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

Mathieu Denoël[a], Sylvie Libon[a], Patrick Kestemont[b], Catherine Brasseur[c], Jean-François Focant[d], Edwin De Pauw[c]

[a]*Laboratory of Fish and Amphibian Ethology, Behavioural Biology Unit, Department of Biology, Ecology, and Evolution, University of Liège, 22 Quai van Beneden, 4020 Liège, Belgium*

[b]*Research Unit in Environmental and Evolutionary Biology, University of Namur, 5000 Namur, Belgium*

[c]*Center for Analytical Research and Technology, CART, Chemistry Department, Laboratory of Mass Spectrometry, University of Liège, 4000 Liège, Belgium*

[d]*Center for Analytical Research and Technology, CART, Chemistry Department, Organic and Biological Analytical Chemistry, University of Liège, 4000 Liège, Belgium*

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 μg 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

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.

Keywords: Amphibian decline, Behavioral endpoints, Biomarkers, Endosulfan, Survival, Video tracking

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

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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 and 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 and 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 and 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 and Amiard-Triquet, 2009). Several studies focused on the behavioral effects of endosulfan in amphibians (Berrill et al., 1998, Rohr et al., 2003 and 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 and 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 and 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 and Selderslaghs et al., 2010) or invertebrates (Nørum et al., 2010 and 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): NaHCO3: 48 mg L^{-1}, CaSO4 2H2O: 30 mg L^{-1}, MgSO4 7H2O: 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 LC50 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 and 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-

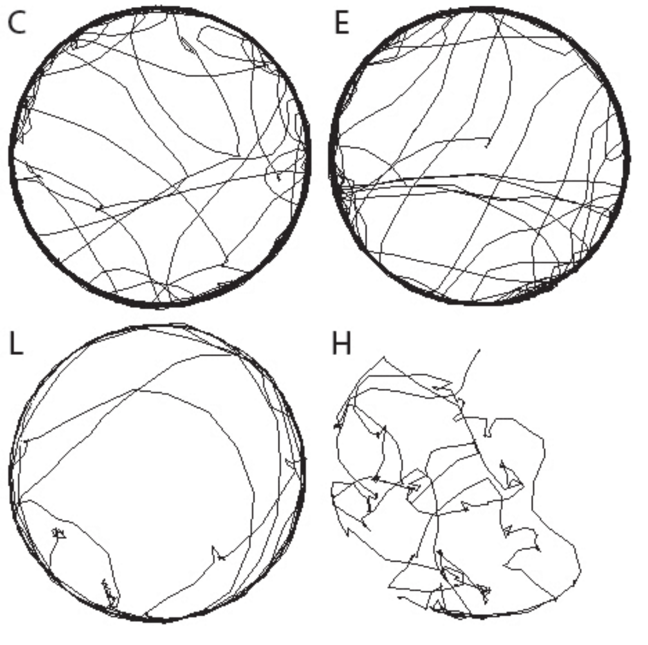

Fig. 1. Representative video tracks of tadpoles. C: control; E: solvent-control (ethanol); L: low endosulfan concentration (5 µg L-1); H: high endosulfan concentration (50 µg L-1).

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 SupelcleanTM 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, PA, USA). The gas chromatography oven ramp temperature was started at 80 °C for 2 min, then increased to 200 °C at a rate of 30 °C min^{-1}, then to 260 °C at a rate of 5 °C min^{-1} and held for 5 min. The mass spectrometry transfer line temperature was 280 °C. The ionization was performed on negative chemical ionisation (NCI) mode with an ion source temperature of 140 °C, an ionization voltage of 200 V and methane at 1 mL min^{-1} as the reagent gas. The collected mass range was 30–700 amu with a scan rate of 2.5 spectra s^{-1} and detector voltage of 2300 V.

2.3. Behavioral experiments, measures and statistics

After 48 h of endosulfan exposure, during a single day, tadpoles were transferred one by one to separate white plastic tanks (10.5 cm in diameter) filled with clear water. They were left 30 min to acclimatize to their new tank before recording each of them for 15 min with a video-camera (Sony HDR-HC3) placed just above the tanks. The video streams were digitalized into AVI files with Microsoft Windows Movie Maker 5.1. in real time during each trial from a control computer room, with no personnel present in the laboratory room during the video-recording. The tadpoles were returned to their original tanks directly after each video recording. Survival was recorded daily within a period of 21 d after the first day of exposure.

After the experiment, we used video-tracking software (Ethovision XT7, Noldus Information Technology, Wageningen, The Netherlands) to obtain both X and Y coordinates of the central point of each tadpole across time, i.e., every 0.2 s for 15 min. We used greyscale contrast to identify the tadpoles from their background. Each observation tank was calibrated into the video tracking system, i.e., in terms of size and position of the arenas (Noldus Information Technology, 2009). This allowed to express the results in metric units. The 64 computed video tracks (see four representative tracks in Fig. 1) were visually analyzed to confirm the absence of erroneous detection. There was no position lost in the tracks. The video tracks delivered 288 000 data points (i.e., coordinates), from which were calculated the following behavioral endpoints:

- distance moved (during the experiment, but converted into cm s^{-1} to make this measure independent of the duration of the experiment);
- distance to zone (mean distance to the periphery of the tanks: short distances mean that the tadpole is close from the periphery);
- swimming activity (the proportion of time the tadpoles exhibited a swimming movement);
- mean speed (i.e., during swimming activity).

The images of each tadpole were extracted from the video files and then calibrated and measured (total length) using an image analyzer software (Able Image Analyzer 3.6, Mu Labs, Slovenia).

We used generalized linear models (MANCOVA) to evaluate the effects of the four treatments on the four behavioral endpoints. Arcsine and log transformations were used prior to this analysis for proportion and other continuous data to fit a normal distribution, respectively. The treatment was included as a fixed factor. Because of the potential effect of tadpole size on locomotor performance (Denoël et al., 2010), tadpole length was included as a covariate in the model. Clutch of origin (random factor) and its interaction with treatment had no significant effect on behavioral patterns. Consequently, it was not included in subsequent analyses. Dunnet contrast-tests were used to test each specific treatment against controls. Univariate logistic regressions were applied to determine the suitability of each behavioral markers (at day 3) as an indicator of survival probabilities (at day 21). Survival at day 21 was analyzed by a Gehan survival test. All analyses were done in Statistica 10.1 (Statsfot, 2010).

3. Results

Treatment, but not size, had a significant effect on the exhibition of behavioral endpoints (Wilk's λ = 0.164, $F_{12,148}$ = 14.663, $p < 0.0001$ and λ = 0.961, $F_{4,56}$ = 0.567, p = 0.69 respectively). There was a significant effect on each of the four behavioral endpoints: Distance moved ($F_{3,59}$ = 64.239, $p < 0.0001$), distance to zone ($F_{3,59}$ = 48.841, $p < 0.0001$), swimming activity ($F_{3,59}$ = 28.019, $p < 0.0001$), and mean speed ($F_{3,59}$ = 7.056, $p < 0.001$) (Fig. 2). Post-hoc contrast-tests showed that tadpoles from the highest concentration treatment moved less, further from the periphery, swam less often (all $p < 0.0001$) and reached a slower speed than controls ($p < 0.01$). The same effects were found for the tadpoles exposed at the low concentration (all $p < 0.0001$), except that these tadpoles moved at a similar speed to controls (p = 0.91). Solvent had no significant effect on the four

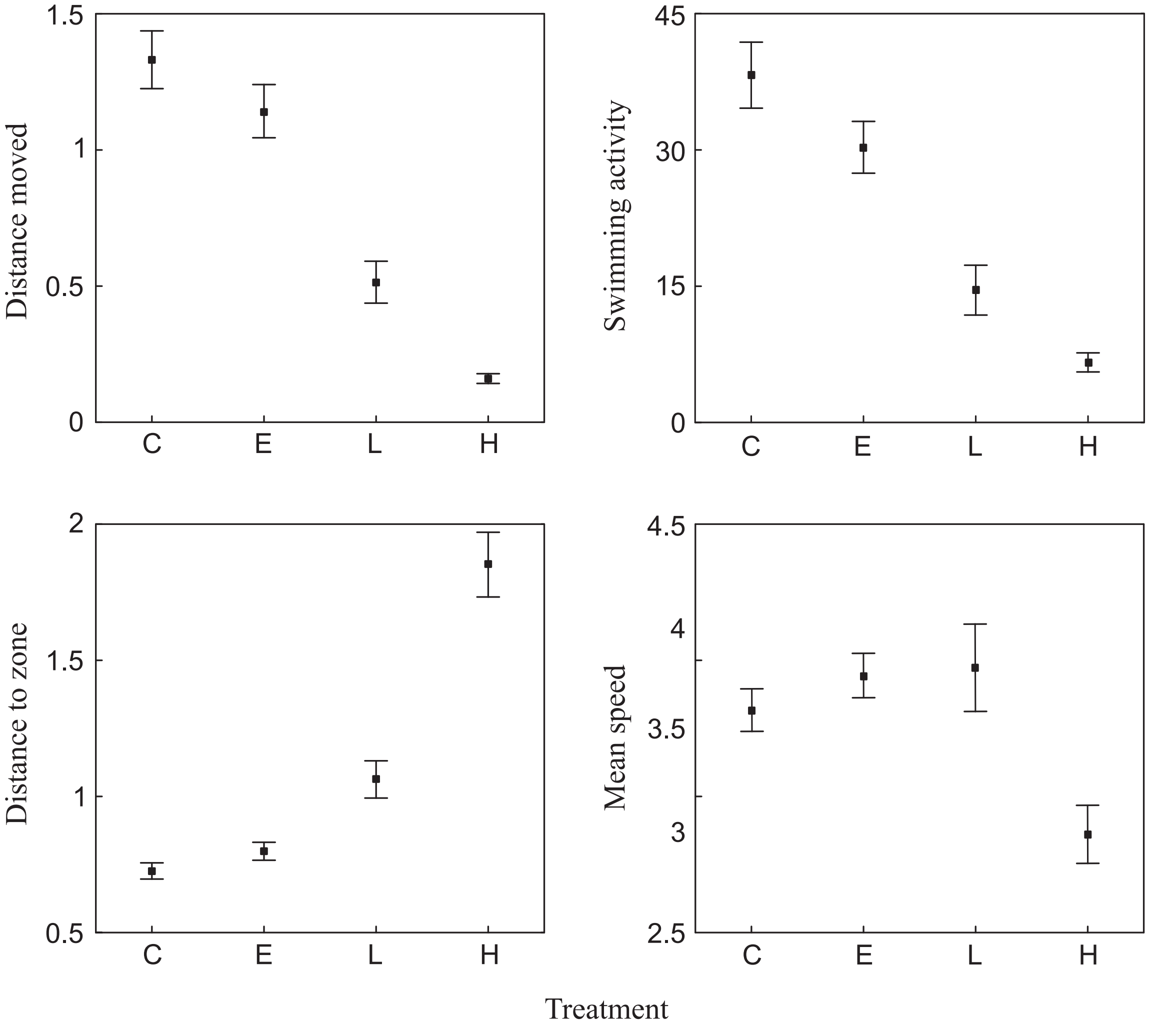

Fig. 2. Effect of treatment on behavioral endpoints (day 3): distance moved (cm s-1), distance to zone (i.e. from periphery, cm), swimming activity (%), and speed (during swimming activity, cm s-1). C: control, E: solvent-control (ethanol), L (low endosulfan concentration: 5 µg L-1), H (high endosulfan concentration: 50 µg L-1).

behavioral endpoints (all $p > 0.19$). Representative tracks of the four treatments are shown in Fig. 1.

Survival at 3 weeks was affected by treatment (Gehan survival test, $\chi2 = 21.458$, 3 df, $p < 0.0001$). Only tadpoles exposed to the higher concentration had a lower survival than controls (Wilcoxon–Gehan test = −3.265, $p < 0.01$). Ten out of 16 (63%) died during this period, whereas only one out of 16 (6%) in each control died. Only two tadpoles out of 16 (12%) at the lowest concentration died. There were no significant effects of the low concentration on survival (Wilcoxon–Gehan test = −0.652, $p = 0.52$). All tadpoles were alive at day 3 when behavioral endpoints were measured.

Survival probability was significantly predicted by the four behavioral endpoints (Fig. 3): Distance moved (logistic regression, $t_{62} = 2.720$, $p < 0.01$), distance to zone ($t_{62} = 3.315$, $p < 0.01$), swimming activity ($t_{62} = 2.422$, $p < 0.05$), and mean speed ($t_{62} = 2.326$, $p < 0.05$). Tadpoles that moved over shorter distances, further from the periphery, that swam less often and at a lower speed at day 3 had a higher probability of dying at 3 weeks than tadpoles that moved over longer distances, closer to the periphery and swam more often and at a higher speed.

4. Discussion

The results obtained in the present study show that endosulfan affects amphibians at the two tested environmental concentrations (5 and 50 µg L^{-1}). Using recent developments of video-tracking technologies, this study provides quantitative evidence of the negative effects of endosulfan on amphibian tadpoles. Varied behavioral endpoints associated with locomotion were changed after a short-term exposure to endosulfan, whereas survival was only decreased after a longer exposure. Behavioral endpoints were also good markers of survival.

Four different behavioral endpoints were computed from video tracks of amphibian tadpoles. Endosulfan affected all of them. It caused a reduction of the mean swimming speed, but also depressed different traits related to activity and space use. Contaminated tadpoles spent less time swimming and traveled shorter distances. The video-tracking analysis showed that the effects were even more complex as tadpoles moved not only less but also differently. Control tadpoles typically moved more along the walls of the tanks, whereas contaminated tadpoles stayed more in open areas. All these beha-

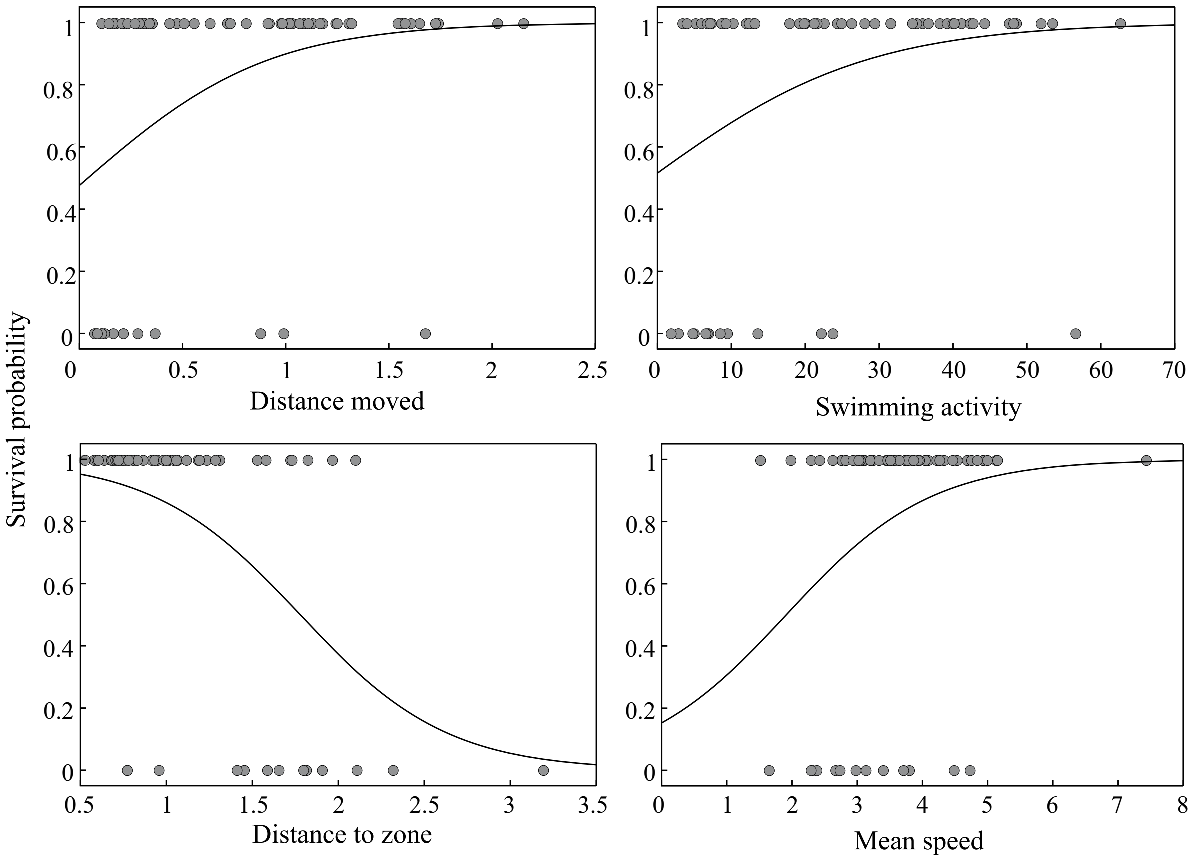

Fig. 3. Survival probabilities (day 21) in relation to behavioral endpoints (day 3): distance moved (cm s^{-1}), distance to zone (i.e. from periphery, cm), swimming activity (%), and speed (during swimming activity, cm s^{-1}).

vioral results confirm the effectiveness of these four endpoints in ecotoxicological studies. More specifically, they confirm the toxicity of endosulfan at environmental concentrations within the range of those used in the present study (Berrill et al., 1998, Brunelli et al., 2009, Jones et al., 2009 and Denoël et al., 2012) and detail the locomotor patterns that are affected. Behavior is an essential trait of organisms and alteration of the normal repertory is a sign of disturbance (Genuis, 2008), thus indicating an ethical and conservation issue in the use of endosulfan at the vicinity of water bodies. In addition, any dysfunction at this level is expected to indirectly impair fitness through varied processes such as a less foraging and greater vulnerability to predators (Lehman and Williams, 2010). The short distances traveled and the lower swimming activity of contaminated tadpoles show that they explore smaller portions of their habitat, thus possibly gathering less food. This is in accordance with results on feeding found in previous studies. Most particularly, using the same amphibian model in another study, we found that contaminated tadpoles ingested less food (Denoël et al., 2012). Here, by using locomotion measurements, we showed that such feeding depression can be in part due to a reduction of movements. On the other hand, the fact that contaminated tadpoles used more open habitats and exhibited slower speed suggests that they may be more visible to predators and may be less efficient in escaping them, respectively. Although a lower activity of tadpoles could also be advantageous in decreasing their detectability by predators (Marquis et al., 2004), the reduced performance of tadpoles exposed to endosulfan makes them more vulnerable to predation by dragonfly larvae (Broomhall and Shine, 2003). Future studies should thus explore more in depth the combined effects of pesticide and predatory risk on the effects of pesticides such as endosulfan. For instance, Janssens and Stoks (2012) recently found that endosulfan increased activity levels of damselflies but affected their escape performance during attacks.

The effect of endosulfan depended on its concentration. Tadpoles exposed at the lowest concentration showed three out of the four behavioral scores (all, but swimming activity) that were intermediate between the scores of controls and those of the high concentration treatment group. This dose-dependence is well known, particularly for survival estimates having deeper and faster effects at the highest concentrations (see e.g., Jones et al., 2009). However, in the present case, this also shows that endosulfan has a complex mode of action at the same concentration in that it does not alter all behavioral traits similarly. Speed, activity, and space use imply different mechanisms. Reaching high speed and sustaining long activity bouts are dependent on muscle efficiency. The lower scores found in contaminated tadpoles are in line with the reduction of acetylcholinesterase activity in muscles as found in other organisms (Tu et al., 2009). At a low concentration, tadpoles exhibit normal speed, but as they travel shorter distances than tadpoles contaminated at a high concentration, this shows they cannot swim for long periods of time. Moving in specific portions of the habitat is not directly related to performance capacity, but rather to cognitive patterns. The induced change in space use in presence of endosulfan could be associated with its direct neurotoxic effects (Scremin et al., 2011).

Tadpoles exhibited behavioral alterations before any mortality occurred as survival decreased only after 6 d of experimentation. By linking individual patterns of behavior early in development to survival of the same individuals at a later time, it was possible to show that the four behavioral endpoints were efficient markers. Low values for distance moved, speed and swimming proportion, and high distances to the periphery mean a higher probability of a mortality event later in life. Being predictive, behavioral endpoints do not only report on immediate sublethal effects but also on long-term effects on fitness (Amiard-Triquet, 2009). These two characteristics make the use of behavior in short-term experiments particularly relevant. Less time-consuming and thus less costly, this makes it possible to evaluate the sublethal toxicity of more chemicals. This is essential as the number of new toxicants is continuously increasing. Nonetheless, behavioral endpoints are still rarely taken into account in environmental regulations (Amiard-Triquet, 2009). The fact that both behavior and population changes through survival decrease could be related indicate that behavioral endpoints could meet criteria and should be considered in toxicity assessment.

The solvent (ethanol) used in conjunction with endosulfan did not affect locomotion. On one hand, this indicates that the observed results on both behavior and survival are caused by the pesticide itself. On the other hand, this suggests that, at the concentration used (33 $\mu L\ L^{-1}$), ethanol has no negative effects over the short-term for locomotor behavior of common frog tadpoles, and over a longer time period (3 weeks) for survival. This is in agreement with the conclusions of Marquis et al. (2006) and Jones et al. (2009) that found that ethanol concentrations (1 $\mu L\ L^{-1}$–1 $mL\ L^{-1}$) do not affect survival after short-term exposition (2 and 4 d).

Behavior is an important trait of the integrity of organisms: not only because it includes vital activities such as feeding and breathing but also because it implies, through locomotion, the use of environmental resources and is at the basis of interactions between organisms. In this perspective, laboratory studies in behavioral ecotoxicology are an aid in understanding how chemicals such as pesticides affect individual behavior (Amiard-Triquet, 2009). Through straightforward visual analyses, various behavioral effects have been highlighted, providing a better understanding of the mode of action of various substances (Brunelli et al., 2009, Egea-Serrano et al., 2011 and Denoël et al., 2012). It was shown that looking at such causes through short-term experiments is a valuable complement to long-term and mesocosm studies to understand toxicity on specific components of organisms (Denoël et al., 2012). As for other fields such as proteomics (Gillardin et al., 2009), behavioral ecotoxicology is benefitting from new technologies. In particular, video tracking has helped to gather large amounts of quantitative data than can illustrate specific locomotion patterns (Delcourt et al., in press). The new possibilities offered by video-tracking software (e.g., Ethovision) now make it possible to go even further in characterizing more behavioral endpoints from video recording (Denoël et al., 2010 and Selderslaghs et al., 2010). In the present study, we obtained new quantitative data to clearly depict varied patterns such as distance moved, space use, speed, and swimming activity in amphibian tadpoles. Both traditional and video tracking techniques are complementary. Some endpoints can be assessed by the two techniques, but with greater detail in video tracking (one-time position in an arena versus mean position across time, activity scores versus continuous sampling for instance). Although speed could be determined manually, video-tracking analysis allows to obtain fastly mean values over long bouts of swimming activity. Finally, some complex behaviors such as feeding have been determined only using visual techniques (Brunelli et al., 2009 and Denoël et al., 2012). Because embryonic and larval amphibians are increasingly use as models for ecotoxicological research (Sparling et al., 2010), such tools are expected to offer new possibilities and should be integrated into laboratory designs. A major advantage is that video-tracking offers a wide array of quantitative analyses and can be supplemented to other experiments without affecting them given that behavioral endpoints can be remotely recorded. Future research should continue to produce new behavioral markers to improve our understanding of the toxicity of chemicals, in conjunction with other biological fields such as neuroscience and physiology (Weis et al., 2001, Scott and Sloman, 2004, Amiard-Triquet, 2009, Almeida et al., 2010 and Tu et al., 2010).

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Corresponding author:

Tel.: +32 043665084; fax: +32 043665113. E-mail address: Mathieu.Denoel [a] ulg.ac.be (M. Denoël).