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Endosulfan effects on *Rana dalmatina* tadpoles: Quantitative developmental and behavioural Analysis

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Abstract

Endosulfan is an organochlorine pesticide that was recently labeled as a persistent organic pollutant, but it is still widely employed, particularly in developing countries. The goal of this study is to evaluate the acute (LC50) and chronic effects (developmental and behavioural traits) of this insecticide on *Rana dalmatina* tadpoles after exposure to ecologically relevant concentrations (0.005, 0.01, and 0.05 mg/L) by applying video-tracking techniques to evaluate the quantitative effect of endosulfan on amphibian behavioural patterns. The 96 h LC50 value was 0.074 mg endosulfan/L. Tadpoles chronically exposed to 0.01 and 0.05 mg endosulfan/L underwent high mortality rate, decreased larval growth, delayed development, and increased incidence of malformations, and they did not reach metamorphosis by the end of the experiment. Moreover, tadpoles exposed to these concentrations exhibited several abnormalities in swimming patterns, such as shorter distance moved, swirling, resting, and unusual use of space. The exposure to 0.005 mg endosulfan/L did not cause any significant effects on behaviour, larval growth, or development, but we observed a significant decrease in both survival and time to metamorphosis. We showed that developmental abnormalities are dose-dependent and that the pesticide effects could differ depending on the endosulfan concentration and the species tested. We also validated the hypothesis that behavioural analysis, along with the use of new analytical methods, could be a useful tool in amphibian ecotoxicological studies.

Keywords Temperature · Environment · Phenotypic plasticity · Courtship behavior · Newt

The intensive use of agricultural pesticides led to the widespread presence of these compounds in all of the compartments of the environment; such contamination may produce harmful or toxic effects in freshwater communities and is one of the most important pressures on aquatic ecological systems (Rabiet et al. 2010; Relyea and Hoverman 2006). Amphibians are highly sensitive to the action of pollutants due to their physiology and life habits; for these reasons, they are considered good bioindicators of environmental quality and have been successfully used to study the impact of chemicals on aquatic and agricultural ecosystems (Pollet and Bendell-Young 2000; Venturino et al. 2003).

In recent decades, decreased richness and abundance of amphibian species has become a global phenomenon, and agricultural contaminants, such as pesticides, are thought to contribute to this decline (Houlahan et al. 2000; Houlahan and Findlay 2003; Mann et al. 2009). Agrochemicals can seriously affect amphibian populations by decreasing survival, impairing growth and larval development, and causing morphological and behavioural alterations (Bernabò et al. 2008, 2011a, b; Brunelli et al. 2009; Peltzer et al. 2008; Taylor et al. 2005).

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-ethano-2,4,3-benzodioxathiepin-3-oxide) is an organochlorine pesticide that was recently labeled as a persistent organic pollutant and therefore banned, except for specific uses, particularly in developing countries (United Nations 2011; United States Environmental Protection Agency (USEPA) 2010). It is also found in surface waters worldwide at levels ranging from 0.01 to ≤1.7 mg/L (Carriger and Rand 2008; Dalvie et al. 2003; Ernst et al. 1991; Srivastava et al. 2009). Endosulfan may affect nontarget organisms, such as fish (Ballesteros et al. 2009; Beyger et al. 2012; Ezemonye et al. 2009; Stanley et al. 2009) and amphibian species (Berrill et al. 1998; Broomhall 2002, 2004; Broomhall and Shine 2003; Ezemonye and Tongo 2010a, b; Jones et al. 2009; Kang et al. 2008). Recently, we showed that both acute and chronic exposure to endosulfan may seriously affect sur-

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vival, development, and gill morphology as well as cause severe deformities and behavioural alterations in *Bufo bufo* tadpoles (Bernabò et al. 2008; Brunelli et al. 2009, 2010). In addition, a recent behavioural analysis of *R. temporaria* showed that multiple behavioural end points are affected by endosulfan at environmentally realistic concentrations (Denoël et al. 2012). Some differences were also outlined in the timing of effects depending on the behaviour analysed. This and other previous studies highlighted that changes in behavioural patterns could be used as biomarkers to detect the effects of pollutants, such as pesticides (Broomhall 2005; Brunelli et al. 2009; Denoël et al. 2012). Moreover, new analytical tools have been standardized for evaluating behavioural effects of pesticides on model taxa, such as fishes (Eddins et al. 2010; Kavitha and Venkateswara Rao 2008; Rao et al. 2005), but they have not been used as extensively in amphibians. For instance, only one study applied video-tracking techniques to analyze behaviour after tadpole exposure to chemicals (Denoël et al. 2010). Video-tracking methods provide a detailed quantitative analysis of behavioural patterns and could be successfully used to analyse behavioural patterns that the human observer is unable to accurately estimate (Noldus et al. 2001; Winandy and Denoël 2011).

Based on these previous works, we tested the hypothesis that long-term exposure (i.e., up to metamorphosis in control treatments) to environmentally realistic concentrations of endosulfan would be detrimental on several markers, i.e., growth, development, and locomotory patterns. More generally, we aimed at showing the usefulness of (1) behavioural patterns as a powerful tool to detect detrimental effects in amphibians and (2) video-tracking systems as tools to quantitatively determine such effects.

In our study, the evaluation of endosulfan toxicity was performed by evaluating both acute toxicity (LC50) and chronic effects (behaviour, growth, development, metamorphosis, and incidence of deformities) of this compound on *R. dalmatina* tadpoles, thus providing valuable information regarding possible consequences of a prolonged exposure to pesticides.

Materials and Methods

Tadpole Maintenance

Three *R. dalmatina* egg clutches were collected from a wetland pond in a natural area located near Cosenza (Calabria, Southern Italy, 39°22'42"N, 16°05'58"E, 1,000 m a.s.l.). In the laboratory, eggs were randomly assigned to 50 L aerated tap water-filled glass aquaria (60 × 35 × 30 cm). Determination of the developmental stages was performed according to Gosner (1960). Both acute and chronic tests started when tadpoles reached the first larval stage (Gosner stage 25). Throughout all experiments, the water temperature was maintained at 18 °C ± 1 °C and median pH 7.3, conductivity 679 µS, and dissolved oxygen 5–7 mg/L. The experiment was conducted in 12:12 h light-to-dark cycles. Water-quality parameters (pH, conductivity, temperature, and dissolved oxygen) were recorded before and after renewal of the test solutions.

Acute Exposure

To assess the sensitivity of the species, we identified lethal concentrations of endosulfan by estimating the LC50 value (i.e., the concentration at which 50 % of tadpoles die) at 96 h of exposure. Endosulfan (purity 99 %; Chem Service, West Chester, PA, USA) was dissolved in dechlorinated tap water to obtain the following nominal concentrations: 0.050, 0.060, 0.070, 0.080, 0.090, 0.10, 0.20, and 0.30 mg/L. For each experimental unit, 10 tadpoles of similar size were randomly transferred to 15 L glass tanks (39 × 24 × 19 cm). The control group was maintained in tap water. During the experimental period, the presence of mortality was monitored daily, and dead animals removed. A static exposure system was used in accordance with standard procedure guidelines [American Society for Testing and Materials (ASTM) 1997]. During the 96 h exposure, the animals were not fed. Three replicates were used for each treatment and the control.

Chronic-Exposure Conditions

The exposure period was 56 days, i.e., from Gosner stages 25 to 46. The end of the experiment was determined when all tadpoles from both the control and the low-concentration groups completed metamorphosis (complete tail resorption). During this time, animals in the high- and medium-concentration groups did not reach metamorphosis.

The three concentrations of endosulfan used for chronic exposure trials were chosen to encompass both our 96 h LC50 values as well as previous field (Ernst et al. 1991) and laboratory data (Brunelli et al. 2009; Jones et al. 2009; Denoël et al. 2012). Chronic exposure was performed by dissolving endosulfan in tap water to obtain three nominal concentrations: 0.005, 0.01, and 0.05 mg/L, hereafter referred to as "low-," "medium-," and "high"-concentration group, respectively. The control group was maintained in tap water. A static-renewal exposure system was used according to standard procedure guidelines (ASTM 1997) with complete renewal of the water volume every 3 days. For each concentration, including the control, two replicate tanks were used. Thirty tadpoles of comparable body dimension were randomly assigned to 30 L glass tanks (78 × 24 × 19 cm) containing treatment solution. Tadpoles were fed boiled organic spinach *ad libitum* three times a week throughout the exposure period until the start of metamorphosis (Gosner stage 41). At Gosner stage 42 (forelimb emergence), tadpoles were removed from exposure tanks and transferred in 5 L plastic tanks (30 × 15 × 18 cm) (containing both treatment solution and dry areas) until the final stage of complete tail resorption. During metamorphic climax, tadpoles were not fed because metamorphosing tadpoles live off of fat stored in their tails (Hourdry et al. 1996).

Mortality, Growth, Development, and Metamorphosis

Developmental stage was determined weekly on a subsample of six randomly selected tadpoles per tank under a stereomicroscope (Leica MZ APO, Leica Microsystems, Wetzlar, Germany). Initial body weight (BW) and the snout-vent length (SVL) were recorded at the beginning of the experiment and then weekly as an index of growth. Each tadpole was towel-dried and weighed to the nearest milligram, then put on a sheet of waxed millimeter graph paper to measure SVL (i.e., total tadpole length minus tail length). Mortality and the presence of deformity were recorded daily throughout the tests, and dead animals were removed. Survival to metamorphosis (Gosner stage 46) as well as mass and time to completion of metamorphosis (from the first day of exposure) were recorded for each individual.

Behaviour

Behavioural observations were conducted to analyze swimming activity of *R. dalmatina* tadpoles after 7, 14, 21, and 28 days of endosulfan exposure. For observations, eight tadpoles from the control and each treatment group were randomly selected and transferred individually to circular observation chambers (diameter 8 cm) filled with tap water or the respective endosulfan concentrations. After 30 min to acclimatise, tadpole movement was recorded using a videocamera (Sony DCR-DVD92E) placed perpendicular to the focal plane for 15 min. The recorded video were transferred to a computer and automatically processed by video-tracking software Ethovision XT 7 (Noldus Information Technology, Wageningen, The Netherlands). Video tracking software transforms tadpoles into pixels in digitalized arenas (i.e., observation chambers) and then gives the positions of tadpoles at defined times (every 0.2 s in our set-up) (Denoël et al. 2010). The arenas were calibrated by a diameter of 8 cm corresponding to the diameter of the observation chambers. The grey-scaling method was used to detect tadpoles in the arenas. This method defines the animal as all connecting pixels that are darker than the background (Noldus et al. 2002). To evaluate the swimming activity of *R. dalmatina* tadpoles, we analysed the following behavioural patterns: total distance moved (cm), immobility (s), and use of space (cm) as defined by distance to zone, i.e., to the border of the tank. In our set-up, the immobile threshold was 20 %, and below this value tadpoles were considered immobile. Tadpole tracks (i.e., line of connections between individual positions) were obtained for all observations at the end of the recording. Visual observations were conducted daily to investigate the qualitative effects of endosulfan on *R. dalmatina* tadpoles (swirling behavior).

Statistical Analysis

The LC50 value was determined according to Finney's Probit Analysis LC50 Determination Method (Finney 1971) using software developed by the USEPA (LC50 Software Program Version 1.00, CEAM Distribution Center, Washington, DC; 1999). Behavioural data were analysed by Statistica 8 (StatSoft, Tulsa, OK, USA). Other data were determined using Graph Pad Prism 5.00 (GraphPad, San Diego, CA, USA). For all data, a significance level of 0.05 was used. Data from the two replicates were statistically compared for all end points using Mann–Whitney test. Because no significant differences were apparent (all $p > 0.05$), data were pooled into one data set per exposure group for further analyses. All data were tested for normality and equality of variance assumptions using Kolmogorov–Smirnov and Levene's tests, respectively. If assumptions of normality and homogeneity of variance were met, one-way analysis of variance (ANOVA) was performed; otherwise, data were analysed using nonparametric tests. $\chi 2$ test was used to compare mortality in the endosulfan-exposure groups with control groups (number of living tadpoles vs. number of dead tadpoles) until Gosner stage 42. Subsequently, we used $\chi 2$ test to identify differences in the number of metamorphosed individuals (Gosner stage 46) between the control and the low-concentration groups (i.e., number of tadpoles that reached metamorphosis vs. the number that did not). The effects of endosulfan on BW, SVL, and developmental stage were analysed by Kruskal–Wallis test, which was performed for all testing periods (every 7 days) followed by Dunn's multiple comparison post test to compare endosulfan exposure groups with the control. Mann–Whitney test was used to calculate time to completion of metamorphosis (in days) in the low-concentration and control groups. Behavioural data were normalized and analysed using ANOVA followed by unequal N honestly significant (HSD) post hoc test to assess differences between endosulfan exposure groups and the control.

Results

Acute Exposure

The nominal 96 h LC50 value for endosulfan in *R. dalmatina* tadpoles was 0.074 mg/L. Table 1 lists the relation between endosulfan concentration and mortality rate. In all groups, the mortality rate was <50 % during the first 24 h period. Only one tadpole died in the control group, and no morphological changes were observed in the control for the 96 h acute toxicity test. Estimated LC50 values and 95 % confidence limits for the 96 h endosulfan exposure are listed in Table 2.

Mortality, Growth, Development, and Metamorphosis

Endosulfan produced significantly higher mortality in the high-concentration group after 21 days of exposure [$\chi 2 = 9.317$, 1° of freedom (df), $p < 0.01$] and until the end of experiment (all $p < 0.001$) than occurred in the control (Fig. 1). At the end of the experiment, all contaminated tadpoles at the high concentration died. In the medium- concentration group, significant mortality occurred starting from day 42 ($\chi 2 = 6.667$, 1 df, $p < 0.05$) until the end of experiment (all $p < 0.001$) compared with the control. A significant difference in survival between the control and low-concentration groups was observed after day 42 of exposure ($\chi 2 = 6.667$, 1 df, $p < 0.05$) (Fig. 1).From day 7 of exposure until the end of experiment, endosulfan caused significant effects on BW and SVL in both the medium- and high-concentration groups compared with the control ($p < 0.001$) (Fig. 2a, b). At the low concentration, a lower BW and SVL than the control were observed at just day 7 of exposure ($p < 0.05$), but no significant differences were detected afterward (all $p > 0.052$; Fig. 2a, b).

In the medium- and the high-concentration groups, development was significantly delayed (p

Table 1 Relation between several concentrations of endosulfan and mortality rate of *R. dalmatina*

Concentration (mg/L)	No. exposed	No. of dead tadpoles	Death in the bioassay	Expected death	Estimated death
0.000	30	1	0.0333	0.0000	0.0312
0.050	30	8	0.2667	0.2431	0.3299
0.060	30	13	0.4333	0.4151	0.4007
0.070	30	15	0.5000	0.4839	0.0477
0.080	30	17	0.5667	0.5527	0.5369
0.090	30	18	0.6000	0.5871	0.5894
0.10	30	22	0.7333	0.7248	0.6351
0.20	30	27	0.9000	0.8968	0.8710
0.30	30	27	0.9000	0.8968	0.9442

Table 2 Estimated LC values and CIs

Point	Concentration (mg/L)	95 % CIs	
		Lower	Upper
LC 1.00	0.009	0.003	0.017
LC 5.00	0.017	0.007	0.027
LC 10.00	0.024	0.012	0.034
LC 15.00	0.030	0.016	0.040
LC 50.00	0.074	0.059	0.087
LC 85.00	0.184	0.145	0.279
LC 90.00	0.228	0.173	0.381
LC 95.00	0.315	0.222	0.611
LC 99.00	0.574	0.352	1.490

CI confidence interval

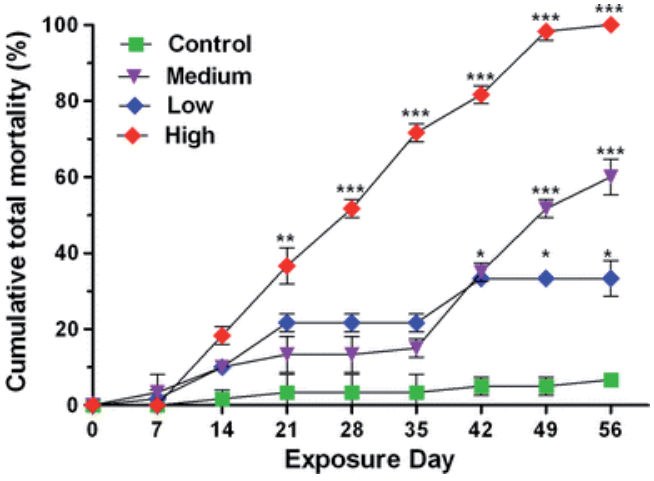

Fig. 1 Cumulative mortality (%) in *R. dalmatina* tadpoles exposed to endosulfan (0.005, 0.01, and 0.05 mg/L) from Gosner stage 25 through 46 showing mortality compared with the control (Fisher's exact p test); * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$. Bars represent mean ± SE

< 0.001); this decrease in developmental stage over time implies that no tadpoles reached metamorphosis (Fig. 3a, b). Until the beginning of metamorphosis (Gosner stage 42), development was similar in tadpoles from the low-concentration and control groups (Fig. 3a, b) with only a slightly significant difference ($p < 0.05$) at day 7 of exposure. After the beginning of metamorphosis, tadpoles in the low-concentration group took significantly less time to complete development compared with the control (Mann–Whitney U test = 68, $p < 0.001$) (Fig. 4).

The frequency of tadpoles that reached metamorphosis in the low-concentration group differed significantly from that of the control group ($\chi 2$ = 6.667, 1 df, $p < 0.01$); in fact, 67 % of tadpoles successfully completed metamorphosis, whereas in the control group metamorphosis was 94 % (Fig. 4). Conversely, no significant difference was shown in metamorphic mass between tadpoles exposed to the low concentration and those in the control group ($p > 0.05$).

The highest endosulfan concentration caused several malformations after only 5 days of exposure in some individuals, and after day 19 the incidence of

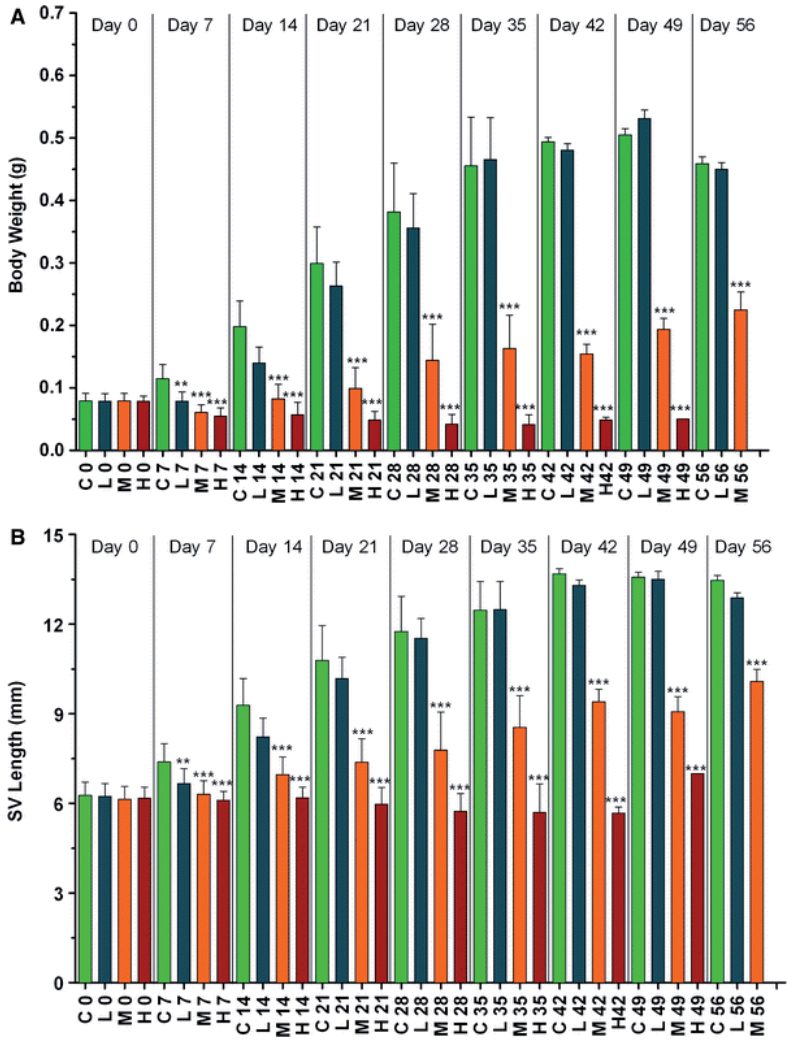

Fig. 2 BW (g) (a) and SVL (mm) (b) of R. dalmatina tadpoles during the exposure time (C = 0 mg endosulfan/L; L = 0.005 mg endosulfan/L; M = 0.01 mg endosulfan/L; H = 0.05 mg endosulfan/L) showing that the treated group differs from the control * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Kruskal–Wallis test followed by Dunn's multiple comparison test). The bars show mean ± SD

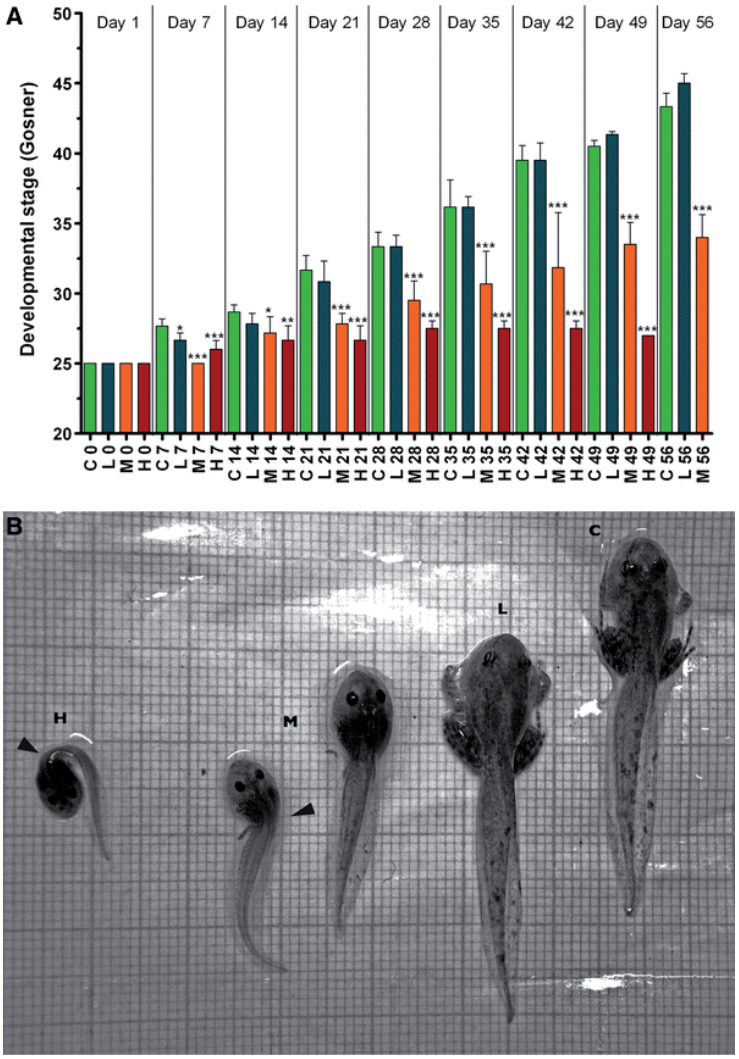

Fig. 3 a Developmental Gosner stage of *R. dalmatina* tadpoles during the exposure time (C = 0 mg endosulfan/L; L = 0.005 mg endosulfan/L; M = 0.01 mg endosulfan/L; H = 0.05 mg endosulfan/L) showing that the treated group differs from the control * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Kruskal–Wallis test followed by Dunn's multiple comparison test). The bars show mean ± SD. b Dorsal view of representative developmental stage of tadpoles exposed to endosulfan and from the control group after day 49 of exposure. Note the presence of skeletal deformity in the medium- and high-concentration groups (arrowheads)

deformity was 100 %. After 11 days of exposure, in the medium-concentration group, the appearance of deformities was also recorded, and after day 40 of exposure the incidence of deformity was 42 %. The deformities mainly observed were bloated heads and skeletal malformations (Fig. 3b). None of the tadpoles belonging to the control and low-concentration groups showed malformations.

Behaviour

Already after day 2 of exposure, some individuals exposed to both medium and high endosulfan concentrations showed convulsive irregular movements (swirling) associated with body twisting followed by resting with either the ventral body up or on the flank. After day 20 of exposure, tadpoles in the two highest concentrations showed complete immobility and swirling if forcibly moved. Behavioural analysis performed by Ethovision showed that endosulfan exposure caused significant effects on behavioural patterns.

We first analysed the distance tadpoles moved, thus showing that treatment had a significant effect on this behavioural pattern ($F_{3,28}$ = 16.859, $p < 0.0001$ at day 7; $F_{3,28}$ = 13.356, $p < 0.0001$ at day 14; $F_{3,28}$ = 20.376, $p < 0.0001$ at day 21; and $F_{3,28}$ = 15.947, $p < 0.0001$ at day 28). Post hoc models showed that the two highest concentrations had a significant negative effect on distance moved at all time points, whereas the low concentration did not affect the ability to cover distance compared with the control group. The significance was constant at all time points in the high-concentration group; however, in the medium-concentration group, it was variable during the different testing periods (Fig. 5a).

The mobility of tadpoles was also significantly affected by endosulfan exposure ($F_{3,28}$ = 11.1, $p <$

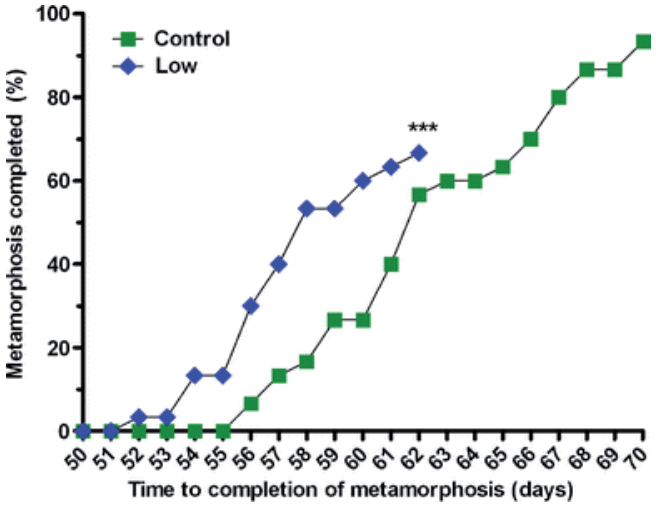

Fig. 4 Metamorphosis completed (%) and time to completion of metamorphosis (days) in *R. dalmatina* tadpoles from low and control groups. *** $p < 0.001$ (Mann–Whitney test)

0.0001 at day 7; $F_{3,28}$ = 8.7, p < 0.001 at day 14; $F_{3,28}$ = 15.4, p < 0.0001 at day 21; and $F_{3,28}$ = 6.3, p < 0.01 at day 28). In particular, tadpoles exposed to the two highest endosulfan concentrations showed significantly decreased mobility compared with the control group (Fig. 5b).

Evaluation of the use of space showed a significant effect on tadpole capacity to use space ($F_{3,28}$ = 26.393, p < 0.0001 at day 7; $F_{3,28}$ = 6.229, p < 0.01 at day 14; $F_{3,28}$ = 21.326, p < 0.0001 at day 21; and $F_{3,28}$ = 17.918, p < 0.0001 at day 28 day). Tadpoles in the medium- and high-concentration groups exhibited significant differences compared with the control group; no significant difference was detected in the low-concentration group compared with control (Fig. 5c).

Discussion

We showed that the exposure to environmentally relevant concentrations of endosulfan (ranging from 0.005 to 0.05 mg/L) may negatively affect survival, growth, development, and metamorphosis and also cause behavioural and morphological alterations, which is in agreement with previous studies on amphibians (Bernabò et al. 2008; Brunelli et al. 2009, 2010; Denoël et al. 2012; Jones et al. 2009; Kang et al. 2008, Rohr et al. 2003; Sparling and Fellers 2009).

We found that the 96 h LC50 value for *R. dalmatina* tadpoles was 0.074 mg endosulfan/L, which is comparable with data previously reported in literature. Indeed, the 96 h LC50 values range from 0.002 to 0.4 mg endosulfan/L across several anuran species; for example, 0.123 mg/L in *B. melanostictus*, 0.43 mg/L in *B. bufo*, 0.021 mg/L in *Pseudacris regilla*, 0.12 mg/L in *P. crucifer*, 0.115 mg/L in *R. temporaria*, 0.015 mg/L in *R. cascadae*, and 0.003 mg/L in *R. clamitans* (Bernabò et al. 2008; Berrill et al. 1998; Denoël et al. 2012; Gopal et al. 1981; Jones et al. 2009; Vardia et al. 1984). According to Jones et al. (2009), it seems that members of Bufonidae spp. are the least susceptible, whereas members of Hylidae spp. are intermediate in sensitivity and members of Ranidae spp. are the most susceptible. In addition to species differences in sensitivity, it has been well established that the acute toxicity of pesticides may also differ depen-

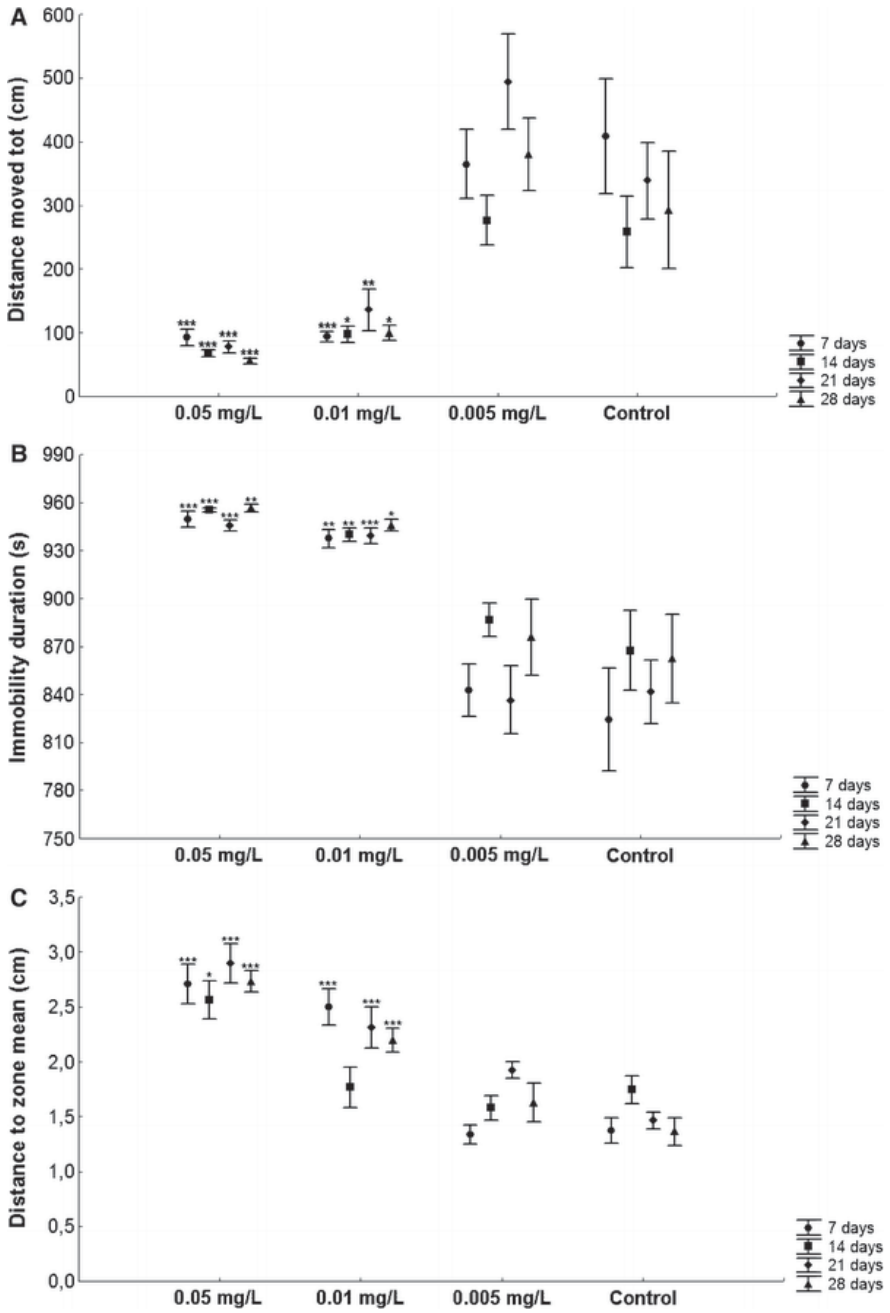

Fig. 5 Distance moved (a), immobility (b), and distance to zone, i.e., to the border of the tank (c) of *R. dalmatina* tadpoles exposed to endosulfan (0.005, 0.01, and 0.05 mg/L) and of the control group after 7, 14, 21, and 28 days of exposure. The bars show mean ± SE, and asterisks show the treated groups that differ from the control * p < 0.05; ** p < 0.01; *** p < 0.001 (post hoc test, Unequal N HSD)

ding on variations in testing protocols (Berrill et al. 1998; Bridges and Semlitsch 2000; Jones et al. 2009).

Chronic expositions of concentrations of 0.01 and 0.05 mg endosulfan/L caused harmful effects to *R. dalmatina* tadpoles. We observed a higher mortality rate, decreased larval growth, and delayed development after contamination with endosulfan. Moreover, tadpoles that were exposed at these concentrations did not reach metamorphosis by the end of the experiment. At the lowest tested concentration (0.005 mg/L), endosulfan did not affect larval growth but did slightly speed up metamorphosis time of tadpoles. In nature, tadpoles can have longer larval periods when environmental conditions are favorable to maximize size at metamorphosis, which is correlated with adult fitness traits (Semlitsch et al. 1988; Smith 1987). Instead, tadpoles living in an unfavorable, unpredictable, and ephemeral environment can accelerate development and metamorphose sooner but often have smaller size (Bridges 2000; Greulich and Pflugmacher 2003). Decreased larval growth and development rate, along with increased incidences of deformities, may negatively affect future fitness (Altwegg and Reyer 2003; Boone and Semlitsch 2002; Semlitsch et al. 1988).

Moreover, we confirmed a strong correlation between the incidence of deformities and endosulfan exposure in amphibians; in fact, an exposure to both 0.01 and 0.05 mg endosulfan/L caused severe deformities—such as skeletal malformation, asymmetric and bent tail, and bloated head—in *R. dalmatina* tadpoles. Similar malformations have been reported, in both field and laboratory studies, after exposure to endosulfan and other pesticides (Bernabò et al. 2011b; Berrill et al. 1998; Brunelli et al. 2009; Harris 2000; Kang et al. 2008; Rohr et al. 2003).

Exposure to a concentration of 0.01 mg/L endosulfan is able to disrupt developmental and behavioural patterns in *R. dalmatina* tadpoles, whereas the same concentration did not affect swimming activity and metamorphosis in *B. bufo* tadpoles (Brunelli et al. 2009). It seems that also after a chronic exposure, a species-specific difference exists among anuran species, leading us to suppose a greater ability of Bufonidae spp. to counteract pesticide effects.

We also evaluated the quantitative effects of a chronic endosulfan exposure on amphibian behaviour by using video-tracking analyses. This made it possible to demonstrate the advantages of this method compared with standard visual methods. We found that tadpoles exposed to the two highest endosulfan concentrations (0.01 and 0.05 mg/L), but not at the lowest (0.005 mg/L) concentration, exhibited several abnormalities in swimming patterns, such as shorter distance moved, resting, and unusual use of space. Motor impairments have also been visually reported in amphibians after exposure to endosulfan and other pesticides (Berrill et al. 1998; Bernabò et al. 2011b; Brunelli et al. 2009; Denoël et al. 2012). They were also reported in long-term exposure in association with the presence of deformities (Brunelli et al. 2009). In natural environments, alterations in swimming activity, such as slower and irregular swimming, resting position (ventral up or resting on the flank), could be problematic by increasing the susceptibility to predation and decreasing the time invested in feeding, which in turn has a negative effect on the larval growth-and-development rate (Horat and Semlitsch 1994; Jung and Jagoe 1995; Relyea and Hoverman 2006).

In conclusion, we have shown that (1) environmental concentrations of endosulfan are harmful to the life stage of amphibians; (2) all organismic traits studied were affected by endosulfan; (3) a dose-dependent response was found for some of the traits, thus underlying the importance, when evaluating the effects of a pesticide, to perform an analysis at multiple levels because the effects could be different depending on the concentration and the species tested; (4) the use of new analytical methods, such as video-tracking in behavioural sciences, could be an useful tool in amphibian ecotoxicological studies (see also Denoël et al. 2010).

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