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Cytogenetic and developmental toxicity of bisphenol A and bisphenol S in *Arbacia lixula* sea urchins embryos

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Abstract

Bisphenol S (BP-S) is one of the most important substitutes of bisphenol A (BP-A), and its environmental occurrence is predicted to intensify in the future. Both BP-A and BP-S were tested for adverse effects on early life stages of *Arbacia lixula* sea urchins at 0.1 up to 100 μM test concentrations, by evaluating developmental and cytogenetic toxicity endpoints. Embryonic malformations and/or mortality were scored to determine embryotoxicity (72 h post-fertilization, p-f). Cytogenetic toxicity was measured as mitotic activity endpoints and chromosome aberrations score in embryos (6 h p-f).

Both BP-A and BP-S exposures induced embryotoxic effects from 2.5 to 100 μM test concentrations as compared to controls. Malformed embryo percentages following BP-A exposure were significantly higher than in BP-S-exposed embryos from 0.25 μM to 100 μM (with a ~5-fold difference). BP-A, not BP-S exhibited cytogenetic anomalies at 25 and 100 μM. Our results indicate an embryotoxic potential of bisphenols during critical periods of development with a potent rank order to BP-A vs. BP-S.

Introduction

Bisphenol-A (BP-A) is an industrial chemical that has been used extensively to produce certain plastics and resins (Corrales et al. 2015). Current literature has raised concern about BP-A's implications in several human chronic diseases (Rezg et al. 2014) and/or ecotoxicological complications (Corrales et al. 2015). These toxicologic impacts prompted different authorities to interdict this plasticizer from different industrial applications (Trasande 2014). Several countries have substituted the parental analogue with bisphenol S (BP-S) under the "BP-A-free" label to indicate the safety of new products and reassure the consumer (Fig. 1). However, the recent literature raised some doubts about the safety of "BPA-free" plastic products and raised concern about their possible physiological disruptor properties and/or ecotoxicological effects (Mornagui et al. 2019; Qiu et al. 2019; Rezg et al. 2018; 2019; Wu et al. 2018; Wan et al. 2018; Zhou et al. 2019). BP-S is used in consumer products present in daily life such as food containers, canned foods, personal care products, paper products, manufactured plastics, and in other industrial applications (Liao and Kannan 2012; 2014). Although the impact of microplastics and BP-A on marine wildlife is reported (Shahul Hamid et al. 2018; Xu et al. 2020), the adverse effects of BP-A alternatives as emergent pollutants are less well understood.

Bisphenols pass in aquatic environments through effluents discharged from wastewater treatment, as well as directly from manufacturing industries, leachate discharges, and degradation of plastic litter (Corrales et al. 2015; Ying et al. 2009). Recently, BP-A and BP-S were detected as the predominant molecules in effluents of wastewater treatment plants in the US (Xue and Kannan, 2019). Furthermore, BP-S has been detected in aquatic organisms and surface water samples from major rivers in many countries reaching, e.g., 7.2 μg/L in Adar, India (Yamazaki et al. 2015). As the usage of BP-A is predicted to decline further, environmental emissions of BP-S are likely to intensify in the future (Liu et al. 2021; Yu et al. 2015).

Sea urchins are an ecologically relevant animal group, and valuable models frequently used for toxicity bioassays (Goldstone et al. 2006; Oral et al. 2017; Pagano et al. 2017). To the best of our knowledge, no data in the literature describes the toxicity of BP-S on sea urchins embryos. Thus, the aim of this study was to evaluate embryotoxicity and cytogenetic toxicity for both BP-A and BP-S in sea urchin embryos.

Materials And Methods

Chemicals

Bisphenol A (BP-A; 4,4'-Isopropylidenediphenol; CAS 80-05-7, Purity 99%) and Bisphenol S (BP-S; 4,4'-Sulfonyldiphenol; CAS 80-09-1, Purity 98%) were obtained from Sigma-Aldrich Co.

Sea urchins

A. lixula, which is distributed in shallow rocky reefs along the Mediterranean coasts and are important grazers in sublittoral benthic communities, was used as test organism (Guidetti and Mori, 2005). Specimens were collected by hand from the coastal side in Seferihisar, Izmir, Turkey (38.152331, 26.823245). Twenty liters of seawater were bottled from the sea urchin habitat. Specimens and water samples were transferred to the laboratory in icebox, then water samples were filtered with a 0.45 µm filter. Cytogenetic and developmental toxicity assays were carried out as described previously (Oral et al. 2017; Pagano et al. 2017). Cytogenetic toxicity tests were completed in polystyrene test beakers and contained 3 replicates whereas embryotoxicity tests were carried out in 6 replicates.

The choice of test concentrations was made according to Bošnjak et al. (2014) and based on the prediction that environmental emissions of BP-S are likely to intensify in the future (Liu et al. 2021; Yu et al. 2015). For this purpose, we selected concentrations ranging from 0.1 to 100 µM. Thus, the test concentrations of both chemicals were 0.1, 0.25, 1, 2.5, 10, 25, and 100 µM for both developmental and cytogenetic toxicity experiments.

Developmental and cytogenetic toxicity control groups consisted of untreated and healthy embryos (30 embryos/ml) in 10 ml of filtered seawater. Test chemicals were dissolved in dimethyl sulfoxide (DMSO), therefore a DMSO (0.1% v:v) control group for each test was applied as well.
Embryological analysis

For embryotoxicity tests, BP-A or BP-S were placed at the bottom of each culture plate well [Falcon™ Tissue Culture Plates (6 wells, 10 ml/well)], and then suspended in 9 ml FSW. Thereafter, 1 ml of zygotes (10 min post-fertilization, p-f) was added to BP-A or BP-S and incubated at 18°C in the dark for 72 h. After a 72-h incubation, 10^{-4} M chromium sulfate was added to the culture wells, resulting in loss of larval mobility, and the larvae were scored on well bottom by an inverted microscope (100×) (Pagano et al. 2017). Embryonic/larval developmental defects were scored in 100 random embryos of each test group to determine the embryotoxic effects of the test chemicals, as shown in Fig. 2. Embryonic malformations (P1: skeletal malformations and gastrointestinal tract abnormalities; R: half size of a normal larvae; P2:

pre-pluteus stage blockage ; D: early embryonic death) were scored to determine the toxic effects of both test chemicals at embryonic level. Embryotoxic effects were determined by P1+P2+D sum.

Cytogenetic analysis

Cytogenetic tests were carried out 6 h p-f and the embryos were fixed in Carnoy's solution (ethanol, chloroform, acetic acid; 6:3:1 V:V:V). Fixative was replaced with absolute ethanol right after fixation. 24 h after fixation, absolute ethanol was renewed and the samples were ready to be observed under a light microscope (1000×) with oil immersion. Mitotic activity (numbers of metaphase and anaphase) and chromosome aberrations (chromosome bridges, lagging chromosomes, multipolar spindles, free chromosome sets, fragmented chromosomes) as shown in Fig. 3, were scored in each embryo, thus allowing to assess both quantitative endpoints and mitotic anomalies.

Statistical analysis

All datasets gathered from the bioassays were statistically analyzed in IBM SPSS v20. Results of bioassays are given as mean ± standard error in the charts. Homogeneity of variances was checked by Levene's test. Differences between each concentration group and the controls were determined by two-tailed Independent Samples t-test. A normality test was performed and the significance of the difference among the groups was evaluated by One-way Analysis of Variance (ANOVA). Kruskal-Wallis and Mann-Whitney U Tests were applied where ANOVA assumptions were not fulfilled. Differences were considered significant when $p<0.05$.

Results

Embryotoxicity

BP-A started to induce embryotoxic effects with 29% of malformed embryos at 1 μM concentration, as shown in Fig. 4. Compared to the control groups, malformed embryo percentages significantly differed at 2.5 μM ($p<0.01$, Tamhane's). 10, 25, and 100 μM concentrations of BP-A affected all embryos in the test groups ($p<0.001$, Tamhane's). Malformed embryo rates in embryos exposed to BP-S showed significant differences at 2.5 μM compared to the control groups ($p<0.05$, Tukey's). 10 and 25 μM concentrations were at a close embryotoxic level (20.5 to 21%) and differed from the controls ($p<0.01$, Tukey's). Malformed embryo rates raised to 23% at 100 μM concentration ($p<0001$, Tukey's). Malformed embryo percentages in BP-A vs. BP-S significantly differed at all tested concentrations ($p<0.05$ up to $p<0.001$) (Student's t tests). EC_{50} was calculated based on the nominal concentrations and it was found as 3.48 μM (95% Confidence Interval: 1.84 to 6.53 μM) for BP-A and 0.77 μM (95% Confidence Interval: 0.24 to 6.16 μM) for BP-S. Altogether, developmental toxicity of BP-S was significantly increased vs. controls, yet significantly lower than BP-A-induced developmental toxicity.

Cytogenetic toxicity

Figure 5 displays the cytogenetic results for BP-A plasticizer and its substitute BP-S. Mitotic activity in the embryos exposed to BP-A was inhibited at 25 ($p<0.05$, Student's t) and 100 μM ($p<0.01$, Student's t)

concentrations. At the concentrations of 25 and 100 μM, mitotic activity significantly differed for BP-A vs. BP-S ($p<0.05$, Student's t) (Fig. 5a). Also the data in Fig. 5b showed that the number of embryos lacking mitotic figures (% Interphase Embryos, IE) was significantly increased in embryos exposed to BP-A (25 and 100 μM) vs. controls ($p<0.05$). BP-S-exposed embryos failed to exhibit significant IE decrease vs. controls, and differed from BP-A-induced decrease of IE values ($p<0.05$, Student's t). The metaphase/anaphase (M/A) ratio, as shown in Fig. 5c, was significantly increased in BP-A-, not in BP-S-exposed embryos. As shown in Fig. 5d, a significant difference was observed in average total mitotic aberrations in embryos exposed to 25 to 50 μM BP-A compared to controls ($p<0.05$, Mann-Whitney U test), and compared to BP-S-exposed embryos, which failed to show significant rate of mitotic anomalies.

Discussion

Several studies have reported on pleiotropic toxic effects of BP-A in aquatic vertebrates and invertebrates at environmental doses (Canesi and Fabbri 2015; Crain et al. 2007; Kang et al. 2007). BP-A-induced embryotoxicity was noted previously in sea urchins (Cakal Arslan and Parlak, 2008), in zebrafish (Tse et al. 2013), in Xenopus (Gibert et al. 2011), and rodents (Chen et al. 2013). It has been reported that BP-A can alter echinoderm physiology, reproduction, and development at environmental concentrations (Bošnjak et al. 2014; Roepke et al. 2005), which can reach 17.2 μg/L (Crain et al. 2007). BP-A can induce aberrant division of the cell nucleus, leading to defective embryo development through the first cell division and retardation, along with general errors in cytoskeletal functioning in mitosis (Bošnjak et al. 2014).

The present report confirms BP-A-induced developmental and cytogenetic toxicity, while the replacement chemical (BP-S) fails to alter *A. lixula* early life stages, except for a moderate change in mitotic activity (metaphase/anaphase ratio, Fig. 5a). BP-A is more potent than BP-S in particular, at 10, 25, and 100 μM (~5 fold), indicating the sensitivity of *A. lixula* embryos to these specific bisphenols during a critical developmental period. Similar effects were also noted within *Daphnia magna* and in Zebrafish embryos and larvae (Liu et al. 2021). The present data suggest that BP-S raises lesser toxicity than BP-A, and confined to developmental and mitotoxic effects, however below the analogous effects obeserved for BP-A.

The toxicity order for different bisphenols reflects that they may operate *via* distinct mechanisms. Before 2013, BP-S had been detected in freshwater and sewage sludge, but rarely found in marine surface sediment. However, recent literature showed that BP-S concentration in aquatic environments started to increase progressively (Wu et al. 2018). This observation may indicate that BP-S compounds begin to be extensively used all over the world at different degrees with countries (Liu et al. 2021). BP-S is less biodegradable than BP-A in aquatic environments, which may lead to its accumulation in biota, and thus might be a persistent micropollutant affecting ecosystems (Danzl et al. 2009); Herrero et al. (2018) reported detrimental effects of BP-S on the transcriptional rate of genes in the model species *Chironomus riparius*.

In accordance with our data, several studies have reported that BP-A can induce DNA damage as well as structural and numerical chromosomal aberrations *in vitro* (Santovito et al. 2018; Xin et al. 2015) and *in vivo* (Izzotti et al. 2009). A recent study describes no cytogenetic effects for both BP-A and BP-S in human HepG2 cells (Hercog et al. 2020). Also, it has been reported that BP-S, compared to BPA, has a lower acute toxicity, similar or less endocrine disruption, similar neurotoxicity and immunotoxicity, and lower reproductive and developmental toxicity (Qiu et al. 2018).

Conclusions

Taken together, our results indicate an embryotoxic potential of bisphenols during critical periods of sea urchin development with a potent rank order to BP-A vs. BP-S. We thus show that BP-A alternative, BP-S induces lower toxic effects than BP-A with significantly lower severity, though suggesting possibly health concerns regarding the use of this BP-A alternative.

Declarations

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Competing interests

The authors declare that they have no known competing interests that could have appeared to influence the work reported in this paper.

Author contributions

Raja Rezg: Conceptualization, Writing - Original Draft.

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Bessem Mornagui: Resources, Review & Editing.

Giovanni Pagano: Methodology, Validation, Review & Editing, Supervision.

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Figures

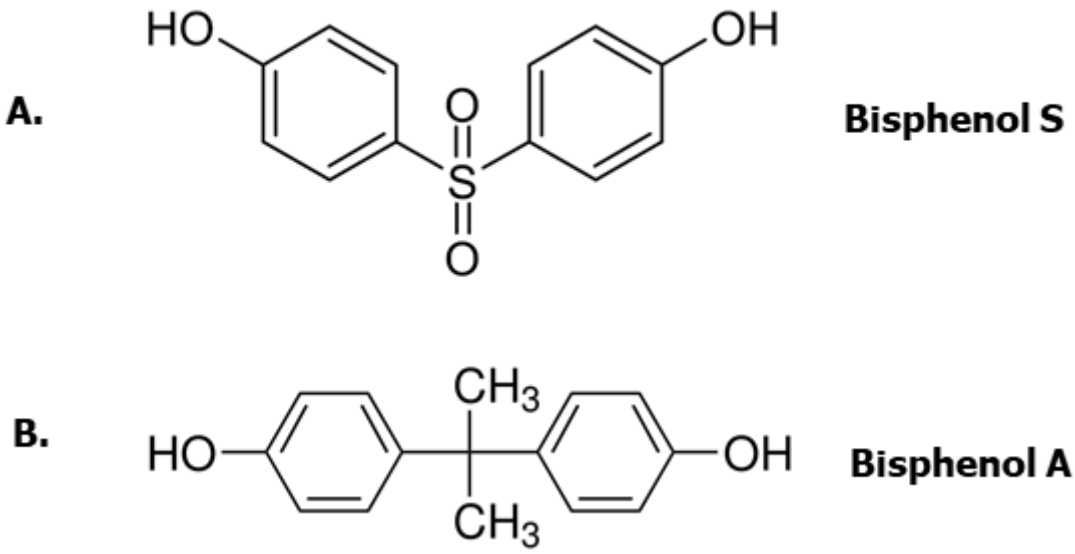

Figure 1

Chemical structures of bisphenol S (A) bisphenol A (B).

Figure 2

Examples of developmental defects; N: Normal pluteus, P1: plutei with skeletal malformations, P2: blockage at pre-pluteus stages.

Figure 3

Mitotic aberrations: A: chromosome bridge; B: lagging chromosome; C: scattered chromosomes; D: fractured chromosomes; E: multipolar spindle.

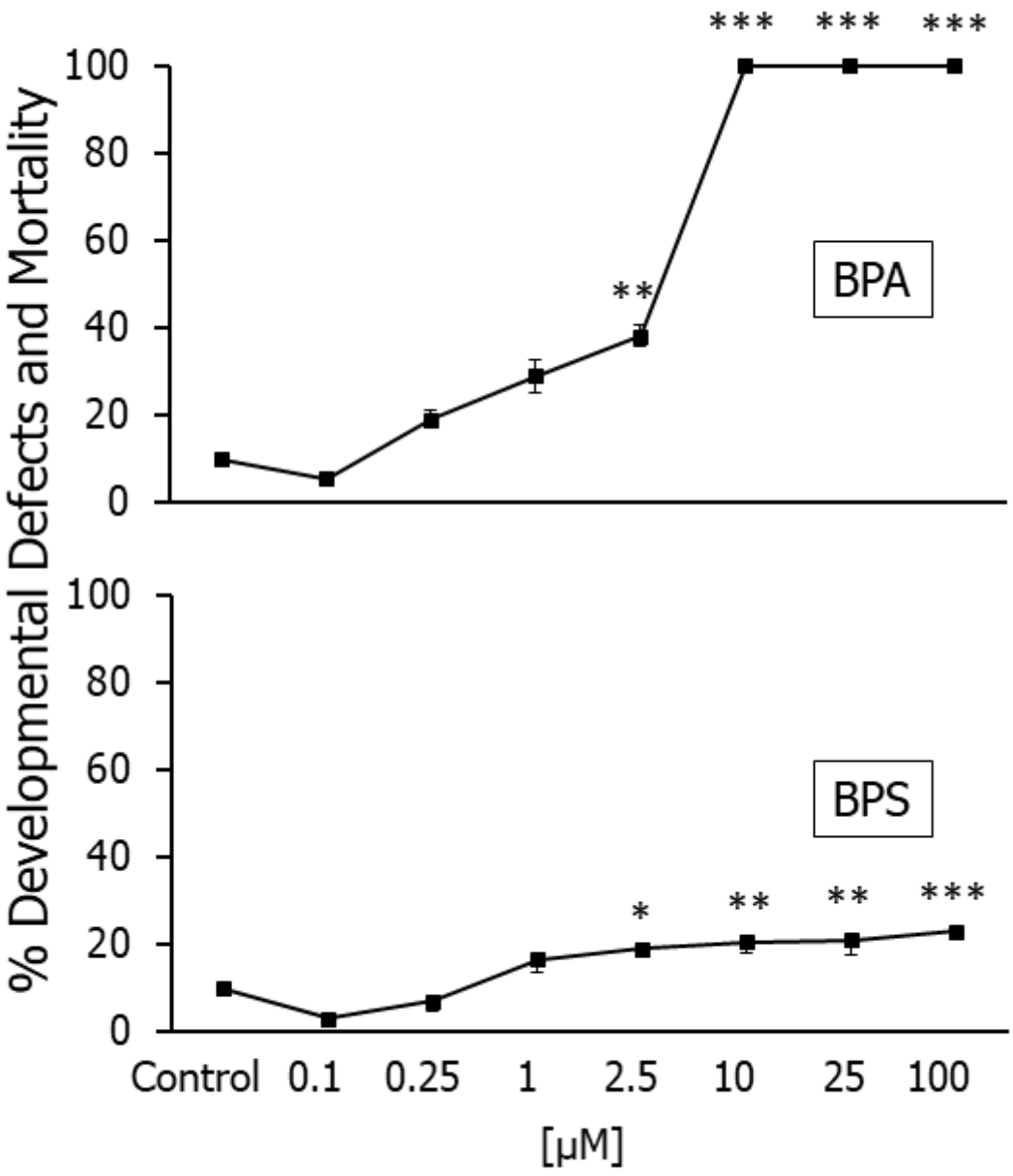

Figure 4

Average affected embryo percentages in embryotoxicity tests (*: p<0.05, **: p<0.01, Tamhane's, Tukey's).

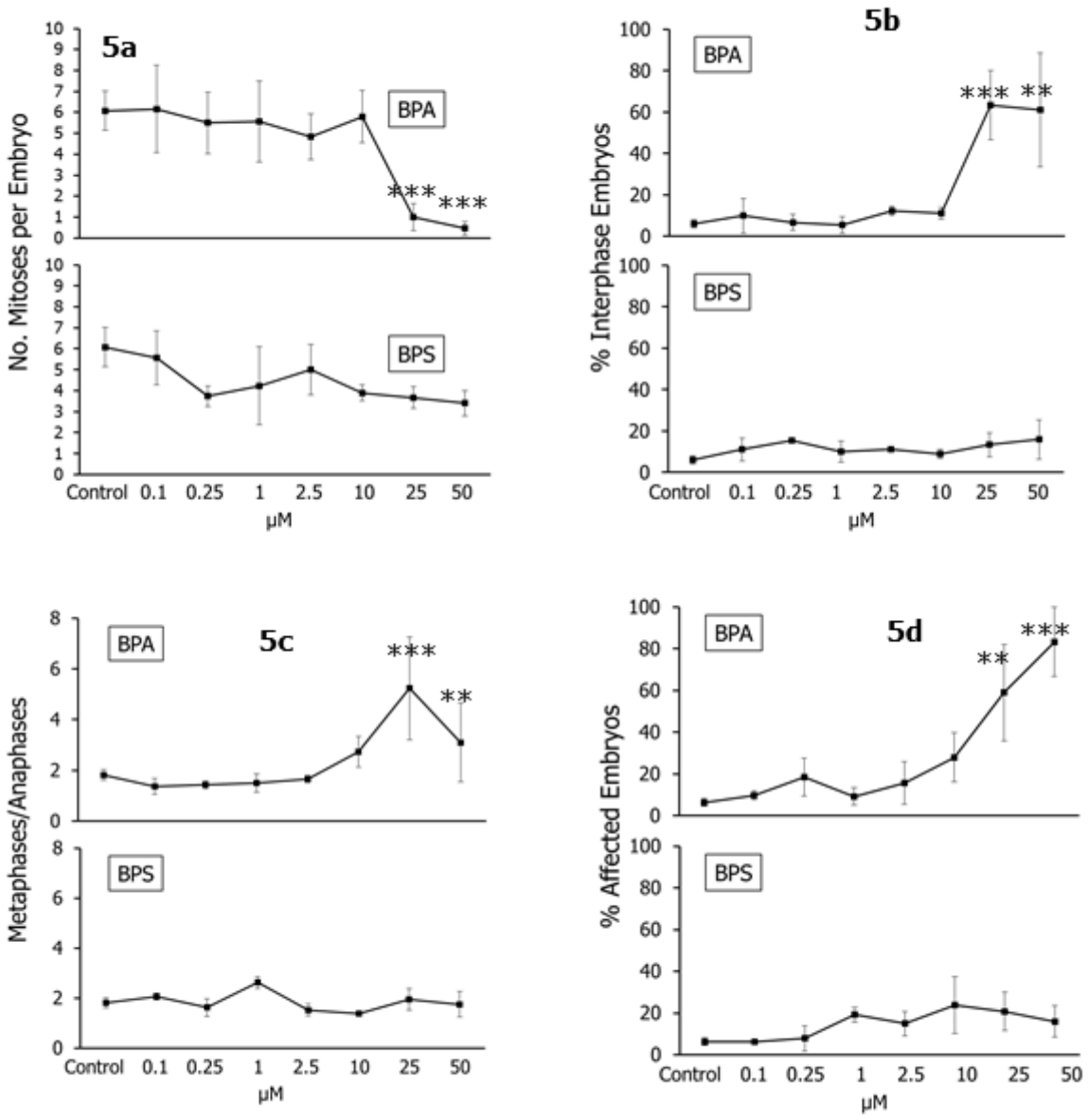

Figure 5

a: Mean No. mitoses per embryo (*: p<0.05, **: p<0.01, ***: p<0.001, Tukey's)

b: % Interphase Embryos (*: p<0.05, **: p<0.01, ***: p<0.001, Student's t and Mann-Whitney U tests).

c: Metaphase/Anaphase ratio (*: p<0.05, **: p<0.01, ***: p<0.001, Student's t).

e: % Mitotic aberrations per embryo values in 6-h exposed *A. lixula* embryos (*: p<0.05, **: p<0.01, ***: p<0.001, Tukey's).