 27 d of exposure, tadpoles were snap frozen on ice and each individual's brain and tail were dissected out for further ELISA assays and measurement of gene expression (9–13 tadpoles per group).

Physiological analyses (serotonin, dopamine and GABA) were performed on 12, 9, 9 and 10 tadpoles' brain samples from control, ethanol control, 0.1 and 1 μg L^{-1} of endosulfan groups, respectively. The molecular analyses were performed on 13 brain samples from control, 11 from ethanol control, 9 from the lowest endosulfan concentration and 10 from the highest one, respectively.

The concentrations of serotonin, dopamine, and GABA were measured in the brain by ELISA (Genway Biotech Inc., San Diego). Tissues were weighed and homogenized for 45 s in buffer (Tris–HCl 25 mM, pH 7.4, 1 mM EDTA, 1 mM EGTA) in a ratio 1:10. The homogenate was centrifuged at 11 000 g for 30 min at 4 °C. A part of the supernatant was filtered with 0.22 μm filters (Millex®GP) and stored at −80 °C until serotonin assay. The rest of the supernatant was centrifuged again at 14 000 rpm for 3 min at 4 °C for dopamine and GABA assays as their subcellular distribution is narrower than serotonin's one (Chomczynski and Sacchi, 1987). Assays were carried out in duplicate following manufacturer instructions.

2.4. Acetylcholinesterase activity (AChE) in tail muscles

Acetylcholinesterase assays were carried out on 20, 15, 15, and 15 tail samples from control, ethanol control, 0.1 and 1 μg L^{-1} of endosulfan groups, respectively. AChE activity was measured using a modified version of a colorimetric technique described byEllman et al. (1961). The reaction mixture was prepared by homogenizing the tails in 50 mM of sodium phosphate buffer pH 7.4 containing the reaction substrate AChI (Acetylthiocholine iodide) and DTNB (dithiobisnitrobenzoate) at a final concentration of 30 mM and 167 μM, respectively. The homogenate was centrifuged at 10 000 rpm for 10 min at 4 °C. Fifty μL of sample PMF were added into the spectrophotometer cuvette to start the reaction. The optic density was read at 412 nm for 7 min using the software SWIFT II Reaction Kinetics. Activity is expressed as nM of hydrolyzed AChI min^{-1} mg^{-1} proteins after the measurement of the protein quantity in the homogenate using the Bradford assay (Bradford, 1976).

2.5. Total RNA extraction, reverse transcription and real-time PCR

RNA was submitted to a reverse transcription step with the kit *Revertaid TM H Minus First Strand cDNA Synthesis* (Fermentas, Burlington, Canada) in order to obtain cDNA. Real-time PCR analysis was performed using a SYBR Green PCR Master Mix (Applied Biosystem, Warrington, UK) using 600 nM L^{-1} of primers and cDNA diluted 50 times. Amplification parameters were as follows: each of the 40 cycles consisted in 15 s of denaturation at 95 °C, 1 min annealing/extension at 56–60 °C depending on gene. As a housekeeping gene, EF1 α revealed no differences among treatments and allowed normalization of gene expression. Primer sequences and melting temperatures are presented in Table 1. A melting curve analysis was performed to verify that a single PCR product was generated. Negative RT samples were performed to check the absence of genomic DNA.

Table 1. Forward and reverse primers sequences coding for receptors of serotonin, dopamine, GABAa, acetylcholine, and for GABA transporter 1, monoamine oxidase a (Mao-), glutamate decarboxylase, arylalkylamine N-acetyltransferase (AANAT1), and DOPA decarboxylase.

	Forward	Reverse	Efficiency (%)	Annealing tempera-ture
EF1 α	5′gaccatctcctttgcaccgtc3′	5′tccgatgttgaaccctgggaa3′	103	60
Serotonin 5HT1a	5′gcagtcgccaatgattcgcaggagc3′	5′gggtaagggtagagatgccatgtgg3′	88	60
Serotonin 5HT2b	5′-aacaccaccttcagcgcaagc3′	5′-accaacactggatagtctgcg3′	92	59
Dopamine D1	5′gagctgtggtctattcatgccagc3′	5′cagcacttcatcttcggggcaa3′	101	60
GABAa	5′tttgcagcgaaaactcaccgtcga3′	5′gtgatggcgaatttgtcccgtttcg3′	78	60
AchNicotinic α5	5′acctcagtccttgtatccctgacc3′	5′gccatagcattgtgtgttgcag3′	80	62
GABA transporter 1	5′ctgtccaacataacccagggtgga3′	5′cccaagatgggaaaacgtagctgc3′	113	60
Monoamine oxidase a	5′atcgtctaccaatggggtctgtc	5′caggttcagtaccatctggcttgg3′	110	60
Glutamate decarboxylase	5′agaaactgcacagggttgctc3′	5′agtcgatgtcggacttggtcg3′	102	60
AANAT1	5′gtcctcatgtgcgaagacttcctg3′	5′ggcatgaccttgaactgggtactg3′	99	60
DOPA decarboxylase	5′gcattacagacaggtcccagttcc3′	5′ggtggcatactcttcggacca3′	100	60

2.6. Developmental and behavioural analyses

Behavioural variables were monitored using visual determination (feeding) and video-tracking (locomotion patterns). Both are sensitive indicators of toxicity (Denoël et al., 2010, Egea-Serrano et al., 2011 and Denoël et al., 2013a). Feeding frequency was determined during 30 s focal observations of each tadpole at three different periods (period 1: d 1–4, period 2: d 9–12, period 3: d 17–20) during the 27-d experiment. Observations were carried out around 10:00 am to standardize circadian activity. The observer moved slowly in front of the tanks and remained stationary during the observation periods. This did not cause changes in the behaviour of tadpoles. A score was attributed for each tadpole on the basis of four observations (0 = never fed, 1 = always fed). Secondly, locomotion was analyzed by video-tracking (*Noldus Ethovision XT* 7.0®). Tadpoles were placed into white plastic tanks (diameter: 19 cm) for 50-min acclimation and their movements were recorded with a video camera (SONY HDR-HC3) for 15 min from above at the end of the experiment (d 26). The tracks were sampled at a rate of 5 images s^{-1} in using grey-scaling detection method (Delcourt et al., 2013), similarly as done for tadpoles in Denoël et al. (2013a). Video-tracks with 0.1% or more detection losses and with wrong detections were not considered. Two locomotor patterns were considered on the 57 analyzed tracks (measures calculated at every two successive images, i.e. every 0.4 s, for a total of 20 520 images analyzed): distance moved (in cm), and speed during movement (in cm s^{-1}). Because body size can influence locomotor performance, all tadpoles were measured after the video-tracking (Denoël et al., 2010).

2.7. Statistical analyses

One-way ANOVAs were computed to test the effect of treatment on behavioural and neurophysiological data. Tadpole size was included as a covariable in MANCOVAs for the behavioural analyses. Normality and homogeneity of variances were verified with Levene and Shapiro–Wilk's tests respectively and then the data were subjected to a one-way ANOVA. When the factor "treatment" was significant, a HSD (Honestly Significant Difference) post hoc test with unequal *N* was applied. For all analyses, we chose a level of significance of 0.05. Statistics were performed with the software *Statistica* 5.5 and 10.

3. Results

3.1. Neurotoxicity markers

Serotonin concentrations were significantly affected by the treatments ($F_{3,35} = 9.594$, $p < 0.001$). Compared with values from the control, concentrations of serotonin significantly increased in tadpoles exposed to both doses of endosulfan (HSD post hoc test: $p < 0.05$ and 0.001 for comparisons with low and high concentrations, respectively), while differences were only significant between the higher dose of endosulfan and the ethanol control group ($p < 0.05$, Fig. 1A). Brain dopamine and GABA concentrations were also significantly affected by the treatments ($F_{3,36} = 4.292$, $p < 0.05$ and $F_{3,36} = 3.089$, $p < 0.05$, respectively). The level increased between the values from the low endosulfan treatment compared with values from the ethanol control (HSD post hoc test: $p < 0.01$ for dopamine and $p < 0.05$ for GABA, Fig. 1B and C).

Acetylcholinesterase activities in tail muscle varied between 28 ± 5.5 and 34 ± 7.2 nM min^{-1} mg^{-1} proteins. Although there was a slight general effect of treatment ($F_{3,61} = 3.035$, $p < 0.05$), there were no significant effects of endosulfan on AchE activities (HSD post hoc tests in comparison with ethanol control: all $p > 0.05$) and no other post hoc comparisons were significant.

3.2. Gene expression

No significant changes in the expression of genes coding for brain neurotransmitter receptors (serotonin 5HT1, serotonin 5HT2, dopamine D1, $GABA_a$, nicotinic α5 Ache) or for metabolic enzymes implied in the metabolic pathway of neurotransmitters (monoamine oxidase Mao-A, glutamate decarboxylase, arylalkylamine N-acetyltransferase AANAT1 and DOPA decarboxylase) (ANOVAs, all $p > 0.10$, Fig. 2A–J) were observed after endosulfan exposure, probably partly explained by a high within treatment variability in some genes (*e.g.* MaO). However, the gene coding for GABA transporter 1 was significantly modulated according to the treatments ($F_{3,39} = 4.328$, $p < 0.01$): up-regulated in both contaminated groups when compared with the ethanol control group ($p = 0.05$ for the low dose and $p < 0.05$ for the high dose, Fig. 2F).

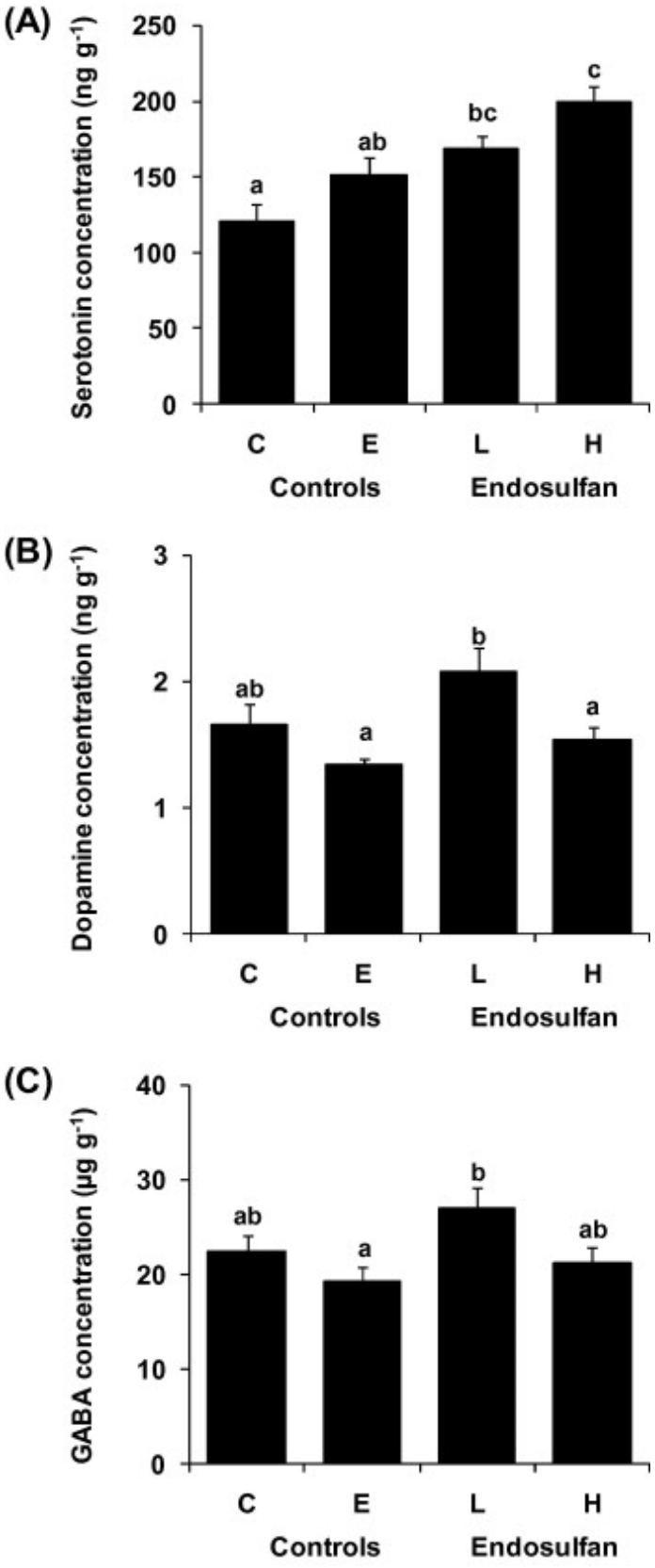

Fig. 1. Effect of endosulfan on concentrations of serotonin (A), dopamine (B) and GABA (C) in the brain of African clawed frog tadpoles. Tadpoles were exposed during 27 d to control (C), ethanol control (E), low endosulfan concentration (0.1 µg L^{-1}) (L), and high endosulfan concentration (1 µg L^{-1}) (H). Means and SE values are represented (n = 9–12). Different letters indicate significant differences between treatments.

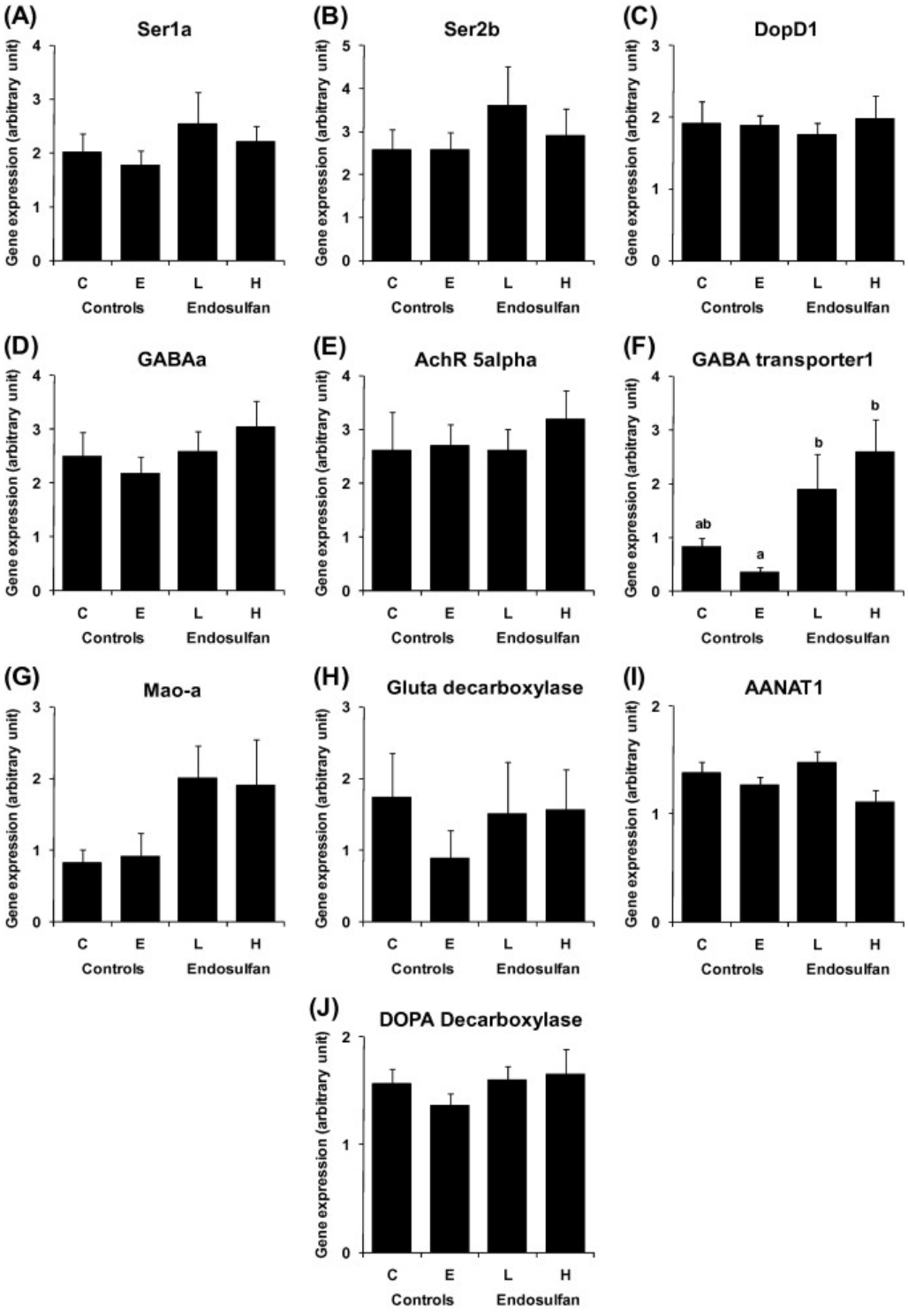

Fig. 2. Effect of endosulfan on the expression of genes linked to neurotransmitter receptivity and metabolism in the brain of African clawed frog tadpoles. Tadpoles were exposed during 27 d to control (C), ethanol control (E), low endosulfan concentration (0.1 μg L^{-1}) (L), and high endosulfan concentration (1 μg L^{-1}) (H). Normalized expression of receptors for serotonin 5HT1 (A: Ser1a), serotonin 5HT2 (B: Ser2b), dopamine D1 (C: DopD1), $GABA_a$ (D), Acetylcholinesterase nicotinic α5 (E: AchR 5alpha), GABA transporter 1 (F), monoamine oxidase a (G: Mao-a), glutamate decarboxylase (H), arylalkylamine N-acetyltransferase (AANAT1) (I) and DOPA decarboxylase (J). Means and SE values are represented (n = 9–13). Different letters indicate significant differences between treatments as observed for the GABA transporter 1.

3.3. Growth and behaviour

After 27 d of contamination, the effect of treatment on body size and weight of tadpoles was only marginally significant (Wilks' $\lambda = 0.864$, $F_{6,168} = 2.128$, $p = 0.05$). The separate main effects on body size and weight were significant ($F_{3,85} = 3.625$, $p < 0.05$ and $F_{3,85} = 3.688$, $p < 0.05$, respectively). There were no significant pairwise differences between ethanol control and endosulfan treatments, but well between the control and the high endosulfan treatment for both the body size and weight (HSD post hoc test, $p < 0.05$) (Table 2).

Table 2. Weight and size of African clawed frog tadpoles exposed during 27 d to control, ethanol control, low endosulfan concentration (0.1 µg L^{-1}), and high endosulfan concentration (1 µg L^{-1}). Observations were made at d 27 following the contamination period. Different letters indicate significant differences between treatments.

Treatment	Size (mm)	Weight (mg)
Control	55.5 + 7.2 [a]	1076 + 257 [a]
Ethanol control	52.1 + 9.3 [ab]	993 + 35 [ab]
Low endosulfan concentration	49.3 + 9.6 [ab]	842 + 303 [ab]
High endosulfan concentration	47.6 + 9.7 [b]	832 + 27 [b]

Feeding behaviour was significantly affected by treatment ($F_{3,90} = 8.807$, $p < 0.001$) and period ($F_{2,180} = 42.030$, $p < 0.001$). The interaction between treatment and period was not significant ($F_{6,180} = 0.031$, $p = 0.83$). At low and high endosulfan concentrations, tadpoles fed less than in controls (HSD post hoc test: all $p < 0.05$) (Fig. 3). There was a significant effect of treatment, but not size, on locomotor patterns (Treatment: Wilks' $\lambda = 0.747$, $F_{2,100} = 2.620$, $p < 0.05$; size as covariable: Wilks' $\lambda = 0.964$, $F_{2,50} = 0.923$, $p = 0.40$) (Fig. 4). There were a significant effect for speed and a marginally significant effect for distance travelled ($F_{3,51} = 3.149$, $p < 0.05$ and $F_{3,51} = 2.698$, $p = 0.06$, respectively). In both cases, there was a trend for lower travelled distance and speed in endosulfan treatment but post hoc (HSD) comparisons were not significant (Fig. 4).

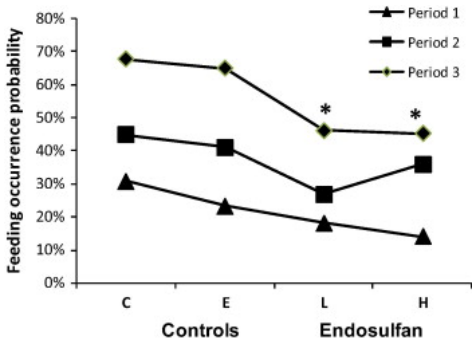

Fig. 3. Effect of endosulfan on feeding behaviour of African clawed frog tadpoles. Tadpoles were exposed during 20 d to control (C), ethanol control (E), low endosulfan concentration (0.1 µg L^{-1}) (L), and high endosulfan concentration (1 µg L^{-1}) (H). Observations were made during three periods of exposure (n = 30–32). The periods correspond to a range of exposure time: period1: d 1–4; period 2: d 9–12; period 3: d 17–20. Asterisks represent treatments that are significantly different from controls as observed between the ethanol control and both doses of endosulfan.

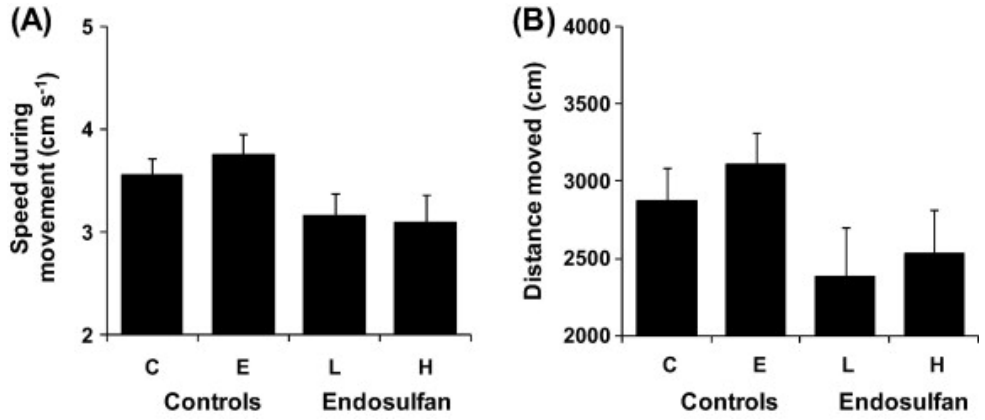

Fig. 4. Effect of endosulfan on the speed during movement (cm s^{-1}, A) and distance moved (cm, B) of African clawed frog tadpoles. Tadpoles were exposed during 26 d to control (C), ethanol control (E), low endosulfan concentration (0.1 μg L^{-1}) (L), and high endosulfan concentration (1 μg L^{-1}) (H). Means and SE values are represented (n = 9–20).

4. Discussion

The combination of different approaches allowed highlighting the neurotoxicity mechanism of an organochlorine pesticide, endosulfan. Specifically, we showed that low environmental concentrations of endosulfan altered neurotransmitters and caused behavioural disorders in an amphibian. This extends previous results on the toxicity of endosulfan in investigating the physiological and molecular effects in the brain of vertebrates and in providing putative explanations for the alterations of feeding and locomotion. This points out the need for inter-disciplinary studies to understand the consequences of pollutants on organisms (see also Yu et al., 2013).

Endosulfan induced a significantly higher level of serotonin, reaching up to 200 ng g−1. This observation is in agreement with several other studies conducted on vertebrates (Paul et al., 1994, Lakshmana and Raju, 1994 and Cabaleiro et al., 2008). Cabaleiro et al. (2008) suggested that the effects of endosulfan on serotonin concentrations in rat brain could be caused by a direct action on the brain, as endosulfan can cross the hemato-encephalic barrier. Mechanisms that modify monoamine concentrations in brain are still poorly understood. According to the absence of endosulfan effect on receptor and metabolic enzyme gene expression, we believe that monoamine receptivity and synthesis are not markedly affected by endosulfan in tadpoles. Brain is rich in lipids and, therefore, represents a target for endosulfan, as this latter can easily dissolve into synaptic membranes and induce serotonin level changes (Lakshmana and Raju, 1994). It has been shown in mice that serotonin is responsible for an increase of free radicals that can cause an oxidative stress and, consequently, cellular damages (Bist and Bhatt, 2009). A serotonin increase could thus represent a cell protection response against the pollutant. The serotonergic system is also implied in a large range of physiological regulations such as sleeping, thermoregulation, mood, reproduction, locomotion and appetite (Meguid et al., 2000). Many studies have shown that serotonin is directly released from hypothalamus while the animal is eating, in order to make it stop. This increase of serotonin enhances satiety and inhibits feeding. Our results on food acquisition are in agreement with previous studies on amphibian tadpoles which showed that feeding rates were decreased or even suppressed after endosulfan exposure (Denoël et al., 2012 and Broomhall and Shine, 2003).This is also consistent with the

high observed level of serotonin and suggests that the decrease of feeding activity may be linked to the increase of serotonin concentration in the brain. In *Xenopus*, serotonin plays also a role in the swimming duration and intensity as it boosts electrical pulses (Sillar et al., 1998). However, in our study, the change in brain serotonin level was not accompanied with an increase of locomotion as contaminated tadpoles moved at lower speed than control tadpoles. This may be due to the fact that Sillar et al. (1998) used high doses, at least 400-fold higher than the ones used in the present paper. Previous studies showed also that convulsions, which can result from uncorrected neural discharges, were not produced at low environmental endosulfan concentrations (Brunelli et al., 2009 and Denoël et al., 2012). Finally, exposure to SSRI (Selective Serotonin Reuptake Inhibitor) causes a growth reduction of 35% in *X. laevis* (Conners et al., 2009) but no effects of endosulfan were found on growth rates in the present study whereas previous research highlighted the presence or absence of effects.

Compared with serotonin, dopamine concentrations were very low in tadpoles' brain. After an exposure to a low concentration of endosulfan, they were higher than in controls, again confirming that endosulfan affects the neurophysiology of the tadpoles. In rat brain, the administration of endosulfan conducted to similar changes of serotonin and dopamine levels (Lafuente and Pereiro, 2013). In mammals, dopamine is also considered to be a physiologically relevant mediator of feeding behaviour as the mesolimbic dopamine reward system in human plays a key role in transforming subjective "liking" to motivational "wanting" of palatable food (Rui, 2013). The exposure to several pesticides induces a release of dopamine in the extracellular part of brain cells (Faro et al., 2009), which supports that this endosulfan effect on neurotransmitter content in the brain is conserved among vertebrates. Similar responses were observed for GABA which is in accordance with interconnected regulations of these neurotransmitters in time and space (Liu et al., 1997). In *Xenopus* tadpoles, GABA level in the brain was 100–10 000 fold higher than serotonin and dopamine levels, thus supporting that GABA is also a major inhibitory neurotransmitter in this species. Cabaleiro et al. (2008) also observed increased GABA levels in rats after 30 d of exposure to 0.61 and 6.12 mg endosulfan^{kg-1} while the levels significantly decreased after 60 d. According toBist and Bhatt (2009), modifications of GABA after exposure to lindane (another organochlorine) in mice would be correlated with alteration of GAD (Glutamic Acid Decarboxylase). In mammals, it is also well known that endosulfan acts as an antagonist of $GABA_a$ receptor and inhibits chloride flux through cell membrane, the neuron being subjected to uncontrolled electrical pulses. This phenomenon of receptor antagonism could be responsible for an increase of GABA because contaminated animals would compensate antagonist effects by increasing the quantity of ligand (Cabaleiro et al., 2008). In tadpoles, the negative results on glutamate decarboxylase and GABA receptor do not support involvement of these actors in the signaling pathway altered by endosulfan action. However, the effect on GABA transporter gene expression suggests that endosulfan stimulates GABA sequestration in presynaptic nerve terminals, inducing the increase of synaptic GABA release and in turns the whole GABA content in brain. All together, we further demonstrate that endosulfan greatly targets the GABA signaling in the brain of tadpoles and we postulate that this action is commonly observed in vertebrates.

AChE activity assayed in tail muscles was not affected by the exposure to low concentrations of endosulfan. Responses depend on species and on tested concentrations (Dutta and Arends, 2003). For example, in bluegill sunfish *Jenynsia multidentata*, exposure to 0.07 μl L^{-1} of endosulfan induced an inhibition of AChE activity (Ballesteros et al., 2009). Many studies reported an inhibition of this enzyme in X. laevis when exposed to organophosphorous compounds (Bonfanti et al., 2004 and Colombo et al., 2005). More research is particularly needed on the links between acetylcholinesterase and locomotor performances. Our results on

locomotion patterns are in line with those recently found on the common frog *Rana temporaria* (Denoël et al., 2013a) and the agile frog (Lavorato et al., 2013) but at a lower extent. This could be explained by the lower concentration used here (0.1 and 1 μg L−1 versus 5 and 50 μg L−1 in Denoël et al., 2013a and Denoël et al., 2013b and 10 and 50 μg L−1 in Lavorato et al., 2013). Determining AchE activity at concentrations inducing larger locomotor effects would thus help at determining its possible role in lowering swimming performance in tadpoles.

In conclusion, endosulfan is demonstrated to affect neurotransmitter levels in the brain in addition to effects on foraging, and locomotion. Environmentally realistic concentrations of endosulfan may thus present deleterious effects on amphibian tadpoles at a variety of scales. More research should be conducted to find out other neurologic markers of endosulfan exposure and to check *in situ* if the presence of endosulfan is linked with such physiological disorders. On the other hand, this study exemplifies the need for research programs operated at multiple levels of analysis to understand the complex mode of action of pollutants.

Authors' contributions

VP and MD carried out the contamination study and the behavioural observations. VP and SM carried out the physiological analysis. ED performed the endosulfan assay. VP, SM, MD and PK wrote the manuscript. PK and MD managed the project and all authors revised the manuscript. All authors read and approved the final manuscript.

Funding sources and policy ethics

This work was financially supported by the F.R.S.–FNRS (Fonds de la Recherche Scientifique), project FRFC n° 2.4.507.08. MD is a Research Associate at F.R.S.–FNRS. This is a publication of the Applied and Fundamental Fish Research Center (AFFISH-RC). The experiment was carried out in an agreed laboratory, and the research project was accepted by the ethical commission of the university. The capture permits were provided by the Service Public de Wallonie.

Acknowledgements

We thank the two anonymous reviewers for their comments on the manuscript and Marie-Claire Forget for technical assistance.

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Article history: Received 28 March 2014, Received in revised form 23 July 2014, Accepted 29 July 2014

Handling Editor: Tamara S. Galloway