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TOXICITY OF ENDOSULFAN ON EMBRYO-LARVAL DEVELOPMENT OF THE SOUTH AMERICAN TOAD, *RHINELLA ARENARUM*

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Running title: Toxicity of endosulfan on *Rhinella arenarum*

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Abstract: Endosulfan (ES) is a widely used pesticide despite its extreme toxicity to a variety of taxa and its worldwide ban. In this context, the aim of this study was to evaluate the acute and chronic toxicity of ES on the embryo-larval development of the common South American toad, *Rhinella arenarum*. The results showed that lethal and sublethal effects increased with concentration and exposure time. The sensitivity to ES increased during the larval period, with the complete operculum stage (S.25) being the most sensitive (504-h LC50=0.01 mg ES/L; LC10=0.004 mg ES/L). ES exposure caused morphological abnormalities such as general underdevelopment, edema, gill malformations and cellular dissociation, as well as neurotoxicity. Our results also showed that larvae exposed to concentrations of 0.005 and 0.01 mg ES/L completed metamorphosis earlier than controls, but with underdevelopment. The 240-h Teratogenic Index (TI) was 6.13, implying a high risk for embryos to be malformed in the absence of significant embryonic lethality. Considering that the Hazard Quotients for chronic exposure were over 1, the level of concern value, and toxicity endpoints obtained in this study for *Rhinella arenarum* occurred at concentrations lower than the levels of ES reported in the environment, this pesticide should be considered a potential risk for this species.

Keywords Developmental toxicity; Amphibians; Ecological risk assessment; Endosulfan

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INTRODUCTION

Due to the worldwide expansion of the agricultural frontier, large amounts of pesticides are increasingly polluting ecosystems. Endosulfan (ES) is a synthetic organochlorine compound used as insecticide and acaricide. The routes of exposure in wildlife are through contact (skin, hair) and ingestion, and at high temperatures, by inhalation. Its mechanism of action is the over stimulation of the central nervous system, inhibiting calcium and magnesium ATPase [1]. Technical grade ES is a mixture of two stereoisomers, alpha and beta, in a ratio of 7:3 [2]. The half-life at 14°C of alpha and beta stereoisomers in the Parana River, Santa Fe (intensive agricultural area in Argentina) is 50 and 33 days, respectively [3]. The United States Environmental Protection Agency (USEPA) has classified ES as category 1b, "highly hazardous", and is considered a Persistent Organic Pollutant (POP) because of its high toxicity on living organisms, persistence in the environment, high potential for bioaccumulation, and long distance migration capacity from its application sites. The use of ES was recently banned by the United Nations Association, following the recommendation of the Scientific Committee, but with exemptions [4], so it is still largely used at a world-wide, particularly in some developing countries. The impact of this pesticide on wildlife in Argentina is thought to be very significant, considering that 5.5 million liters of ES was applied in 2010 to control insects on soybean, alfalfa, cotton, sunflower, and corn crops [5].

Argentina has approximately 60 species of amphibians, and the toad we studied, *Rhinella arenarum*, occurs widely in the areas where ES is used. Generally, amphibians are an important taxa affected by many pesticides that are sprayed on agricultural fields [6]. The pesticides accumulate in temporary ponds and result in high concentrations during spring and summer, coinciding with amphibians breeding and highly sensitive stages of development [6]. Moreover, their skin and egg membranes are highly permeable to pollutants. Many studies have examined the toxic effects of pesticides on the most sensitive life stages of amphibians, the embryo-larval development [7-9]. It was reported that ES causes lethality in many species of amphibians with 96-h LC50 (lethal concentration) values ranged from 1.3 to 120 μg/L [10], as well as sublethal effects such as delay in the time to complete metamorphosis [11], malformations of gills [12] and neurotoxicity (hyperactivity, whip like convulsions, narcosis, paralysis) [13,14]. Amphibian populations are declining in size and distribution worldwide due to

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agrochemical contamination as well as other anthropogenic and natural changes in their ecosystem [15]. Within this context, the importance of amphibians in both aquatic and terrestrial ecosystems can have large-scale consequences through alterations of food webs [16].

It is noteworthy the levels of ES ranging from 0.1 to 100 g/L have been reported in ground and surface water in intensive agricultural areas in the Western Cape (Africa) [17] with exceptional levels of up to 500 g/L occurring after runoff events [18]. Moreover, there is abundant information confirming the presence of ES at places far away from the application site [19].

The aim of this study was to evaluate the lethal and sublethal effects of ES at embryonic and larval stages of *Rhinella arenarum*, an amphibian species with a wide distribution throughout South America, using the standardized bioassay, AMPHITOX [20]. Stage-dependent sensitivity to ES was also evaluated.

An ecological risk assessment of ES on this native species was performed using the Hazard Quotient (HQ) approach [21]. The HQ is the ratio of the expected environmental concentration (EEC) [22, 23] and the level at which no adverse effects are expected (LC 10). The EEC of a pesticide is a theoretical concentration based on a worst-case scenario for exposure of non-target aquatic and terrestrial habitats interspersed within or adjacent to proposed use areas.

MATERIALS AND METHODS

Acquisition of Rhinella arenarum embryos

To examine the potential effects of ES on the embryo-larval development of *R. arenarum,* three mating pairs of adults weighing approximately 200–250 g per animal, were acquired in a non-impacted site, Lobos (Buenos Aires province, Argentina: 35º 11´ S; 59º 05´ W). Toad care, breeding, embryo acquisition and analysis were conducted according to the methods described in the AMPHITOX protocols [20]. Briefly, AMPHITOX is a standardized test employing amphibian embryos that can be used to evaluate toxicity for acute, short-term chronic, chronic, and early life stage exposure to hazardous substances and samples. By plotting the LC10, LC50 and LC90, toxicity profile (TOP) curves from 24 h to 504 h of exposure can be obtained allowing the visualization of concentration and time exposure thresholds. By employing the early-life-stage test it is also possible to evaluate malformations. Ovulation of females was induced by means of an intraperitoneal injection of a suspension of one

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homogenized toad pituitary gland in 1 mL of AMPHITOX solution (AS) per female preserved according to Pisanó [24], plus 5000 IU human chorionic gonadotropin (hCG). The composition of AS was: NaCl 36 mg/L, KCl 0.5 mg/L, $CaCl_2$ 1 mg/L and $NaHCO_3$ 2 mg/L, prepared in distilled water. Oocytes were fertilized *in vitro* using a testicular macerate homogenate suspended in AS resulting in a spermatozoid suspension of 10%. The sperm viability was confirmed by observing the spermatozoid morphology and movements under optic microscope. Embryos were staged according to Del Conte and Sirlin [25] as follow: blastula (S.4), gastrula (S.11), neurula (S.13), muscular activity (S.18), gill circulation (S.20), opercular folds (S.23) and complete operculum (S.25). The eggs quality and fertility were inspected and considered acceptable if the fertility rate was greater than 75% and embryo survival at the neurula stage was greater than 70%. For embryos used before hatching (S.18), the jelly coat was dissolved by immersing egg ribbons in a solution of 2 % thioglycolic acid at pH 7.2 containing 1.35 mL of saturated sodium hydroxide (NaOH) solution in 100 mL AS. This step was followed by a thorough wash of the embryos. Embryos were kept in AS and maintained at 20 ± 2ºC. The AS was replaced entirely every three days and monitored weekly to ensure that the pH was at acceptable levels (7±0.5).

Toxicity bioassays

For treatments during embryonic stages, ten embryos were randomly placed in triplicate 10 cm-diameter glass Petri dishes containing 40 mL of test. For bioassays with larvae, ten early larvae (S.25) were placed in triplicate 20 cm-diameter glass Petri dishes containing 150 mL of test. The toxicity bioassays were performed under the conditions summarized in Table 1.

After 24 h pulse treatments, embryos were thoroughly washed and kept in AS until 504 h post-exposure. Organisms were maintained at 20 ± 2ºC, and a 12:12 h light:dark photoperiod. Tadpoles were fed with 3 granules of balanced fish food TetraColor®. Test solutions were entirely replaced every 48 h.

Lethal and sublethal effects were evaluated each 24 h, comparing the abnormal effects with the normal development and behaviour of controls. The sublethal effects evaluated were: developmental delay, cellular dissociation, irregular surface, persistent yolk plug, underdeveloped gills, microcephaly, wavy tail and edemas. Neurotoxicity endpoints included spasmodic contractions, alterations in swimming and narcosis. Also feeding behavior was qualitativley assessed. Additionally, we evaluated ES effect on metamorphosis. Abnormalities and

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neurotoxic effects were observed under a binocular stereoscopic microscope (Zeiss Stemi DV4), photographed and recorded with a Sony DSC-S90 digital camera, and identified according to Bantle et al [26]. Embryos with significant adverse effects and controls were fixed in formalin 4%, dehydrated in a gradient of ethanol, prepared for scanning electron microscopy (SEM) by means of the critical point drying technique [27] and observed in a Philips XL-30 operated at 10 Kw for ultrastructure evaluation.

Test solutions

Test solutions were made using technical-grade ES (PS81, Supelco) with a purity of 99%. A primarly stock solution containing 1,000 mg ES/L was made by dissolving ES in analytical grade acetone. The exposure concentrations were prepared by diluting the stock solution with AS. Acetone concentration in test solutions was always lower than 1.1% [28]. Both AS and acetone treatments, were simultaneously maintained as controls. The concentration of ES in stock solution was analyzed by HPLC-ESI-MS (negative mode), the identity of the compound was confirmed by SCAN detection and the ions m/z=405 and m/z=407 were used for quantification [29]. The solution was analyzed daily and was found to be stable over the exposure time. The error between nominal and measured concentration of the stock solution did not exceed 5%.

Data analysis

Lethal and Effective Concentrations (LC and EC) were statistically estimated by the USEPA Probit Program [30], being EC based on malformations. To examine statistical differences between the LC values obtained, a comparison was made, considering the difference statistically significant when the higher LC/lower LC ratio exceeded the critical value (95% confidence interval) established by APHA [31]. The Teratogenic Index (TI) was calculated as LC50/EC50, establishing a TI>1.5 as a high risk for embryos to be malformed in the absence of significant embryonic lethality [28]. We conducted a two-way analysis of variance (ANOVA) to evaluate the effect of ES concentration and exposure time on lethality and metamorphosis. Tukey's tests were used to compare treatment means where significant ($p < 0.05$). For this analysis was used the GraphPad Prism software version 6.03.

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Ecological risk evaluation

The Expected Environmental Concentration (EEC) for ES was based upon 10% of the maximum application rate given on manufacturers labels. The maximum application rate allowed for a commercial formulation (Endosulfan 35%, 350 g/L active ingredient [32]) is 2.5 L of the product per hectare resulting in a maximum application concentration of 4370 mg/L/ha active ingredient. The EEC was calculated assuming a water depth of 15 cm and an area of 1 m^2 [22, 23]. The Hazard Quotient (HQ) was calculated as EEC/LC10, and compared to the USEPA level of concern (LOC) [21]. The LOC is a policy tool that the Agency uses to interpret the hazard quotient and analyze the potential risk to non-target organisms and the need to consider regulatory action. The LOC value for risk is 1. If the HQ>1, harmful effects are likely due to the contaminant in question.

RESULTS

Because the two controls, AS and acetone solvent, did not differ statistically, both treatments were combined and reported as the 'control' in the rest of the manuscript.

Continuous exposure from blastula stage (S.4)

Exposure of embryos starting at the blastula for 96 h expressed acute lethal effects at concentrations ≥ 15 mg ES/L. Post 96 h, there was an increase in toxicity of ES increased that was coincident with the beginning of the larval stage (Figure 1). The toxicity profile curves based on LCs indicates a significant increase in toxicity with 96 h and 504 h LC10s of 11.55 mg ES/L and 0.007 mg ES/L, respectively. This was equivalent to 587 fold increase in toxicity.

Sublethal effects in embryos noted during the initial 24 h exposure, included cellular dissociation, irregular surface, persistent yolk plug and delayed development. As development advanced, underdeveloped gills, microcephaly, wavy tail and marked edema were observed (Fig. 2). Moreover, they exhibited neurotoxicity such as spasmodic contractions. As exposure increased, neurotoxic effects included erratic swimming and loss of balance. These effects over time evolved into general weakness up to total absence of spontaneous movement, or even after light/mechanical stimulus. The NOEC 240 h for sublethal effects was 0.01 mg ES/L and the TI for ES at 240 h was 6.13.

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Continuous exposure from complete operculum stage (S.25)

Early *R. arenarum* larvae treated with concentrations greater than 1 mg ES/L showed lethal effects after 48 h exposure, reaching 100% lethality at the end of the acute period. TOP curves show that this developmental stage was more sensitive to ES than blastula stage. Moreover, while 96-h LC10 was 0.45 mg ES/L, it was only 0.004 mg ES/L at 504 h, evidencing a toxicity increase of more than 100 times (Fig. 3).

Sublethal effects were observed from 0.01 mg ES/L onwards and consisted in delayed development and wavy tail, but also neurotoxicity as erratic swimming, spasmodic contractions, and fewer movements up to narcosis. Furthermore, essential functions such as feeding were deeply affected by the pesticide, observing that the 3 granules of food were intact after 24 hours, while in plates of control larvae, the feces of the granules eaten were present. These effects were concentration-dependent.

Of the larvae exposed to 0.005 mg ES/L, 83% of the larvae survived when they began the metamorphosis process (not significantly different from the control group, $p > 0.05$), whereas only 46% completed the process (Fig. 4). Before death, larvae exhibited general underdevelopment and lethargic behaviour and starvation. In the case of larvae exposed to 0.01 mg ES/L, only 16% died before the metamorphosis and 30% completed it. Interestingly, larvae exposed to 0.005 and 0.01 mg ES/L began the metamorphosis earlier than control group. However, these recently metamorphosed toads showed general underdevelopment compared with controls.

24 h-pulse exposure at different developmental stages

Figure 5 shows the differential sensitivity of embryos and larvae to ES. Sensitivity of embryos was lower in early embryonic stages but increased in late stages, particularly from larval stage (i.e. 336-h LC10 value for S.4 and S.25 were 3.75 and 0.3 mg ES/L respectively). Although there was no acute and short-term chronic lethality was observed during exposure at S.4, reaching larval stage it significantly increased up to 5 times in 10 days (240-h LC50=16.05 mg ES/L, 504-h LC50=3.62 mg ES/L). There was no significant lethality ($p > 0.05$) for embryos exposed from early stages (S.4-S.18), even at 20 mg ES/L for acute and short-term chronic exposures, so LC50 could not be obtained. The most sensitive stage was S.25 with the sensitivity to ES remaining relatively constant from 96 h until 504 h (96-h LC50=0.62 mg ES/L; 504-h LC50=0.53 mg ES/L).

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The main sublethal effects observed at 336h post exposure on larvae at all concentrations were edema, wavy tail, lateral and dorsal axis incurvation, underdeveloped opercular folds, underdeveloped gills, persistence of the oral adhesive apparatus, different degrees of cellular dissociation, persistence of cilliar cells, and neurotoxicity as spasmodic contractions and erratic swimming, up to absence of movements. Figure 6 shows the ultrastructure of malformed larvae caused by ES 24 h-pulse exposure during muscular activity stage (S.18).

Ecological risk evaluation

A risk evaluation analysis for continuous exposures to ES that were initiated at the blastula and complete operculum stages was performed. The Estimated Environmental Concentration (EEC) for ES was calculated as 10% of the maximum application rate allowed (4370 mg AI/L/ha), so the EEC was 0.0437 mg ES/L/m^2. Using this value, the hazard quotient (HQ=EEC/LC10) for both treatments were estimated (Table 2). The results for both stages highlight that HQ values for acute and short-term chronic exposure periods were below the LOC value, while for chronic exposure the HQ were above the LOC value.

DISCUSSION

The results obtained in this work demonstrate lethal and sublethal effects produced by ES on *Rhinella arenarum* embryo-larval development. This study also revealed that larvae were almost 26 and 3 times more sensitive than embryos at acute and short-term chronic exposures to ES respectively. Embryos and larvae exposed to ES showed differences in their toxicity pattern in that the larvae were highly sensitive to the pesticide from the beginning of the exposure while embryos sensitivity increased coinciding with neuromuscular development, the main target organ of this pesticide. Moreover, the 24 h-pulse exposures confirmed this tendency in that S.25 was the most sensitive stage and embryos from early stages (S.4-S.18) were more resistant even at higher ES concentrations. Although the 24 h-pulse concentrations were high relative to those used in the continuous exposures, the pulsed experimental design may simulates environmental conditions such as overspray and accidental spills that result in high but transient concentrations. This information is useful to evaluate the differential sensitivity throughout the development of a species. The general pattern of the stage-dependent sensitivity obtained in this study is on line with that reported by Harris et al [33] where *Rana pipiens* larvae at metamorphic stage could not survive ES concentrations that were sublethal for embryos.

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Toxicity values of ES for *R. arenarum* obtained in this study are in the range of those determined in studies with other amphibian species. Considering acute lethality, 96-h LC50 for *Bufo bufo* was 0.43 mg ES/L [12], while it was 0.015 mg ES/L and 0.13 mg ES/L for *Rana clamitans* [33] and *Hypsiboas pulchellus* [13] respectively. In reference to sublethal effects, Broomhall and Shine [34] observed developmental delay in *Litoria freycineti* tadpoles exposed to 0.03 or 1.3 μg ES/L. Exposure of *Bufo Bufo* tadpoles to 0.05 and 0.1 mg ES/L resulted in increased incidences of mouth and skeletal malformations, reduced body weight [11] while alterations in the ultrastructure and cell composition of gills, indicating impaired gas exchange and osmoregulation in the gills were observed in tadpoles exposed to 0.2 mg ES/L [12]. The teratogenic potential of this pesticide represented by a 240-h TI of 6.13 is 4 times the threshold level to be considered a high risk for embryos to be malformed in the absence of significant lethality [28]. Furthermore, we confirm and extend results of previous studies on neurotoxicity, as lack of correct equilibrium and posture lying on the lateral side, swirling, non-feeding behavior and extensive paralysis [35, 14]. The neurotoxicity effect could be associated with the increased synaptic concentrations of several neurotransmitters, including the decreased acetylcholinesterase activity as observed in wild frogs exposed to ES [36], as well as the neuronal degeneration in cerebral targets, such as the mesenencephalon and hypothalamus reported in ES exposed fish [37]. Behavioral markers have relevant potential as early warning systems when other toxicity parameters such as mortality are absent.

It is well known that thyroid hormones regulate the metamorphosis process in amphibians [38]. Adverse scenarios such as pesticide exposures can induce the activity of these hormones [39], accelerating larval development and completing metamorphosis earlier, a phenomenon characteristic of amphibian larvae called phenotypic plasticity [40]. However, these larvae have reduced weight and length, decreasing their terrestrial fitness [41]. The results obtained in this study on metamorphosis are consistent with this hypothesis, showing that exposed larvae at concentrations as low as 5 g ES/L completed the metamorphosis earlier with general underdevelopment. These developmental disorders can make them more vulnerable to predation or other environmental stressors such as infectious agents, invasive species and changes in physical and chemical parameters of the environment, influencing the physical condition of the animals or their reproductive success [42].

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Comparing the ES environmental levels reported between 0.1-100 g/L [17], reaching exceptional levels of 500 g/L [18] to the EEC obtained in this study of 43.7 g/L, we can affirm the realistic prediction of this parameter. Also, taking into account the 504-h LC10 as low as 4 g ES/L determined in this study, the measured and predicted ES concentrations exceed the levels that allow the survival of *R. arenarum* larvae. Based on this risk assessment scenario, HQs could be greater than ten times the level of concern, highlighting that ES represents a threat for *R. arenarum* embryo-larval development.

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Figure 1. Toxicity Profile (TOP curves) of *Rhinella arenarum* continuously exposed to ES starting at the blastula stage (S.4). Error bars indicate 95% confidence interval.

Figure 2. Stereoscopic microscopy pictures of *Rhinella arenarum* larvae continuously exposed to ES starting at the blastula stage (S.4), at 240 h. All the embryos exposed exhibited general underdevelopment and marked edema, and from 0.3 mg ES/L exhibited wavy tail.

Figure 3. Toxicity Profile (TOP curves) of *Rhinella arenarum* continuously exposed to ES starting at the complete operculum stage (S.25).

Figure 4. Cumulative number of *Rhinella arenarum* individuals completing metamorphosis (complete tail resorption) over time, for control and larvae exposed to ES starting at the complete operculum stage (S.25).

Figure 5. The LC50 and its corresponding confidence limit (95%) in ES 24 h-pulse exposure in *Rhinella arenarum* embryos at different developmental stages.

Figure 6. Scanning electron microscopy pictures of *Rhinella arenarum* larvae exposed to 24 h-pulse to different ES concentrations, during muscular activity stage (S.18) fixed at 96 h: lateral and dorsal axis incurvation from 10 mg ES/L, Magnification (M): 21x **(a)**; underdeveloped opercular folds and underdeveloped gills from 5 mg ES/L, malformed, thicker and shorter gill filaments than control group, and increasing the effect with concentration, M:68x and 200x **(b and c)**; persistence of the oral adhesive apparatus at 5 and 10 mg ES/L, M: 200x **(d)**; axis incurvation and thicker fin at 15 and 20 mg ES/L, M:45x **(e)**; different degrees of cell surface dissociation, persistence of cilliar cells in specific regions of the epithelium and general disorganization of the epithelium, M:375x **(f)**.

Table 1. Conditions of the bioassays

Developmental stage	Treatment	Exposure duration (h)	Bioassay duration (h)	Exposure concentrations (mg ES/L)
Blastula (S.4)	Continuous	504	504	0.01, 0.05, 0.1, 0.3, 0.5, 0.75, 1, 2.5, 5, 10, 15, 20
Complete operculum (S.25)	Continuous	2568 (107days)	2568 (107 days)	0.005, 0.01, 0.05, 0.1, 0.5, 1
Blastula (S.4) **Gastrula (S.11)** **Neurula (S.13)** **Muscular activity (S.18)** **Gill circulation (S.20)** **Opercular fold (S.23)** **Complete operculum (S.25)**	24 h-pulse	24	504	0.5, 1, 2.5, 5, 10, 15, 20

Table 2. Toxicity and hazard quotient (HQ) of ES for *Rhinella arenarum* at blastula and complete operculum stages.

	Exposure Time (h)							
	96		168		336		504	
Stage	**LC10 (mg ES/L)**	**HQ**	**LC10 (mg ES/L)**	**HQ**	**LC10 (mg ES/L)**	**HQ**	**LC10 (mg ES/L)**	**HQ**
Blastula	11.55	0.004	0.62	0.07	0.025	1.75*	0.007	6.24*
Complete operculum	0.45	0.097	0.22	0.20	0.04	1.09*	0.004	10.93*

LC10 (mg ES/L) and hazard quotient (HQ) values for acute (96 h), short-term chronic (168 h), and chronic (336, 504 h) exposures. *HQ>1, estimates harmful effects due to ES exposure.

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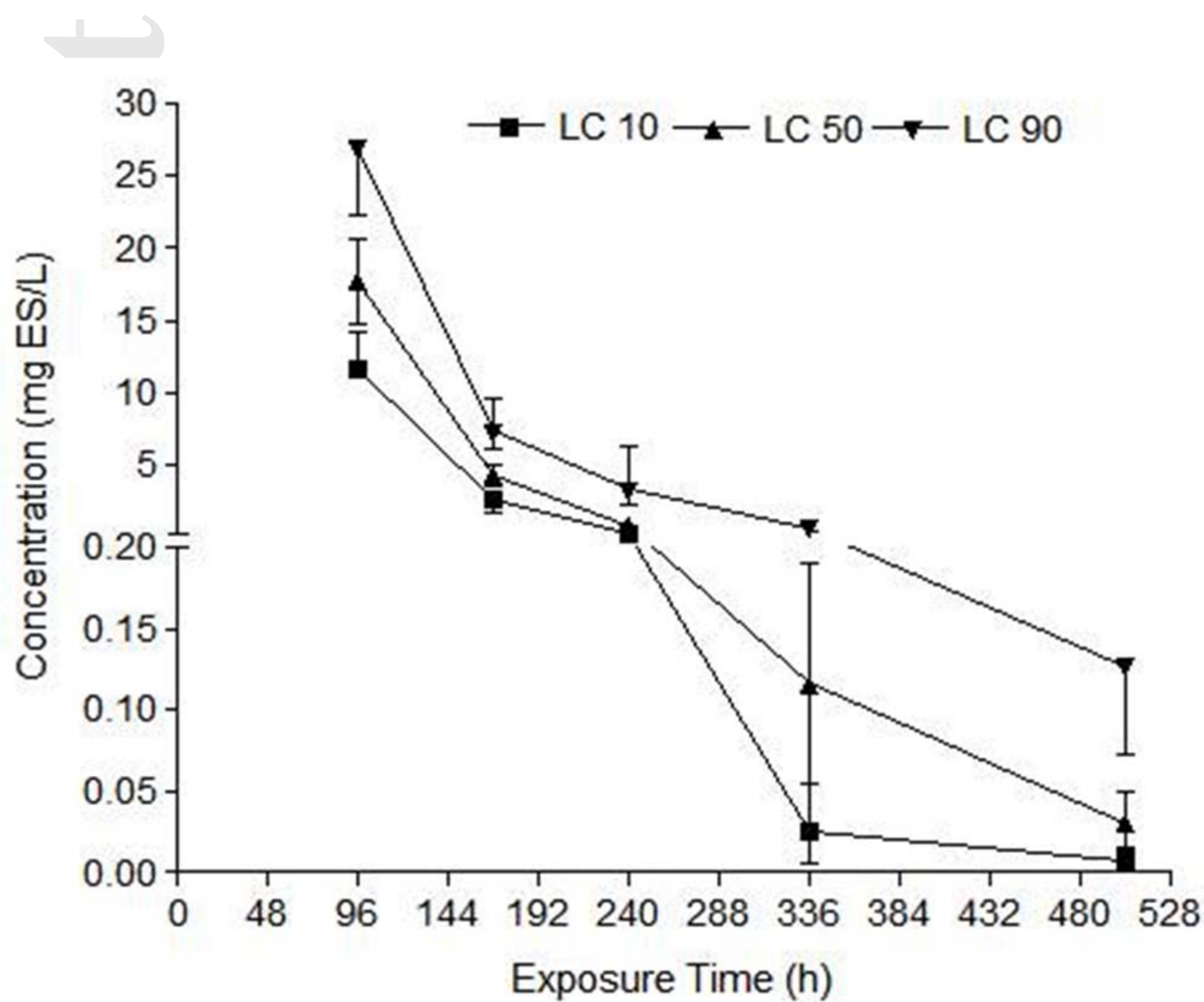

Figure 1

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Figure 2

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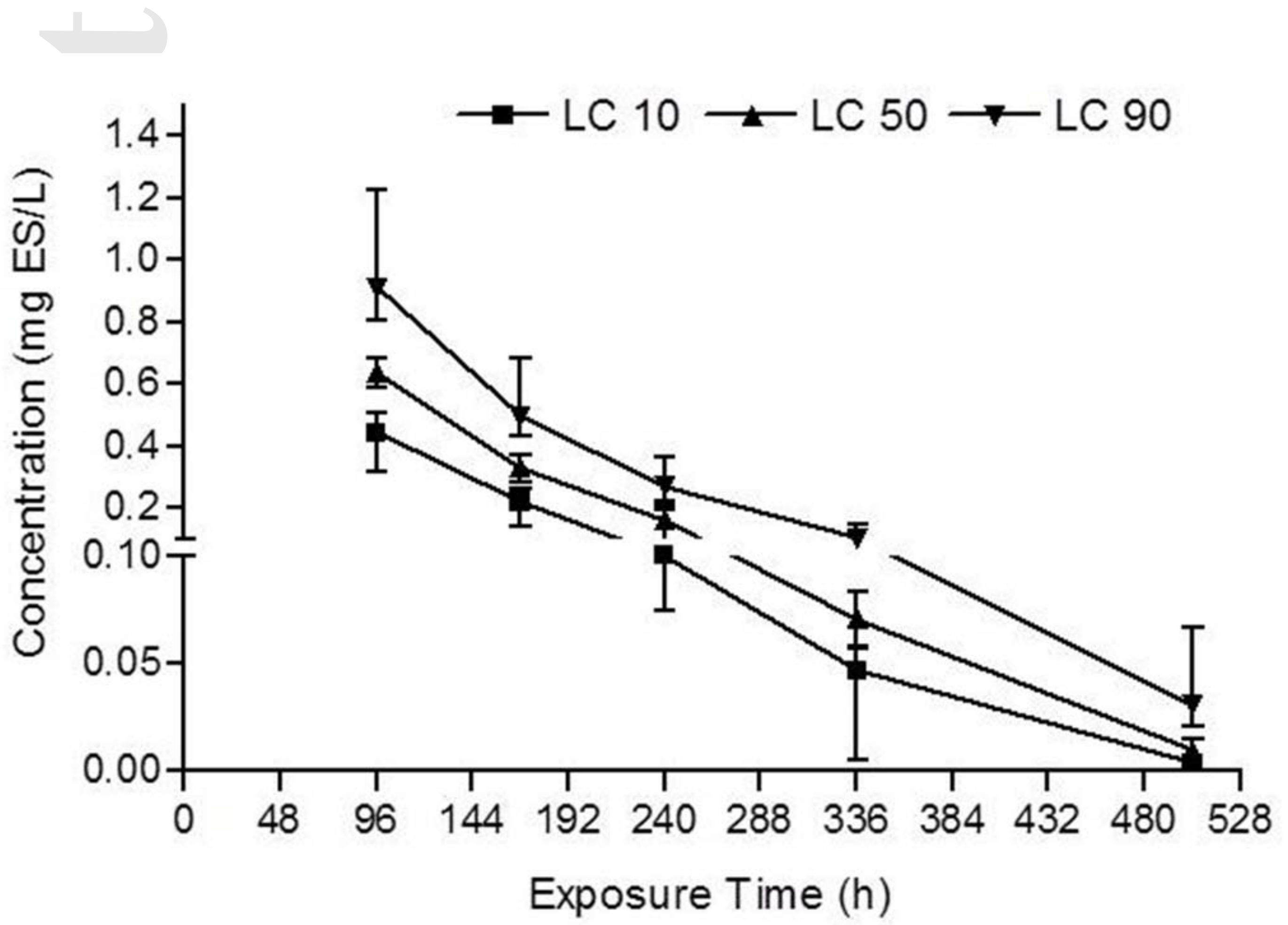

Figure 3

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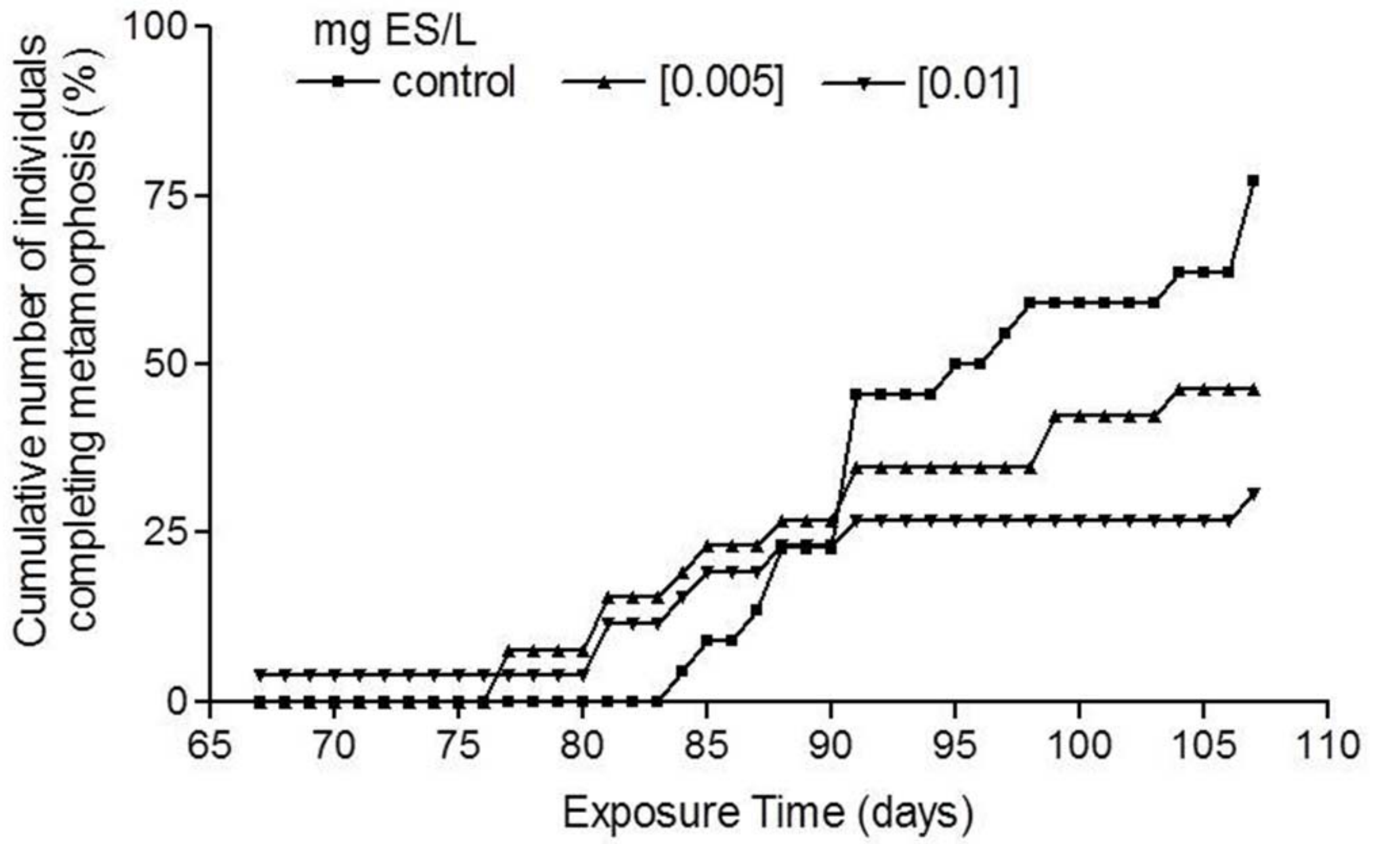

Figure 4

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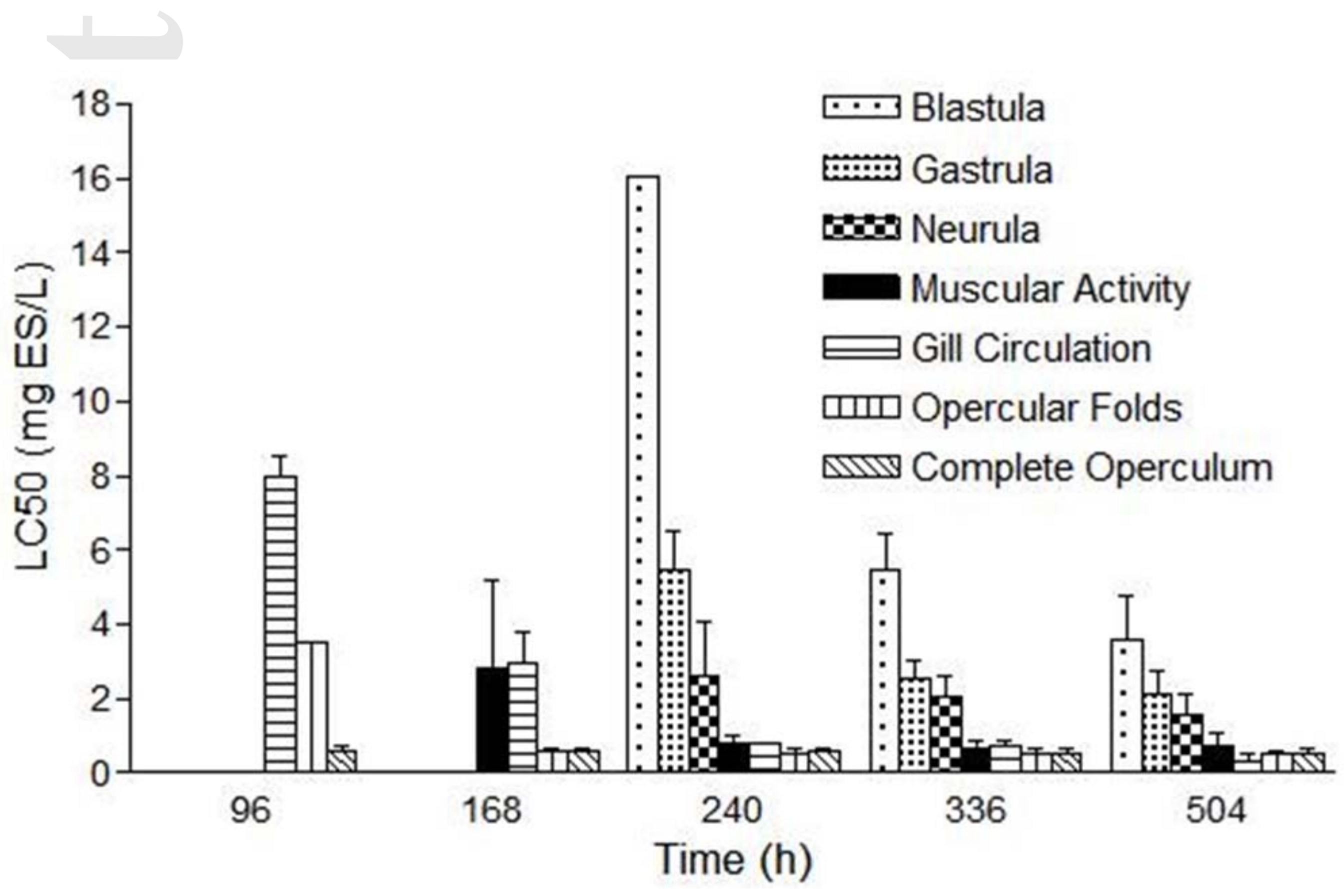

Figure 5

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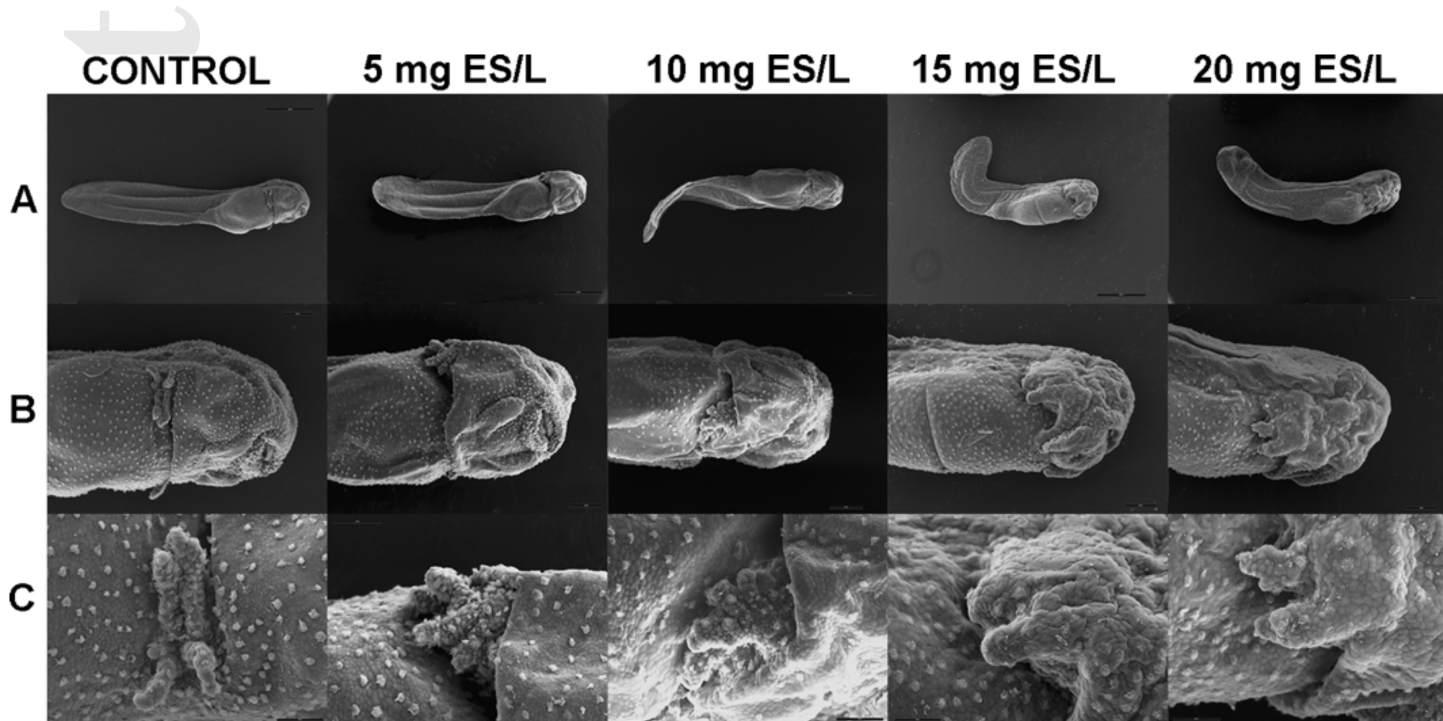

Figure 6a

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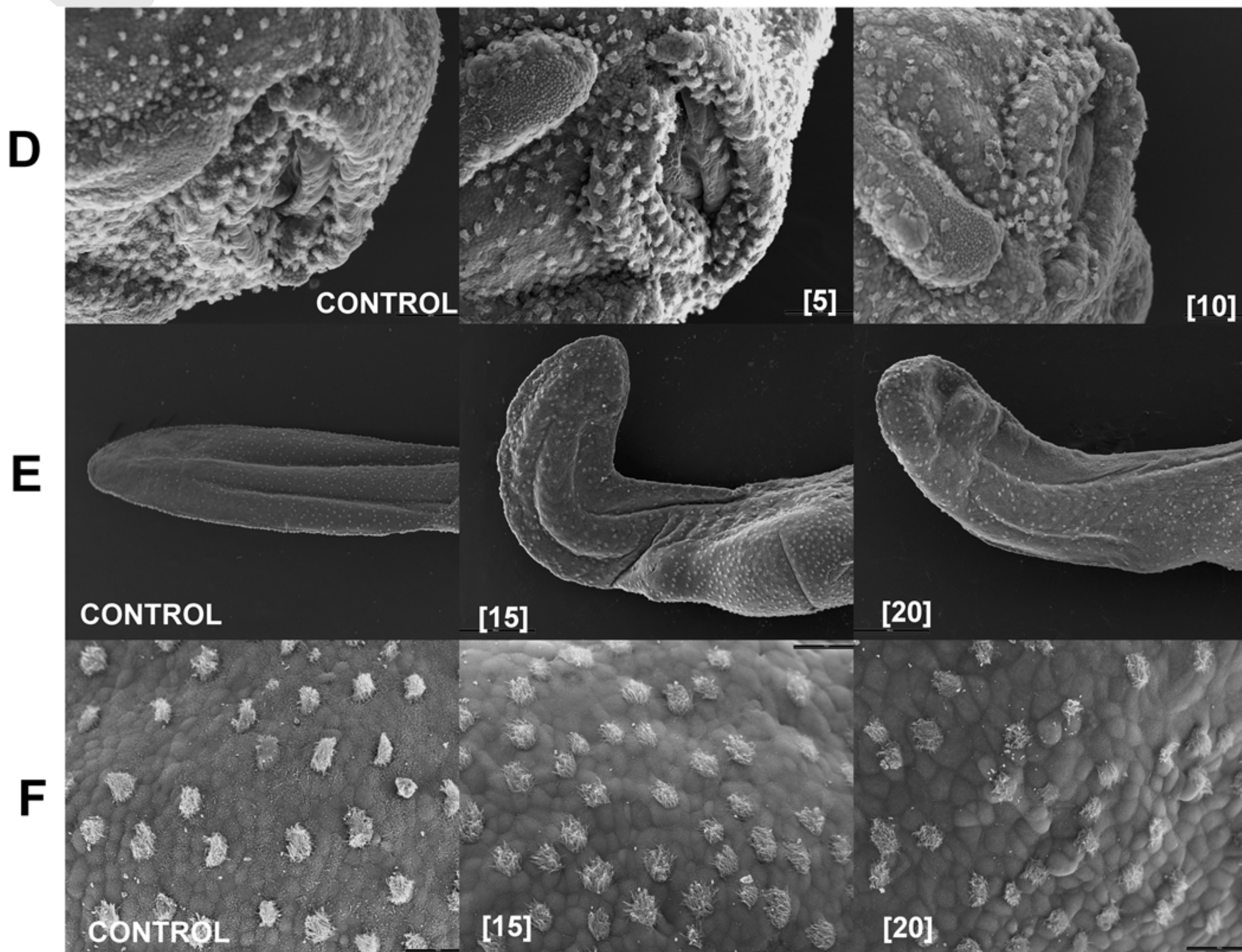

Figure 6b