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Effects of a sublethal pesticide exposure on locomotor behavior: A video-tracking analysis in larval amphibians

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HIGHLIGHTS

- ▶ We evaluate the use of behavioral endpoints as biomarkers of toxicity in amphibians.
- ▶ Endosulfan impairs locomotor behavior before survival.
- ▶ Distance moved, speed, activity, and space use are affected at sublethal concentrations.
- ▶ Behavior predicts ulterior mortality.
- ▶ Video-tracking shows its potential as sentinel of sublethal effects of pesticides.

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ABSTRACT

Organochlorine pesticides such as endosulfan have been shown to have both lethal and sublethal effects on amphibians. In this context, behavioral endpoints have proved their usefulness in evidencing impacts of such chemicals at environmental concentrations that do not necessarily cause mortality. The recent development of video-tracking technologies now offers the possibility of accurately quantifying locomotor behaviors. However, these techniques have not yet been applied to evaluating the toxicity of pesticides in amphibians. We therefore aimed at determining the potential toxicity of endosulfan on endpoints associated with locomotion after short-term environmental endosulfan exposure in *Rana temporaria* tadpoles and at using these data as warning systems for survival alterations after a longer exposure. To this end, we analyzed video-tracks of 64 tadpoles (two pesticide treatments: 5 and 50 $\mu g\ L^{-1}$, one control and one solvent-control) with Ethovision XT 7 software. The highest endosulfan concentration had a significant effect on all four behavioral endpoints. Contaminated tadpoles traveled shorter distances, swam less often, at a lower mean speed, and occupied a less peripherical position than control tadpoles. The lowest endosulfan concentration had similar but lower effects, and did not affect mean speed during swimming. Survival was reduced only after a long-term exposure to endosulfan and was associated with short-term behavioral dysfunctions. These results show that endosulfan strongly affects the behavioral repertory of amphibian tadpoles, but in different ways depending on concentration, thus suggesting that the pesticide has complex modes of action. Given the importance of locomotion and space use in tadpole success in their aquatic environment, these results confirm the toxic action of endosulfan. By highlighting effects before mortality markers, video-tracking systems also show their potential as sentinels of sublethal effects of pesticides.

1. Introduction

Amphibians have been highlighted by the International Union for Conservation of Nature as suffering from a major decline, with 30% of the species threatened (Stuart et al., 2004). This high rate, compared to other vertebrate classes, made them to be considered as part of the sixth world extinction (Wake and Vredenburg, 2008). This can have large-scale consequences through alterations of food webs, given the importance of amphibians in both aquatic and terrestrial ecosystems (Regester et al., 2008). Understanding the causes and mechanisms of such declines is therefore a key to maintaining biodiversity. Among the large number of identified stressors, many pesticides and other chemicals have been

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highlighted for their toxicity at environmental concentrations (Mann et al., 2009; Lehman and Williams, 2010).

Endosulfan is an organochlorine pesticide that was very recently added to the persistent organic pollutant (POP) list of the Stockholm Convention, but with exemptions (UN, 2011). Because of its wide use, it can now be found in most ecosystems, even far from the original sources of use through atmospheric drift, runoffs and drainage (Weber et al., 2010). It is also found in non-target habitats such as wetlands, particularly in proximity of agricultural lands (Ernst et al., 1991; Srivastava et al., 2009). Many amphibian species are dependent on such water resources for breeding and are therefore exposed to concentrations that can be lethal (Broomhall and Shine, 2003; Rohr et al., 2003; Kang et al., 2008; Jones et al., 2009; Shenoy et al., 2009). Even at sublethal environmental concentrations, a wide variety of effects has been highlighted in physiological (Park et al., 2001), morphological (Bernabò et al., 2008), and behavioral (Brunelli et al., 2009) studies.

Behavioral endpoints are in increasing use in ecotoxicological studies (Selderslaghs et al., 2010; Egea-Serrano et al., 2011). They have proved their effectiveness in both chronic and acute studies because of their sensitivity and their usefulness in ecological risk assessment (Weis et al., 2001; Amiard-Triquet, 2009). Several studies focused on the behavioral effects of endosulfan in amphibians (Berrill et al., 1998; Rohr et al., 2003; Brunelli et al., 2009). They showed direct neurotoxic effects, such as convulsions or paralyses. At concentrations lower than those causing such alterations, previous research also determined effects on critical endpoints such as a partial or total inhibition of feeding and air breathing (Broomhall and Shine, 2003; Denoël et al., 2012). There are also data showing alterations of space use and activity patterns (Denoël et al., 2012). All these analyses relied on direct visual observations or manual analyses of image or video-recording data. The development of new technologies now makes it possible to move one step further in behavioral ecotoxicology. This consists in using video-tracking software to automatically analyze video streams of focal organisms (Delcourt et al., in press). These systems transform focal organisms into pixels over space and time, thus delivering a huge number of spatio-temporal data that can be converted into important variables associated with locomotion, such as speed and distance moved (Denoël et al., 2010; Winandy and Denoël, 2011). Because of their novelty and despite their increasing use in other fields, such systems are still rarely used on laboratory organisms. To our knowledge, no studies have been published on the use of such video-tracking software to assess the effect of pesticides in amphibians. However, video tracking recently proved its usefulness in ecotoxicological research of other organisms such as fish (Eddins et al., 2010; Selderslaghs et al., 2010) or invertebrates (Nørum et al., 2010; Porcel et al., 2011), thus offering a vast potential for fast and efficient assessment of chemical toxicity at sublethal concentrations. This is particularly awaited because of the large number of new chemicals released on the market for which there is a need for rapid assessment.

In this perspective, our aim was to use the latest developments in video-tracking analyses to test the following hypotheses (1) endosulfan impairs varied aspects of amphibian locomotion (moved distance, swimming activity, speed, and space use) at the tadpole stage soon after exposure, (2) behavioral alterations occur before mortality events and can be indicators of such events, and (3) new video-tracking techniques are useful tools in ecotoxicology.

2. Material and methods

2.1. Laboratory maintenance

We collected four freshly laid clutches of the common frog (*Rana temporaria*) in a pond in March 2011 (La Mare aux Joncs, Liège Province, Belgium; WGS84 geographic coordinates: 50°34′18″N, 5°30′35″E, elevation: 250 m a.s.l.). Pesticides were neither historically nor recently used close to the site. The clutches were carried to the laboratory (20-min drive) in separate 3-L tanks and then placed in four 25-L tanks at their arrival. In March, we sampled 20 moving tadpoles from each clutch at an early larval stage (Gosner stage 26: Gosner, 1960). They were placed individually in 64 containers (250 mL, which were distributed on the shelves of the laboratory so that each treatment and clutch was at random. The tanks and containers were filled with a reconstituted solution from deionized tap water following APHA recommendations (APHA, 1985): $NaHCO_3$: 48 mg L^{-1}, $CaSO_4$ $2H_2O$: 30 mg L^{-1}, $MgSO_4$ $7H_2O$: 61 mg L^{-1}, KCl: 2 mg L^{-1}. Water was renewed every 5 d with a fresh stock (Hoke and Ankley, 2005). Tadpoles were manipulated gently during water change. They were placed in small containers, filled with the same water but without pesticide, as their experimental tank during water change.

Environmental concentrations of up to 100 μg L^{-1} of endosulfan have been reported in wetlands 30 m from targeted application sites (Ernst et al., 1991). A previous experiment on *R. temporaria* gave a LC_{50} estimated at 115 μg L^{-1} after 4 d of endosulfan exposure (Denoël et al., 2012). As our aim was to determine the sublethal effects on locomotion, we used two lower nominal concentrations of endosulfan pesticide (5 and 50 μg L^{-1}). We also used two additional treatments: a control and a solvent-control, including ethanol (33 μL L^{-1}). The actual mean concentrations (±SE) of endosulfan during the experiment were 4.7 ± 0.3 μg L^{-1} and 48.5 ± 5 μg L^{-1} ($n = 8$ samples, with 4 per treatment), henceforth referred to as 5 and 50 μg L^{-1} (see hereafter for details on the chemical analyses) to fit with nominal concentrations. No endosulfan was detected in the controls. Endosulfan and ethanol were analytical "Dr. Ehrenstorfer" grade purchased from Cluzeau Info-Labo (France). The solvent-control was used because of endosulfan's low solubility in water (Marquis et al., 2006; Jones et al., 2009). The amount of ethanol added was the same as that used in both endosulfan concentration treatments (33 μL L^{-1}). Endosulfan was added every 5 d after the water change and before returning tadpoles to their tanks. Organic spinach leaves previously boiled, frozen and thawed to increase digestibility by tadpoles were given *ad libitum* (one leaf of ca. 0.5 cm^2/tank). The photoperiod followed the natural cycle of the capture place, i.e., 12 h 30 light, 11 h 30 dark. Water temperature and dissolved oxygen were maintained at a mean ± SE of 14.27 ± 0.07 °C and 9.76 ± 0.05 mg L^{-1}, respectively ($n = 36$, taken randomly during the experiment; Thermometer–Oxymeter HQ40d, Hach Lange, Germany).

2.2. Gas chromatography coupled to high-resolution mass spectrometry

To determine the actual endosulfan concentrations in the tanks, water samples were analyzed by gas chromatography coupled to high-resolution time-of-flight mass spectrometry (GC-HRTOFMS). Endosulfan and Mirex (internal standard) were Dr. Ehrenstorfer grade, purchased from Cluzeau Info-Labo (France). Chemical solvents were obtained from Sigma–Aldrich (Germany) for isooctane and VWR (USA) for ethanol and ethyl acetate. The water samples were first extracted following a solid-phase extraction method as described by De la Colina et al. (1996). For this purpose, Supelco Supelclean™ ENVI-18 SPE cartridges were used (1 g, 6 mL) (Supelco, Bellefonte, PA, USA) with a 5 mL volume of isooctane/ethyl acetate (v:v/50:50). The elution fraction was concentrated to 500 μL using a gentle stream of nitrogen, then 50 μL of Mirex was added as the internal standard. The purified extracts were injected on a JEOL AccuTOF GC system (JEOL Ltd., Tokyo, Japan) using a 30 m × 0.25 mm × 0.25 μm Rxi®-XLB column (Restek, Bellefonte,