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Bioaccumulation, behavior changes and physiological disruptions with gender-dependent in lizards (*Eremias argus*) after exposure to glufosinate-ammonium and L-glufosinate-ammonium

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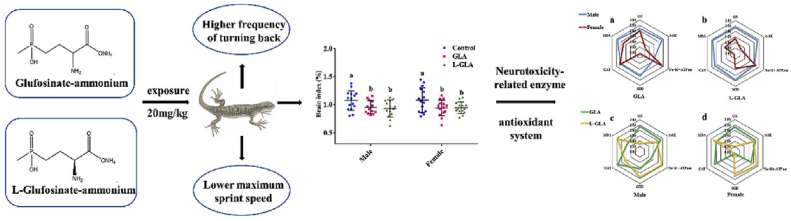
HIGHLIGHTS

- Poor locomotor performance of lizards were observed after GLA and L-GLA exposure.
- The accumulation of GLA is higher than L-GLA in Lizard' brain.
- The neurotoxic effects of GLA and L-GLA on lizards is different.
- Neurotoxic effect induced by GLA and L-GLA is gender-dependent in *E. argus*.

GRAPHICAL ABSTRACT



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ABSTRACT

Reptiles, the most diverse taxon of terrestrial vertebrates, might be particularly vulnerable to soil pollution. Reptiles especially lizards have been rarely evaluated in ecotoxicological studies, and there is a very limited report for effects of soil pesticide contaminants on lizards. In this study, male and female lizards (*Eremias argus*) were exposed to Glufosinate-ammonium (GLA) and L- Glufosinate-ammonium (L-GLA) for 60 days. Slower sprint speed, higher frequency of turning back and reduced brain index were observed in treatment groups. The accumulation of GLA in the brain of lizard was higher than that of L-GLA. Moreover, the activities of neurotoxicity-related enzymes and biomarkers of oxidative stress were also investigated. In summary, the neurotoxic effects of lizards have been observed after exposure to GLA and L-GLA. Based on the result of the Integrated Biomarker Response (IBR), males were more sensitive to contaminants than females. On the other hand, the neurotoxic pathways by GLA and L-GLA triggered were slightly different: GLA mainly acted on glutamine synthetase (GS), acetylcholinesterase (AchE) and Catalase (CAT) and L-GLA aimed at AchE, Na^+/K^+-ATPase, Superoxide dismutase (SOD) and Malondialdehyde (MDA). In summary, the accumulation of GLA and L-GLA in lizard's brain induced neurotoxicity by altering the levels of enzymes related to nervous system and antioxidant activity and further resulted in the decrease of brain index and locomotor performance.

1. Introduction

The application of pesticides was deemed vital to the increase in crop yield (Buchapudi et al., 2011). However, Irrational and unprotected use of pesticides has already led to the pesticides

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residues. Pesticide exposure has been threatened environment, animal health, and biodiversity through the food web (Yazicioglu et al., 2013). Accordingly, various strategies have been proposed to reduce the risks of pesticides. Production of chiral pesticides is among important strategies positively making pesticides safer because of the distinctive properties of chirality (Ulrich et al., 2012). Nevertheless, the enantiomers of many chiral pesticides are various in biological activities, some of which exhibit high activity and the others are low activity or inactivity due to their stereoselectivity (Ye et al., 2010). Therefore, investigating the toxic effects of chiral pesticides on non-target organisms is of great significance for their protection (Qin et al., 2017).

Glufosinate-ammonium (GLA) ((R, S)-2-amino-4-(hydroxyl (methyl) phosphonoyl) butanoic acid), is a broad-spectrum, non-selective and low toxicity organophosphate herbicide with a chiral center and a pair of chiral isomers. Its herbicidal activity was ascribed to the L-Glufosinate-ammonium (L-GLA) (Mao et al., 2014). Owing to the similar structure with glutamate, GLA acts in weeds by irreversible inhibition of glutamine synthetase (GS), which is essential for ammonia detoxification, resulting in the excessive accumulation of ammonia (Ebert et al., 1990). In mice, GLA can also induce toxicity through inhibition of GS and adversely affect the glutamate-utilizing systems at 5 and 10 mg/kg during 10 weeks study (Hack et al., 1994; Calas et al., 2008). Moreover, glutamate is a primary excitatory neurotransmitter in the vertebrate central nervous system (Köles et al., 2015; Xiao et al., 2017). Once the glutamate-utilizing system is destroyed, glutamate could be continual accumulated, *N*-methyl-D-aspartate receptors (NMDARs) are excessively activated and eventually lead to neurotoxic effects (Lantz et al., 2014).

With the greater use of GLA herbicide, higher frequency of mammals and human poisoning accidents were reported (Laugeray et al., 2014; Kim and Min, 2018). In addition, the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2012 found clinical signs of neurotoxicity of GLA in a single-dose toxicity study (200 mg/kg bw) in dogs. Furthermore, GLA produces moderate to severe neurotoxicity including convulsions and memory loss (Lantz et al., 2014). However, in reptile, especially in small lizard *Eremias argus* that is in close contact with the soil, the damage of GLA to its nervous system has never been studied.

In this study, three hypotheses were put forward. First, living in herbicide GLA and L-GLA contaminated soil may influence behavioral performance and change some biomarkers of lizards. Previous study have shown exposure to 1 mg/kg GLA could induce autism in mice (Laugeray et al., 2014) and a woman suffered from anterograde amnesia after GLA intoxication (Kim and Min, 2018). Second, GLA and L-GLA may have different effects on lizards because of their different biological activities and bioaccumulation. As we all know, the enantiomers of chiral pesticides have enantioselective toxicity (Cheng et al., 2015; Chen et al., 2017). Lastly, gender sensitivity is different for GLA and L-GLA. Generally speaking, males are more vulnerable to exogenous pollutants than female (Thomas and Woodley, 2017). To test these hypotheses, we conducted 60 days of soil exposure with a factorial experiment design (control, GLA and L-GLA × male and female) in *E. argus*.

2. Material and methods

2.1. Reagents

96% glufosinate-ammonium (GLA) and 91% L-GLA were all standard compounds obtained from Institute for the Control of Agrochemicals, Ministry of Agriculture (ICAMA). Derivatization reagents, 9-Fluorenylmethyl chloroformate (FMOC-CL) was obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Sodium borate ($Na_2B_4O_7 10H_2O$) and ammonium acetate (CH_3COONH_4) (analytical grade) were bought from Beijing Chemical Work (Beijing, China). Acetonitrile (C_2H_3N) (chromatographic grade) was purchased from Beijing tong guang fine chemicals company (Beijing, China).

2.2. Test lizards husbandry

E. argus mainly distributed in China, Russia, and Korea, which has been considered as an endangered species by the Korean Ministry of Environment in 2005 (Chang et al., 2018). Additionally, some properties of *E. argus* are consistent with species who faced to high extinction risk (Mingo et al., 2016). On the other hand, it is easier to keep this species in the laboratory and obtain their food mealworms. In this study, Adult *E. argus* were obtained from an artificial farm in Hebei Province, China, which has no history of chemical application. Moreover, the lizards from this farm are marketed as Chinese herbal medicine. So, we can guarantee that the artificial farm free of use of herbicides. *E. argus* were randomly separated into three groups: control group, GLA polluted soil group and L-GLA polluted soil group. Lizards in the three treatment groups were fed in three plastic basket, respectively and there are 30 lizards in each basket (15 males and 15 females). The concentration of GLA (racemate) and L-GLA (the active insomer) (20 mg/kg soil weight) was set on the basis of 1% of 2000 mg/kg, which is the acute LD50 data of birds (JMPR, 1991). Because toxicity data of GLA or L-GLA in reptiles and the concentration in natural environment have not been reported so far. The data of reptiles were often replaced by birds in ecological risk assessment (Weir et al., 2010). The 49 × 30 × 12 cm white plastic baskets with 4 cm soil (5 kg, clay, 26.80% ± 0.42%; silt,12.00% ± 0.49%; sand, 61.20% ± 0.57%; organic matter, 22.70% ± 0.26%; moisture content, 4.24% ± 0.02%; and pH, 7.80 ± 0.03) were provided for the cultivation of lizards. The soil used in this study was obtained from a reference site in Changping District, Beijing, China, where has no history of chemical application. This soil samples were analyzed by liquid chromatography–mass spectrometry (HPLC–MS/MS) and the result was undetected. The procedures of contaminating the soil are as follows: firstly, 104 mg GLA and 110 mg L-GLA (100 mg active ingredients) were weighed accurately and dissolved in 20 ml double distilled water, respectively. Secondly, 20 ml water solutions (GLA and L-GLA solutions) were added slowly to 0.5 kg soil and then mixed for 30 min using a mechanical shaker. Lastly, 4.5 kg soil uncontaminated was fully mixed with 0.5 kg contaminated soil by using the mechanical shaker. The same procedure were conducted in the control group with 20 ml double distilled water. Moreover, the temperature of 24 ± 1 °C was more suitable for *E. argus*. A 12 h light-dark cycle (from 07.00 to 19.00 h) was provided with the humidity from 35% to 45%. Lizards were allowed to feed and drink water freely during this study. Three grams of mealworms (about 30 mealworms) were fed to lizards in each treatment group and each lizard could get one mealworm daily. Taking into account animal welfare, we gave them a calcium supplement every week. Animals were adaptively cultured for two weeks before the formal trial. After 14 days exposure visualized neurotoxic behavior in treatment groups was observed, so the behavioral study was conducted. During 60 days experimental period, there were two lizards death in GLA and L-GLA, respectively (2 males in GLA group, a male, and a female in L-GLA group). At the end of the exposure, all lizards were weighed, lost consciousness by freezing anesthesia and sacrificed by decapitation. Then brain tissues were collected, weighed, frozen in liquid nitrogen immediately and then stored at −80 °C until analyses. The brain index (brain weight/body weight × 100%) were calculated. Animal experiments were

approved by the ethical committee for Laboratory Animals Care and conducted under the Guide for the Care and Use of Laboratory Animals (Council, 2011). The animal ethics approval protocol number for this study was CAU2018-0316-4.

2.3. Locomotor performance

The locomotor performance was used to assess lizard's behavior after 14 days of exposure. The wooden board racetrack used in this test is 100 cm long and marked at 20-cm intervals. Owing to lizard's preference of hiding under the dark bunker, there is an opaque light-proof box at the end of the track. Each lizard (eight males and eight females in each treatment group) was raced down the track at 24 ± 1 °C. Lizards were placed in starting point and allowed to acclimate for 2 min before the test. When the beginning of the test, lizards were gently stimulated near the tail with a soft paintbrush to force them to move forward. Once the movement of the lizard is suspended, they will be stimulated again. If a lizard turned back, it was turned forward without changing its relative position on the track. To quantify lizard's locomotor performance, the speed of the fastest 20-cm interval as the maximum sprint speed was recorded by stopwatch. The test was repeated two times with half an hour break between each test. The average maximum sprint speed (AMSS) and the total number of turning back (TNTB) of the two tests were analyzed.

2.4. Accumulation study

The brain tissues (three replicates for each sex in each treatment, two lizards for each replicate) were collected into 1.5 ml Eppendorf tubes. The extraction method referred to the previous report (Yongtao et al., 2016) is shown in supporting information. The accumulation of GLA and L-GLA in lizards' brains was analyzed by liquid chromatography–mass spectrometry (HPLC–MS/MS). ThermoFisher TSQ Quantum Access MAX system (Tewksbury, Massachusetts, USA) was used with C18 column (100 mm × 2.1 mm, 3 mm). Mass spectrometry conditions were as follows: precursor ion was 404 (*m*/*z*), product ions 182 (*m*/*z*) and 208 (*m*/*z*) was used for quantification and qualitation. The collision energies of two product ions of 15 V and 10 V, respectively.

2.5. Biochemical analysis

The brain tissues of six male and six female lizards (there replicates for each sex in each treatment, two lizards for each replicate) were used in the biochemical analysis. Glutamine synthetase (GS) activity was detected by Glutamine synthetase assay kit (Suzhou Ke Ming Biotechnology Co., Ltd). The absorbance was measured by ultraviolet spectrophotometer at 540 nm and the result was expressed as U/mg protein (U = mol/min). The activity of acetylcholinesterase (AchE) was measured referring to a previous report (Ellman et al., 1961). 5-thio-2-nitrobenzoate (TNB) was produced through the reaction of thiol with 5,5-dithiobis-(2-nitrobenzoic acid) DTNB. And it was detected by ultraviolet spectrophotometer at 412 nm. AChE activity was expressed as U/mg protein (U = mol/min). The activity of Na^+/K^+-ATPase was determined using a Na^+/K^+-ATPase assay kit (Nanjing Jian cheng Bioengineering Institude). The absorbance was determined by a microplate reader at 636 nm and the results were expressed as U/mg protein (U = μmol/Pi/hour). Antioxidant enzyme Catalase (CAT) and Superoxide dismutase (SOD) activities were also detected by CAT assay kit at 405 nm and SOD assay kit at 550 nm, respectively (Nanjing Jian cheng Bioengineering Institude). The results expressed as U/mg protein (U = μmol/min). The content of lipid peroxide malondialdehyde (MDA) was also determined by MDA assay kit (Nanjing Jian cheng Bioengineering Institude) using an ultraviolet spectrophotometer at 532 nm. The result was expressed as nmol/mg protein. All of the measurements followed the manufacturer's protocol.

2.6. Integrated biomarker response (IBR)

In view of more than one biomarker responding to toxic chemicals, the IBR assessment is a method that offers both an intuitive graphical comparison and a numeric score of all different biomarker responses (Devin et al., 2018; Ji et al., 2018). The IBR was calculated referring to the previous study (Kim et al., 2010), with some modifications. The detailed method was attached to the supporting information.

2.7. Statistical analyses

Results were expressed as the mean ± SD (standard deviation) and analyzed by SPSS 20.0 software. Values were normally distributed and homoscedasticity conducted by the Kolmogorov-Smirnov test and Levene's test, respectively. Data were tested for sex and treatment interaction effects by two-way analysis of variance (ANOVA) followed by the post-hoc Duncan and then, one-way ANOVAwith post hoc Duncan's method was used to assess statistical differences between different treatments, and the results were considered statistically significant if $p < 0.05$.

3. Results and discussion

3.1. Neurotoxic behavior

After 14 days of exposure, the neurotoxic behavior induced by GLA and L-GLA was observed. The lizards in exposed groups were timider. They pawed and burrowed with higher frequency to hide in the soil or under the shelter. In addition, they jumped anxiously when the shelters were removed. Compared with the GLA induced symptoms including convulsions, amnesia, and autism in rats (Laugeray et al., 2014; Kim and Min, 2018), the toxicity of GLA and L-GLA in lizard was mainly timidity and anxiety, which are transient behaviors that are difficult to quantify. However, these performances were the signals that we could conduct standardized behavioral tests such as locomotor performance at this point in time. To quantify lizards' behavior, locomotor performance including AMSS and TNTB were measured and the result is listed in Table 1 and Table S2. Compared with the control, the AMSS of lizards in the treatment group decreased significantly except for L-GLA females ($df_1 = 2$, $df_2 = 21$, $F = 9.863$, $P = 0.001$ and $df_1 = 2$, $df_2 = 21$, $F = 4.932$, $P = 0.018$ for males and females, respectively). In the process of this test, lizards in the treatment group appeared to be more alert to the surrounding environment and the path they took were mostly curve rather than a straight line like the control group. Therefore, the AMSS of them was reduced distinctly. Locomotor performance especially maximum sprint speed is essential for the predation escaping and foraging of animals (Clemente and Wilson, 2016). Slower sprint speed may cause animals to be at a disadvantage in competing with other competitors. Obtaining fewer food and resources may lead to a poor physical condition of animals (Scales and Butler, 2016). On the other hand, it is more difficult for animals with slower sprint speed to dodge the predator and further result in a higher mortality. For the result of TNTB, higher frequency of turning back was observed in all treatment males compared to the control. However, there is no significant change in females ($df_1 = 2$, $df_2 = 39$, $F = 3.943$, $P = 0.035$ and $df_1 = 2$, $df_2 = 21$, $F = 0.111$, $P = 0.895$ for males and females, respectively). The result indicates that males are more sensitive than females after GLA and L-GLA exposure.

Table 1
Average maximum sprint speed (AMSS) and the total number of turning back (TNTB) among the control group, GLA, and L-GLA group after 14 days exposure. Different letter represent statistical differences between different treatments.

		Control	GLA	L-GLA
AMSS (cm/s)	Male	24.75 ± 8.76^{a}	12.18 ± 5.30^{b}	10.63 ± 4.75^{b}
	Female	14.12 ± 4.95^{a}	7.43 ± 3.27^{b}	10.04 ± 3.61^{ab}
TNTB (times)	Male	0.37 ± 0.48^{a}	1.25 ± 0.66^{b}	1.25 ± 0.82^{b}
	Female	0.25 ± 0.66^{a}	0.25 ± 0.66^{a}	0.123 ± 0.33^{a}

3.2. Brain index and accumulation study

In view of the behavioral changes in treatment group lizards, body shape after 60 days exposure was recorded by photo shown in Fig. S1. The data of body length (snout-to-vent-length (Svl)) and body weight (Bw) of lizards after 60 days exposure were listed in Table S1. The Svl (Fig. S2) of lizards were not change in both sexes after exposed to herbicide because the lizards in this study are all adults ($df1 = 2$, $df2 = 39$, $F = 0.188$, $P = 0.829$ and $df1 = 2$, $df2 = 41$, $F = 0.864$, $P = 0.429$ for males and females, respectively). Bw might be changed after exposure to GLA and L-GLA. However, the change in Bw could not be seen intuitively from the figure since the body shape of *E.argus* is small. Therefore, we compare the changes of body shape by measuring the weight. Compared to control, body weights (Fig. 1a) were significantly reduced in males in two treatment groups ($df1 = 2$, $df2 = 39$, $F = 5.464$ and $P = 0.008$), while it changed slightly in the female. Moreover, brain weight and brain index were investigated after 60 days of exposure. Both male and female brain weights (Fig. 1b) in all treatment groups were declined distinctly compared to the control ($df_1 = 2$, $df_2 = 39$, $F = 16.467$, $P < 0.001$ and $df_1 = 2$, $df_2 = 41$, $F = 2.026$, $P = 0.145$ for males and females, respectively). The result of brain index was exhibited in Fig. 1c. Compared to the control, the brain indexes of lizards in the treatment groups were decreased significantly ($df_1 = 2$, $df_2 = 39$, $F = 3.764$, $P = 0.032$ and $df_1 = 2$, $df_2 = 41$, $F = 3.579$, $P = 0.037$ for male and female, respectively). Brain index was used to evaluate the health condition of brain tissues in lizards exposed to GLA and L-GLA. The decreased organ coefficient indicated that there could be atrophy and other degenerative changes in the tissues (Yuan et al., 2012). Indeed, in our study, the sufficient decrease of brain weight and brain index in all treatment groups revealed the poor condition of the brain and resulted in lizards' neurotoxic behaviors such as slower sprint speed and higher frequency turning back. It is worth noting that the male body weight reduced more in both GLA and L-GLA groups compared to the female. This is in line with our locomotor performance result that males are more sensitive to exogenous contaminants than females.

The occurrence of neurotoxic behaviors and the decrease of brain index were closely related to the accumulation of herbicides in the brain. Therefore, the contents of GLA and L-GLA in the brain tissues were further determined (Fig. 1d). The most prominent finding was that the concentration of GLA was significantly higher than L-GLA in both sexes. The accumulation of GLA and L-GLA ranged from 0.72 to 0.86 mg/kg and 0.51–0.71 mg/kg, respectively. This suggested that there was different bioaccumulation between GLA and L-GLA, which may cause varying degrees of toxic effects. In

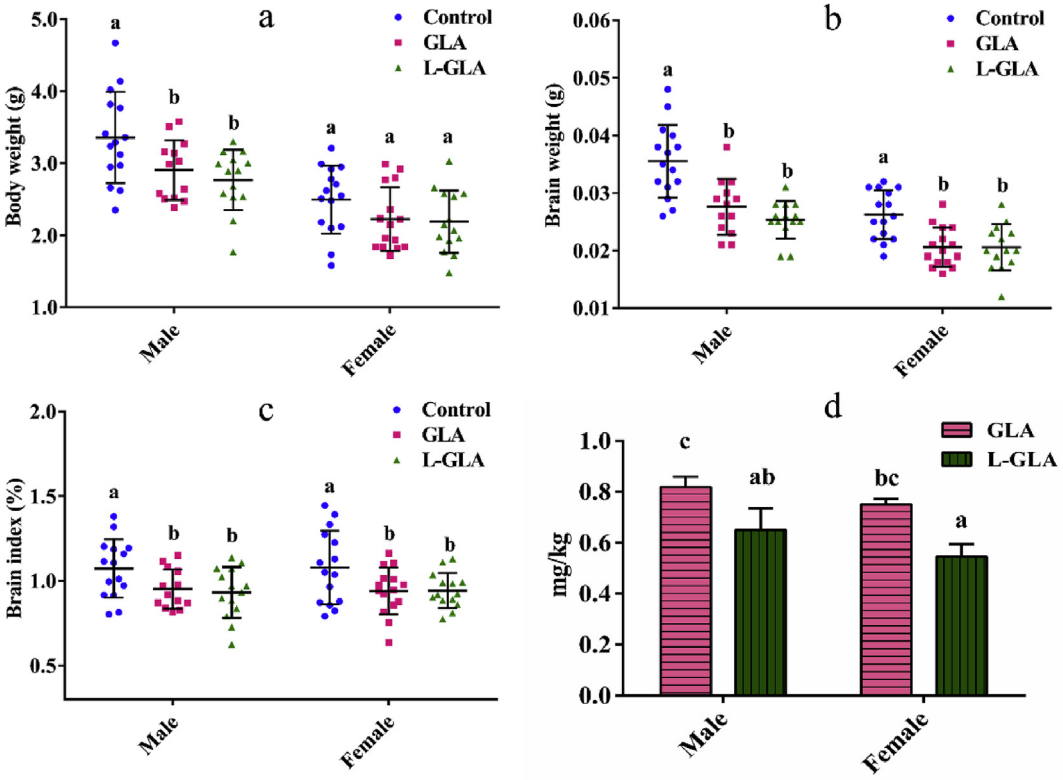

Fig. 1. Body weight (Fig. 1a), brain mass (Fig. 1b), brain weight (Fig. 1c), and the accumulation of GLA and L-GLA in lizards' brains (Fig. 1d). Brain index presented as the ratio of brain weight to body weight among the control group, GLA group and L-GLA group after 60 days exposure. Values represent the means ± SD. Different letters represent a statistically significant.

fact, GLA could not be completely absorbed by animals such as rats, dogs, and livestock. After administration of single (2–500 mg/kg bw) or repeated oral doses (2 mg/kg bw), most of the dose (80–90%) was excreted in the urine and feces (JMPR, 1991), so the accumulation of it in animal brain tissue were often neglected in previous neurotoxicity study. Although it is easily metabolized, it does not mean that it will not be enriched in the organism at all. Thus, it is necessary to investigate the herbicide content in the brain tissues and it was supplemented in our study.

3.3. Determination of neurotoxicity-related enzyme activity

The activity of glutamine synthetase (GS) is shown in Fig. 2a. GS activity was significantly inhibited in the GLA group rather than in L-GLA and dropped by 27.04% in males ($df_1 = 2$, $df_2 = 6$, $F = 9.769$, $P = 0.013$) and 24.94% in females ($df_1 = 2$, $df_2 = 6$, $F = 5.092$, $P = 0.051$). GS was more severely inhibited in males than it in females, although there is no statistical significance according to the two-way (ANOVA). GS, GLA target enzyme, plays multiple roles including ammonia detoxification and converting glutamate to non-toxic glutamine (Nakaki et al., 2000). It is reasonable to consider, therefore, that the inhibition of GS in the brain may lead to the accumulation of ammonia and glutamate and eventually cause neurotoxic effects. Interestingly, inhibition of GS occurred only in the GLA group, but not in L-GLA. The most probable reason is the difference in accumulation between GLA and L-GLA in the lizard brain and the content of GLA was significantly higher than L-GLA. In summary, this result fully reflected that the difference in biological accumulation will be further influenced on biochemical indicators and it supported our hypotheses that different effects of GLA and L-GLA were due to their difference in bioaccumulation.

Acetylcholinesterase (AchE) activity is notably lower in all treatment groups than it in the control and the activity in the GLA group was the lowest among three treatment groups (Fig. 2b). For males, the activity decreased by 46.67% and 38.48% in the GLA and L-GLA groups ($df_1 = 2$, $df_2 = 6$, $F = 125.194$, $P < 0.001$). For females, the activity of AchE declined by 11.97% and 10.29% in the GLA and L-GLA groups ($df_1 = 2$, $df_2 = 6$, $F = 109.550$, $P < 0.001$). The decline of activity in male was lower than that in female ($F = 24.413$, $P_{(\mathbf{Treatment} \times \mathbf{Sex})} < 0.001$). Among all the biomarkers used in environmental studies, AchE is commonly used for evaluation of the neurotoxicity and it may be inhibited when organisms are exposed to organophosphate (Gavric et al., 2015). In this study, GLA and L-GLA led to a significant decline in AchE activity in lizards' brain and it could be a potential neurotoxic mechanism. Moreover, consistent with GS results, AchE declined more in males instead of females.

To a further estimate, the energy metabolism status in brains of lizards, the activity of Na^+/K^+-ATPase was measured (Fig. 2c). The results showed that the activities were lower in all treatment groups than them in the control. For males, the activity reduced by 29.54% and 62.86% in the GLA and L-GLA groups ($df_1 = 2$, $df_2 = 6$, $F = 28.924$, $P = 0.001$). For female, the activity declined by 50.19% and 66.73% in the GLA and L-GLA groups ($df_1 = 2$, $df_2 = 6$, $F = 30.904$, $P = 0.001$). In the vertebrate brain, Na^+-K^+-ATPase, the main consumer of metabolic energy (ATP) is responsible for the regulation of membrane potential, neurotransmitter release and electrical excitability (Stecyk et al., 2017). The inhibition of activity suggested that obstacles to energy metabolism and abnormal ion concentration gradients (K^+ and Na^+) across the membrane (Liu et al., 2017). In general, GLA and L-GLA may decrease the ability of energy generation, and the further induce the neurotoxic effect. In addition, the inhibition of Na^+-K^+-ATPase may be associated with oxidative stress injury (Liu et al., 2017). Therefore, the biomarkers of oxidative stress were also detected.

3.4. Antioxidant system

The Superoxide dismutase (SOD) activities in brains of the males in all conditions showed a sufficient elevation after 60 days exposure. While in females, the increased activity was only observed in L-GLA group (Fig. 3a). Moreover, it rose 45.15% and 68.52% ($df_1 = 2$, $df_2 = 6$, $F = 34.442$, $P = 0.001$) in males' brains in GLA and L-GLA groups. The activity in L-GLA group females grew 20.80% ($df_1 = 2$, $df_2 = 6$, $F = 19.463$, $P = 0.002$). Being the products of aerobic metabolism, reactive oxygen species (ROS) including $O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$, have physiological roles at low levels (Gavric et al., 2015). Under normal condition, the generation and scavenging of ROS are always in dynamic balance. Oxidative damage occurs once the balance was broken down (Jena et al., 2009). The SOD activity growth in this study indicated that an elevation in $O_2^{\cdot-}$ production (Oruç, 2010) and the increase could have been related to increased oxidative stress caused by GLA and L-GLA. It is worth noting that compared with a female, the growth of SOD activity in male was greater ($F = 5.601$, $P_{(\mathbf{Treatment} \times \mathbf{Sex})} = 0.019$) and this is in accordance with our third hypothesis. Despite being a scavenger of superoxide radicals, SOD represented a source of H_2O_2, and could convert $O_2^{\cdot-}$ to H_2O_2 and O_2. Thus, it's necessary to be considered with H_2O_2 reducing enzymes together, like Catalase (CAT) (Regoli and Giuliani, 2014; Cheng et al., 2015). Regarding changes in CAT activity (Fig. 3b), in all treatments, the enzyme activity of male decreased significantly and declined by 20.36% and 13.91% ($df_1 = 2$, $df_2 = 6$, $F = 34.846$, $P < 0.001$) in GLA and L-GLA groups, respectively. However, in females, CAT activity decreased significantly only in the GLA group and reduced by 21.08% ($df_1 = 2$, $df_2 = 6$, $F = 37.034$, $P < 0.001$). CAT is a crucial role in catalyzing H_2O_2 to produce O_2 and H_2O and the decline of CAT is closely related to the rise of SOD. In addition, compared to L-GLA group, the activity of CAT dropped more in GLA group, which may be ascribed to the variation of accumulation between GLA and L-GLA in lizard brain.

The result of membrane lipid peroxidation malondialdehyde

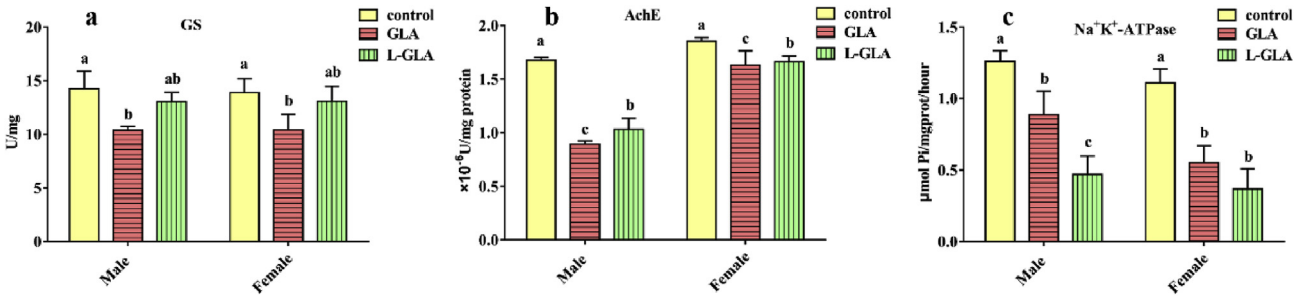

Fig. 2. Results of glutamine synthetase enzyme (GS) (Fig. 2a), Acetylcholinesterase (AchE) (Fig. 2b), and Na^+-K^+-ATPase (Fig. 2c) activity among the control group, GLA group and L-GLA group after 60 days exposure. Results were expressed in means ± SD. Different letters represent a statistically significant.

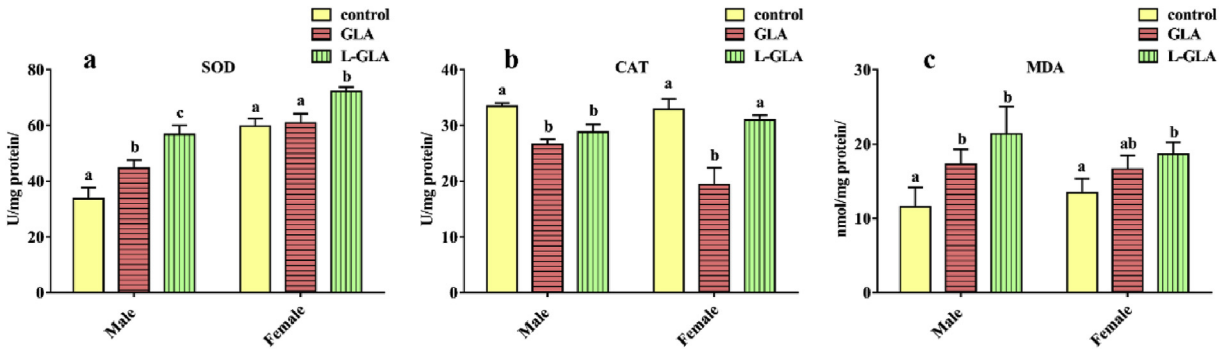

Fig. 3. Results of Superoxide dismutase (SOD), Catalase (CAT) activity and Malondialdehyde (MDA) content among the control group, GLA group and L-GLA group after 60 days exposure. Results were expressed in means ± SD. Different letters represent a statistically significant.

(MDA) was shown in Fig. 3c. Consistent with the antioxidant enzymes results, significant changes were observed in males in all treatment groups. The content of MDA in males rose by 49.71% and 85.17% ($df_1 = 2$, $df_2 = 6$, $F = 8.972$, $P = 0.016$) in the GLA and L-GLA groups, respectively. For females, only 38.91% elevation ($df_1 = 2$, $df_2 = 6$, $F = 6.371$, $P = 0.033$) was observed in L-GLA group. Normally, MDA was considered as the end point for oxidative damage which was produced when membrane lipid was attacked by ROS (Cheng et al., 2015). Our results suggested that male lizards' brains in all treatment groups and females' brains in L-GLA groups suffered from oxidative damage and the antioxidant system in the male was also more sensitive than it in the female. This study revealed that alterations in the activity of antioxidant enzymes and growth lipid peroxidation induced by GLA and L-GLA are one of the most important reasons for the neurotoxic effects on lizards.

3.5. The calculation of IBR

A common challenge in multibiomarkers studies is to go beyond an independent interpretation of each one, and to really assess an overall response. Therefore, IBR including neurotoxicity-related enzyme (GS, AchE, Na^+-K^+-ATPase), antioxidant system (CAT, SOD), and MDA was calculated and shown in Fig. 4 and the IBR score are listed in Table S3. Whether in the GLA group or L-GLA

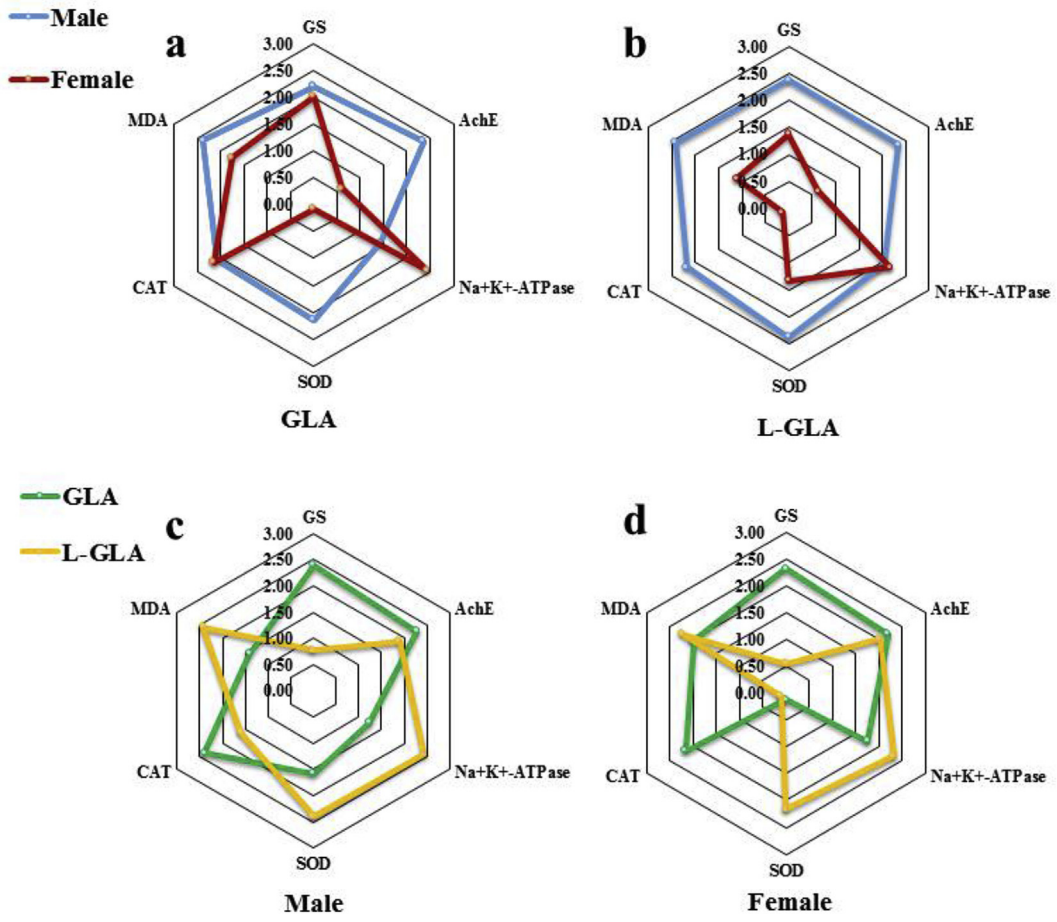

Fig. 4. The radar diagram of Integrated biomarker response (IBR) normalized with the control group for GLA (Fig. 4a), L-GAL (Fig. 4b), male (Fig. 4c) and female (Fig. 4d).

group, IBR (radar diagram area) of males is much larger than that of females (Fig. 4a and b). Larger area indicates stronger biomarker responses and more serious the impact of chemicals on the organism (Kim et al., 2010), and this result is in accordance with our hypothesis that different sensitivities to pollutants due to gender. Males are more sensitive and vulnerable to the exogenous contaminant. Moreover, the male radar diagram is close to a regular hexagon, indicating that the six biomarkers respond roughly equally. However, the response of Na^+-K^+-ATPase activity shows the highest intensity in females. Fig. 4c and b indicate the difference of biomarker responses between GLA and L-GLA group. The different radar chart shape between GLA and L-GLA demonstrates the toxicity difference. GLA mainly acted on glutamine synthetase (GS), acetylcholinesterase (AchE) and Catalase (CAT), while L-GLA pointed at AchE, Na^+/K^+-ATPase, Superoxide dismutase (SOD) and Malondialdehyde (MDA).

4. Conclusion

This study represents the first exploring of the neurotoxic effects of GLA and its monomer L-GLA in *E.argus*. In line with our predictions, changes in biomarkers including neurotoxicity-related enzyme, antioxidant enzymes and lipid peroxidation induced by GLA and L-GLA resulted in changed lizards' locomotor performance especially in males. Additionally, the different radar chart shape indicated that the toxic effects of GLA and L-GLA were different and may due to their various bioaccumulation capacity. Lastly, sex dependence, males more sensitive to GLA and L-GLA than females neatly illustrated by the results of these biomarkers. Other neurotoxicity performance parameters such as predation frequency, learning ability, and exploring behavior should be investigated in further study to better reveal the neurotoxic effects of GLA and L-GLA on *E. argus*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.04.007.

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