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Abstract

In the context of global warming, an important issue is that many pesticides become more toxic, putting non-target organisms at higher risk of pesticide exposure. Eremias argus, a native Chinese lizard, was selected as research organism in present study, because of their characteristics that poikilothermic vertebrate are sensitive to temperature change. The experimental design [(with or without L-Glufosinate-ammonium (L-GLA) pollution × two temperatures (25 and 30 °C)] was used in this study for 90 days to identify the chronic effects of the pesticide–temperature interaction on neuroendocrine-regulated lizard' reproduction. Survival rate, body weight, clutch characteristics, semen quality, testicular histopathology, the content of neurotransmitters and related enzyme activity, the level of sex steroid, the expression of Heat shock protein 70 (HSP70), antioxidant system, the accumulation and degradation of L-GLA were examined. Results showed that L-GLA disrupt reproduction of lizards through hypothalamus-pituitary-gonad (HPG) axis. In addition, temperature can not only change the environmental behavior of pesticides, but also alter the physiological characteristics of lizards. Thus, our results emphasized that temperature is an essential abiotic factor that should not be overlooked in ecotoxicological studies.

Keywords	Eremias argus; L-glufosinate-ammonium; temperature; reproductive disruption; hypothalamus-pituitary-gonad axis
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Cover letter

Dear Editor,

In the context of global warming, an important issue is that many pesticides become more toxic, putting non-target organisms at higher risk of pesticide exposure. *Eremias argus*, a native Chinese lizard, was selected as research organism in present study, because of their characteristics that poikilothermic vertebrate are sensitive to temperature change. A two-factor experimental design [(with or without L-Glufosinate-ammonium (L-GLA) pollution × two temperatures (25 and 30 °C)] was used in this study for 90 days to identify the chronic effects of the pesticide–temperature interaction on neuroendocrine-regulated lizard' reproduction.

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We deeply appreciate your consideration of our manuscript, and we look forward to receiving comments from the reviewers. If you have any queries, please don't hesitate to contact me.

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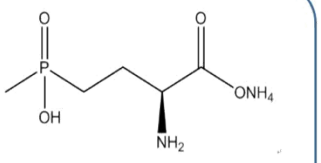
Sincerely,

Dr. Jinling Diao

Highlights

1. L-GLA exposure disrupt reproduction controlled by neuroendocrine system of lizard.
2. Thermal effects influence the reproduction of lizards.
3. High temperature aggravated the reproductive toxicity of L-GLA to lizards.
4. Thermal effects should be taken into account in the future ecotoxicological studies.



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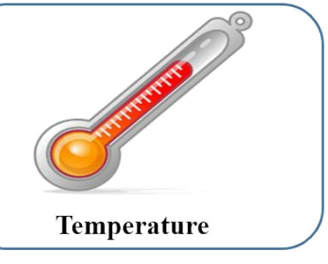

Temperature

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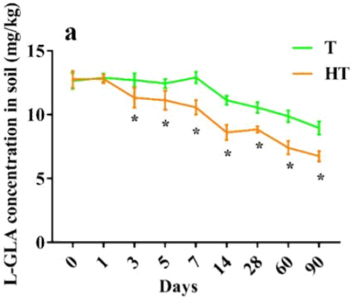

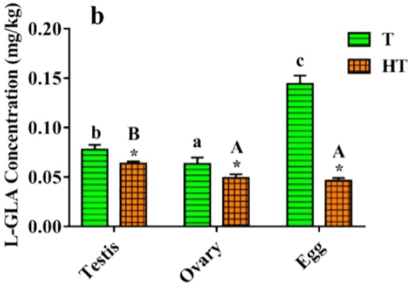

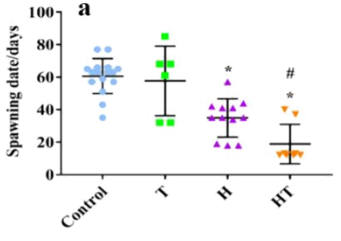

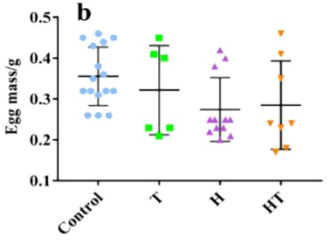

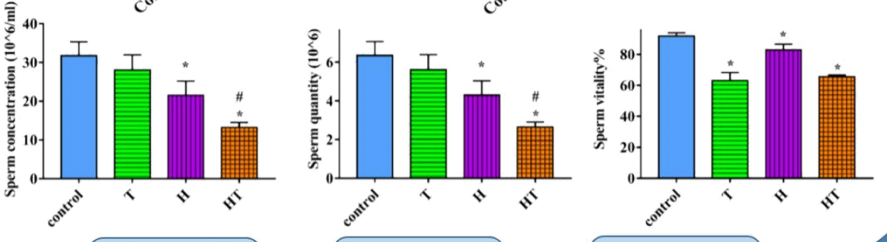

neuroendocrine-regulated

reproductive toxicity

hypothalamus → pituitary → gonad

affect

Effects of L-Glufosinate-ammonium and temperature on reproduction controlled by neuroendocrine system in lizard (*Eremias argus*)

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Abstract

In the context of global warming, an important issue is that many pesticides become more toxic, putting non-target organisms at higher risk of pesticide exposure. *Eremias argus*, a native Chinese lizard, was selected as research organism in present study, because of their characteristics that poikilothermic vertebrate are sensitive to temperature change. The experimental design [(with or without L-Glufosinate-ammonium (L-GLA) pollution × two temperatures (25 and 30 °C)] was used in this study for 90 days to identify the chronic effects of the pesticide–temperature interaction on neuroendocrine-regulated lizard' reproduction. Survival rate, body weight, clutch characteristics, semen quality, testicular histopathology, the content of neurotransmitters and related enzyme activity, the level of sex steroid, the expression of Heat shock protein 70 (HSP70), antioxidant system, the accumulation and degradation of L-GLA were examined. Results showed that L-GLA disrupt reproduction of lizards through hypothalamus-pituitary-gonad (HPG) axis. In addition, temperature can not only change the environmental behavior of pesticides, but also alter the physiological characteristics of lizards. Thus, our results emphasized that temperature is an essential abiotic factor that should not be overlooked in ecotoxicological studies.

Keywords: *Eremias argus*; L-glufosinate-ammonium; temperature; reproductive disruption; hypothalamus-pituitary-gonad axis

1. Introduction

The upward trend in the use of agricultural pesticides during the past half century has been reported (Popp and Nagy, 2013). Due to the lack of strict selectivity of many pesticides to targets, the effect of pesticides on non-target organisms has attracted more and more attention (Li et al., 2016; Martikainen, 1996; Milan et al., 2018). It is generally known that temperature is a crucial abiotic

factor which could influence the environmental behavior of pesticides mainly by affecting their metabolism, degradation and migration, thereby altering the extent of their biological hazards (Laabs et al., 2000; Lourival Costa et al., 2003). According to Intergovernmental Panel on Climate Chang (IPCC) forecasts, the global average temperature will rise by 1.4–5.8 °C in 2100 (Pachauri and Meyer, 2014). An important issue in a warming world is that many pesticides become more toxic, putting non-target organisms at higher risk of pesticide exposure (M et al., 2016; Noyes and Lema, 2015; Noyes et al., 2009). Therefore, in the context of climate change, temperature should be taken into account when exploring the effects of pesticides on non-target organisms.

Temperature not only affects the environmental behavior of pesticides, but also changes the physiological and biochemical traits of animals (Garcia et al., 2011). The growing reality of global warming is focusing scientific attention onto the impacts of ambient thermal variation on organisms (Telemeco et al., 2010). Reptiles, which are non-target organisms of pesticide applications are vulnerable to exogenous pollutants (Mingo et al., 2017). As ectotherms, most of their physiological characters display temperature dependence, including sprint speed, metabolic rate and digestive efficiency (Gilbert and Miles, 2016). Moreover, the effects of temperature on the reproductive activity of lizards is particularly prominent. For example, the sex hormone levels of lizards fluctuate with seasonal temperature changes; the viviparous strategy of lizards is used to target cold climates; and nest temperature affects the sex ratio of embryos (Barry et al., 2010a; Li et al., 2017; Pincheira-Donoso et al., 2017; Rusch and Angilletta, 2017; Tripathy and Rai, 2017).

Although many previous studies on reproductive problems have been reported, most of them focused on the level of the gonad or liver. Regarding the effects of contaminants on reproductive system, a fact that animal's brain control the reproduction system through a strictly regulated

hypothalamus-pituitary-gonad (HPG) axis is often overlooked (Niladri et al., 2009). Gonadotropin-releasing hormone (GnRH) synthesized in the hypothalamus promotes luteinizing hormone (LH) release from anterior pituitary gland. LH, a glycoprotein gonadotropin, promotes gonad development, sex hormone production, follicular maturation and spermatogenesis, thereby regulating the fertility of vertebrates (Kendall and Dickerson, 2010; Niladri et al., 2009). Previous studies have shown that the release of LH is affected by neurotransmitters, including dopamine (Da) and gamma-aminobutyric acid (GABA). Specifically, DA binding to its receptor inhibits LH release, whereas GABA promotes LH production (Sui and Chun-Hong, 2000). Yet, no studies have explored how combined exposure to warming and pesticides influence the lizards (*Eremias argus*) reproduction controlled by neuroendocrine.

Glufosinate-ammonium (GLA), a broad-spectrum and low toxicity organophosphate herbicide with a chiral center and a pair of chiral isomers, can competitively inhibit the glutamine synthetase to interfere with ammonia metabolism, so as to achieve the purpose of weed control (Ebert et al., 1990). In addition to the well-known neurotoxicity of organophosphorus pesticides, the reproductive toxicity has also been reported in vertebrates (Schenk et al., 2010). L-Glufosinate-ammonium (L-GLA), the isomer of Glufosinate-ammonium (GLA), possessed herbicidal activity. So far, there have been few reports on L-GLA ecotoxicology and in our unpublished studies, the toxic effects of GLA and L-GLA on lizards are not identical. Therefore, the purpose of this study were to 1) exploring how L-GLA induces reproductive toxicity by affecting the HPG axis in *E. argus*. 2) Explaining the effects of temperature on the endocrine reproductive system of lizards. 3) Clarifying whether the reproductive toxicity L-GLA induced on lizards is alleviated or aggravated in the context of global warming.

2. Material and methods

2.1 Chemicals and reagents

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

2.2 Lizards husbandry and experimental design

Adult *E. argus* (2-3 years) were obtained from the natural landscape (Inner Mongolia Province, China) and kept in our laboratory for two weeks to acclimate in experimental white plastic incubators (47 × 32 × 23cm) covered with 5cm soil and sand at a 10-14h dark-light cycle.

Female (except for pregnant females) and male lizards were selected according to their snout-vent length (SVL) and body weight（SVL : 4.0-5.0 cm and 4.1-4.9 cm for male and female, respectively; body weight : 2.13-3.06 g and 1.95-3.01 g for male and female, respectively）for this study. The experimental design [(with or without L-GLA pollution × two temperatures (25 and 30 °C)] was used in this study to identify the effects of the pesticide–temperature interaction on neuroendocrine-regulated lizard' reproduction. All lizards were randomly separated into four groups (24 males and 16 females per group): Control group, L-GLA polluted soil group (T), high temperature without L-GLA soil group (H), and high temperature with L-GLA polluted soil group (HT) for 90 days. L-GLA concentration was 13.34 mg/kg soil weight based on predicted environmental concentration (PEC) of GLA because of lack of information on L-GLA application concentration. For GLA of

89 soils a single application and the highest normal application rate (3000 g/ha, China Pesticide
90 Information Network http://www.chinapesticide.gov.cn/) was used when calculating the PEC
91 (based on a soil depth of 5 cm) (Table S1). The temperatures 25 ± 2 °C (for the control and T group)
92 and 30 ± 2 °C (for H and HT group) were fixed based on information regarding the ecological
93 preferences of *E. argus* (Hao et al., 2006) and the humidity ranged from 40% to 50%. Lizards were
94 allowed to feed live mealworms (*Tenebrio molitor*) and drink water freely, and ingest calcium
95 powder every two weeks. In addition, lizards' excreta was cleaned once a week, and the weights of
96 lizards were measured every two weeks. Clutch characteristics (spawning date and egg mass) were
97 monitored regularly during the study. After 14 days exposure (the volume of male lizards semen
98 reached the peak of the year at this time), semen analysis was conducted and Heat shock protein
99 level (HSP 70) was measured after 30 days. Soil (10 g) were collected at 1, 3, 5, 7, 14, 28, 60, and
100 90 days for soil degradation dynamics of L-GLA analysis. At the end of exposure, all lizards were
101 weighed and sacrificed by freezing anesthesia. Brain and gonad tissues were collected and weighed.
102 The right testes of four lizards from each group were fixed in 4% paraformaldehyde for
103 histopathological analysis. The left testes from the remaining animals and brain tissues were
104 collected and stored at -20 °C. Blood was rested at room temperature to get serum and stored at -20
105 °C until analysis. Animal experiments were approved by ethical committee for Laboratory Animals
106 Care and Use of Research
107 Center for China Agricultural University.

108 **2.3 Determination of chemical and neurotransmitters**

109 The contents of L-GLA and neurotransmitters (DA and GABA) were analyzed by high performance
110 liquid chromatography–mass spectrometry (HPLC–MS/MS). The specific method is attached in the

supporting information. The recoveries of L-GLA in testis, ovary, and egg were listed in Table. S2 and the recoveries of DA and GABA were listed in Table. S3.

2.4 Assay of enzyme activity and expression of protein

The neurotransmitter-related enzymes activity: glutamic acid decarboxylase (GAD), gamma-aminobutyric acid - transaminase (GABA-T) and monoamine oxidase (MAO) in brain tissues were measured using a commercially available assay kit (Nanjing Jian Cheng Bioengineering Institute). The levels of gonadotropin and sex steroids in plasma: luteinizing hormone (LH), testosterone (T), estradiol (E_2), and progesterone (Pg) were measured using an assay kit obtained from Shanghai Enzyme-linked Biotechnology Co., Ltd. The content of heat shock protein 70 (HSP 70) in gonads and plasma was determined by an assay kit obtained from Shanghai Enzyme-linked Biotechnology Co., Ltd.

2.5 Data analysis

All values were presented as means with standard error (means± SD) and analyzed via SPSS v20.0 (IBM, USA). Graphical plotting were realized by GraphPad Prism v6.0 (GraphPad Software, Inc. USA), and. Differences among groups were detected by one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test ($P < 0.05$). The degrees of freedom from the treatments (df_1) and residual degree of freedom (df_2) are also reported. Differences in the survival rate among all the groups were performed via survival analysis with Log-rank test. The degradation in soil and accumulation in lizards of L-GLA analyses were determined using Students t-tests and p-value of ≤ 0.05 was considered significantly different. Data were normally distributed and homogeneity of variance was confirmed by the Levene's test. Dunnett’s T3 test (a non-parametric) was conducted when values did not conform to the parametric assumptions.

3. Results

3.1 Survival rate and body weight

Thermal effect is the main factor affecting the survival of lizards (Fig. 1a), with lower survival rates for lizards under the high temperature (30 ± 2 °C) treatment than those in the control group (25 ± 2 °C) (χ^2 = 4.193, P = 0.041 and χ^2 =5.348, P = 0.021 for H and T group, respectively). L-GLA also affected their survival. Compared to control, lizards in T group with lower survival rates were observed, although it was not statistically significant (χ^2 = 1.148, P = 0.284). Taken together, the survival rate was highest in the control, followed by the T group, and lowest in the HT group but raised in the H group.

Regarding to body weight, male lizards of control gradually gained weight throughout the study (Fig. 1b). However, the trend of the change of the body weight in the two high-temperature treatments (H and HT group) were similar, reaching a peak on 28 days, and then gradually declined. The body weight of the lizard in T group initially fluctuated and decreased after 42 days. It is worth noting that only the weights of the HT group was lower than them in the control significantly on day 56 (F = 3.38, df = 36, and P = 0.030). After 70 days, the body weights of all treatment groups (T, H, and HT) decreased distinctly, and fell to the minimum on the 90 day (F =5.78, df = 36, P = 0.011 for the T group, P = 0.035 for the H group, and P = 0.001 for the HT group). Because females were in the oviposition period, the body weight of them fluctuated greatly, so they were not considered. However, the female characteristics of clutch were analyzed.

3.2 Characteristics of clutch

The characteristics of clutch (spawning date and egg mass) are recorded in the Fig. 2 and Table. S4. The spawning dates (Fig. 2a) of two high temperature treatments (H and HT) were significantly

earlier than that of control group ($F = 22.21$, $df = 38$, $P < 0.001$ for H and HT group), and HT group was earlier than T group ($F = 22.21$, $df = 38$, and $P < 0.001$). These result indicated that exposure to high temperature could advance the spawning period, and the adverse effects of L-GLA on spawning were strengthened at high temperature. In addition, the spawning dates of the control group were relatively concentrated, and most of the eggs were laid during 51-66 days. However, in the T group, the date became more scattered and 6 eggs were produced during 32-85 days suggesting that endocrine cycle of lizard might be changed after L-GLA exposure. For the egg mass (Fig. 2b), the average mass of the three treatment groups (T, H, and HT) were lower than those of control, although there was no significant difference. Due to the adverse effects on spawning were observed, semen analysis in males was conducted in this study (the specific method and of semen analysis was shown in the supporting information) and the results are listed in Fig. S1 and S2.

3.3 Hormone levels controlled by neuroendocrine system

3.3.1 Plasma sex-steroid and Luteinizing hormone (LH)

It is common knowledge that hormonal induction of spawning is a technique that promotes the timed release of egg and sperm for fertilization (Cardone et al., 2008; Pandey et al., 2017a; Vu et al., 2017). Therefore sex hormone including testosterone (T), estradiol (E_2) and, progesterone (Pg) (T for males, E_2 and Pg for females) have been investigated. Fig. 3a-c show the mean levels of T, E_2, and Pg in the control, T, H, and HT groups. The high plasma sex hormone level (T: 2.99 ± 0.21 ng/mL, E_2: 197.38 ± 11.72 pg/mL, and Pg: 4.39 ± 0.07 ng/mL) were observed in control lizards. In all treatment groups, sex hormone levels were decreased distinctively (T: $F = 11.39$, $df = 20$, $P = 0.002$, $P < 0.001$, and $P < 0.001$ for T, H, and HT, respectively; E_2: $F = 20.20$, $df = 20$, $P = 0.08$, $P < 0.001$, and $P < 0.001$ for T, H, and HT, respectively; Pg: $F = 16.17$, $df = 20$, and $P < 0.001$ for T,

177 H, and HT), suggesting that exposure to L-GLA, high temperature or a combination of both could
178 lead to a reduction of sex hormones. On the one hand, the low T level caused the poor semen quality
179 and the weak sexual desire in males (Pandey et al., 2017b). On the other hand, the decrease of E_2
180 and Pg could disturb mating behavior, reduce the quality of ovum, and affect embryonic
181 development (Pandey et al., 2017a).
182 Sex steroids are regulated by pituitary gonadotropins like LH (Pandey et al., 2017a), so the level of
183 plasma LH was determined (Fig. 3d). Compared to control, LH level showed reduction in females
184 in the HT group ($F = 3.42$, $df = 20$; $P = 0.023$). For males, LH in all treatment groups were lower
185 than that in control ($F = 12.45$, $df = 20$; $P = 0.043$, $P < 0.001$, and $P < 0.001$ for T, H, and HT,
186 respectively). Moreover, compared to T, marked decrease of LH level in HT were observed in both
187 sexes ($F = 12.45$, $df = 20$, $P = 0.013$ for male; $F = 3.42$, $df = 20$; $P = 0.045$ for female), suggesting
188 that temperature influenced the toxicity of L-GLA to the lizards. Specifically, high temperature
189 enhances the toxic effects of GLA inducing reduction of pituitary gonadotropins LH level and
190 reduced secretion of LH further caused a decrease in sex hormone levels

191 **3.3.2 Neurotransmitters and related enzymes**

192 Gonadotropins as well as gonadal steroids are under regulation of the neuroendocrine pathway
193 hypothalamus - pituitary - gonadal (HPG) axis (Pandey et al., 2017a). In addition, the neural control
194 of LH is multifactorial, involving a multitude of classical neurotransmitters (Niladri et al., 2009).
195 Previous study has pointed out that dopamine (DA) has a clear inhibitory role in LH release among
196 teleosts, mammals, and amphibians through multiple mechanisms (Chang et al., 1990; Habibi et al.,
197 1989; Quigley et al., 1981; Vu et al., 2017). Conversely, gamma-aminobutyric acid (GABA)
198 stimulates the produce of LH and plays a positive role in reproduction (Martyniuk and Chang, 2010).

Accordingly, the contents of neurotransmitters involving DA, GABA, and enzymes related to their synthesis and metabolism in brain tissues were determined.

Compared to control, an increase in DA content were observed only in the high temperature groups (H and HT) in males (Fig. 4a $F = 21.11$, $df = 16$, $P < 0.001$ for H and HT). Monoamine oxidase (MAO), a single molecular enzyme with multiple binding sites, could catabolized DA through oxidative deamination (Niladri et al., 2009). Here we found High temperature significantly inhibited MAO activity by nearly 57% - 75% and 62% - 67% of controls for males and females, respectively (Fig. S5a) (In males, $F = 14.22$, $df = 12$, $P = 0.002$ and $P < 0.001$ for H and HT, respectively. In females, $F = 13.84$, $df = 12$, $P < 0.001$ and $P = 0.001$ for H and HT, respectively). Additionally, the MAO activity in HT was lower than that in T ($F = 14.22$, $df = 12$, $P = 0.001$ and $F = 13.84$, $df = 12$, $P = 0.003$ for males and females, respectively). Reduced metabolic activity of MAO would result in the accumulation of synaptic dopamine, these results further indicated that high temperature can possibly impede lizard reproduction by promoting dopaminergic neurotransmission.

On the contrary to the antireproductive effects of DA, GABA can stimulate LH release. Our findings suggest that GABA content were decreased significantly in all treatment groups (Fig. 4b) (In males, $F = 19.82$, $df = 16$, $P < 0.001$ for T, H and HT; In females, $F = 13.84$, $df = 16$, $P < 0.001$ for T, H and HT). Synaptic levels of GABA are tightly regulated by two metabolic enzymes: GABA can be catalyzed by glutamic acid decarboxylase (GAD) while catabolized by GABA-transaminase (GABA-T) (Niladri et al., 2009). Here we found the reduction of GAD activity only in the T group males (Fig. S5b) ($F = 2.699$, $df = 12$, $P = 0.01$). For the activity of GABA-T, the obvious decrease were observed in in males in all treatment groups and in females in the HT groups (Fig. S5c) (In males, $F = 5.132$, $df = 12$, $P= 0.030$, 0.027, and 0.011 for T, H, and HT groups, respectively; In

females, F = 5.124, df = 12, P= 0.013). Taken together, the increase in MAO activity and decrease in GABA activity inhibited the release of LH from pituitary in lizard.

3.4 Influence of L-GLA degradation in soil and the accumulation in gonads and eggs under thermal stress

Our findings have revealed the effects of L-GLA and high temperature on the reproduction of lizards regulated by neuroendocrine system. As we well know, temperature not only affects the soil behavior of pesticides, but also changes the metabolic rate in animals. Therefore, soil degradation dynamics of L-GLA during 90 days and accumulation of L-GLA in gonads and eggs were determined (Fig. 5). The nominal exposure concentration of L-GLA in the soil is 13.34 mg/kg while the quantified concentrations were 12.65 and 12.76 mg/kg in T and HT, respectively. Generally, the concentration of the two groups decreased with time. The concentration of L-GLA in HT was a significant decrease especially from the third day to the end of the study compared with the concentration in T group ($t = 3.298$, $df = 36$, and $P = 0.002$) suggesting that the degradation of L-GLA in soil was accelerated by high temperature.

With regard to the accumulation of L-GLA in gonads and eggs, the concentration in T were higher than that in HT (For testis, $t = 3.419$, $df = 12$, and $P = 0.005$; For ovary, $t = 3.389$, $df = 12$, and $P = 0.005$; For egg, $t = 23.43$, $df = 12$, and $P < 0.001$). In the HT group, the highest L-GLA content was found in the testis ($F = 30.335$, $df_1 = 2$, $df_2 = 6$). It is worth noting that in the T group, L-GLA content was increased significantly in egg compared to gonads ($F = 127.034$, $df_1 = 2$, $df_2 = 6$) indicating that maternal GLA exposure may bring the high exposure risk to their offspring.

4. Discussion

4.1 L-GLA exposure disrupt reproduction controlled by neuroendocrine system of lizard

Some studies have shown that vertebrate reproduction is controlled by the brain through a tightly regulated HPG communication axis (Lee et al., 2018; Niladri et al., 2009; Pandey et al., 2017a). However, few studies have reported on the chronic reproductive toxicity effects of pesticide in lizard, especially on the HPG axis. The contents of neurotransmitter (DA and GABA) and the activity of related enzymes (GAD, GABA-T, and MAO) in lizard' brain were measured. After exposure to L-GLA, the content of DA remained basically unchanged while a significant decrease of GABA content was observed. DA and GABA are key regulators of gonadotropin release： DA is the inhibitory neurotransmitter controlling LH release, whereas, LH release could be stimulated by GABA in some species (Nealperry et al., 2008; Niladri et al., 2009; Trudeau et al., 2000). Additionally, the level of GABA is strictly regulated by GAD and GABA-T (Jayakumar et al., 1999). In this study, the activity of GABA-T showing an elevation in males in the T group which should be responsible for the reduction of the content of GABA. The serum LH release was also determined and decrease of LH content in both sex was found. Lower LH levels further reduce sex steroid secretion and the level of Pg, E_2, and T in plasma were decreased significantly. T plays an important role in the process of spermatogenesis and the low T level could be harmful to spermatogenesis (Yin et al., 2016), thus, semen analysis and testicular histopathology were conducted. Indeed, the quality of sperm was declined from the result of semen analysis, a shedding of germ cells and thinning interstitial tissue was observed from histopathology sections. On the other hand, abnormal steroid levels may interfere with the ovulation cycle leading to the dispersion of spawning date. In addition, according to previous study, animals with lower sex hormone content are often insufficient to stimulate the brain to produce sexual desire, which affected the mating frequency and resulted in the fewer offspring (Pandey et al., 2017b). It is worth noting that the accumulation of L-GLA in egg

was much higher than that in testis and ovary indicating that eggs are more likely to accumulate L-GLA, which may threaten the growth and development of offspring. Overall, the present study revealed that exposures to L-GLA caused impairment of the HPG axis and disrupt the reproduction, which may have profound influence on the offspring.

4.2 Thermal effects on reproduction of lizards

In present study, reduced survival rate was found in the high temperature treatment groups and the survival rate became lower and lower with the prolongation of exposure time. Exactly, previous study has emphasized that lizard extinction would be enhanced and mortality of turtle larvae increased as a consequence of global warming (Barry et al., 2010b; Tedeschi et al., 2016). One of the strategies for species survival in a warming world is changing life-history traits such as the timing of reproduction to response to the changed environment (Tedeschi et al., 2016). In this study, spawning date in high temperature group was much earlier than that in the control and T group, which could be an adaptive strategy giving offspring more time to grow and develop before hibernation. In addition, at the molecular level, physiological responses to thermal stress often accompanied by the expression of heat shock protein and the level of HSP 70 (the detailed results of HSP 70 were shown in supporting information) in lizard plasma were elevated distinctly in the H group. Activation of HSP is considered to be a protective mechanism to respond to thermal stress and it would enhance heat tolerance of reptile embryos(Gao et al., 2014). Nevertheless, negative effects caused by high temperature in lizard' reproduction was mainly concentrated on four aspects. First, the damage of high temperature on sperm have been widely reported in many species (Chu et al., 2012; Hurley et al., 2018; Ward et al., 2018). In present study, the sperm qualities including sperm concentration, vitality, and deformity rate were severely affected by thermal stress. Secondly,

the levels of plasma steroids were declined under high temperature through the HPG axis regulation. Thirdly, the increased content of ROS and 8-OHdG (the detailed results were shown in supporting information) indicated that testis and ovary were suffered from oxidative stress after high temperature treatment, which is one of the factors inducing the expression of HSP (Khosravi-Katuli et al., 2018). Finally, according to the previous reports, sexual behaviors are modulated by environmental factors like temperature. Fewer hours of activity of animals and diminished energetic resources under warming world would lead to adverse effects on reproduction, although these factors were not taken into account in this experimental design. Moreover, in most ectothermic species, physiological characteristic exhibit some temperature dependence consisting of endurance, metabolic rate and digestive efficiency(Angilletta et al.; Gilbert and Miles, 2016). In this study, lower concentration of L-GLA in lizard' gonad in the HT group compared to control may be attributed to the higher metabolic rate. Temperature can not only alter the physiological characteristics of animals, but also change the environmental behavior of pesticides (Broznić et al., 2012). Indeed, the faster degradation of L-GLA at high temperature was observed in this study.

4.3 Interaction of temperature and L-GLA on reproductive toxicity of lizards

Previous report expected that the sensitivity of reptiles to a pesticide might vary with temperature because of the property of poikilothermic vertebrate (Talent, 2010). However, previous studies of relationship of temperature and sensitivity to contaminants were mainly focused on acute toxicity (Jegede et al., 2017; Lau et al., 2015). Chronic effect especially on reproduction of lizard has been poorly investigated. This study has shown that temperature influenced the toxicity of L-GLA on lizards (*Eremias argus*) and compared to 25°C, L-GLA has greater damage to lizards' reproduction at 30°C. Specifically, less sperm quantity, higher sperm deformity rate, lower level LH, and more

serious oxidative damage in gonads were observed in the HT group, compared to the T group, which was intuitively shown in the star plots for biomarker responses (Fig. S8 and Table S5. The detailed result of Integrated Biomarker Response (IBR) were shown in the supporting information). Because few studies of this kind had been performed with *E. argus* so far, we also compared with other organism which also could be exposed to pesticides. Jegede et al. observed that organophosphorus pesticide (dimethoate and chlorpyrifos) would pose higher risk to acarus under high temperatures (Jegede et al., 2017). In addition, Edwared et al. pointed out that the toxicity of pesticides on amphibian may be significantly amplified at higher temperatures (Lau et al., 2015). Previous reports and the results in this study re-emphasize that temperature is an important abiotic factor which may alter environmental behavior of pesticides and physiological characteristics of organisms. Therefore, temperature effects should not be overlooked in ecotoxicological studies and derivation of safety limits in environmental risk assessment and management.

5. Conclusion

The current study elucidated that L-GLA could damage the reproduction of lizards through axis. Specifically, after L-GLA exposure, changes of the content of neurotransmitters in lizard' brain inhibited the secretion of gonadotropin LH from pituitary, reduced the levels of sex hormones and finally lead to lower quality of semen and fertilization rate. Moreover, temperature is an important abiotic factor, which can alter the degradation rate of L-GLA in soil and the metabolic rate of organism, thus enhancing the toxicity of pesticides. The results in present study revealed that non-target organisms like lizards were put at higher risk of pesticide exposure in the warming world. Therefore, thermal effects should be taken into account in the future ecotoxicological studies.

Acknowledgments

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Figure captions

Fig. 1. Survival rates (a) and males body weight (b) during the 90 days for control, T, H, and HT group. Bars indicate standard deviation (SD). * represents a significant difference compared to control at each sampling time.

Fig. 2. Spawing date (a) and egg mass (b) during 90 days for control, T, H, and HT group. Bars indicate standard deviation (SD). * represents a significant difference compared to control. # represents a significant difference compared to the T group.

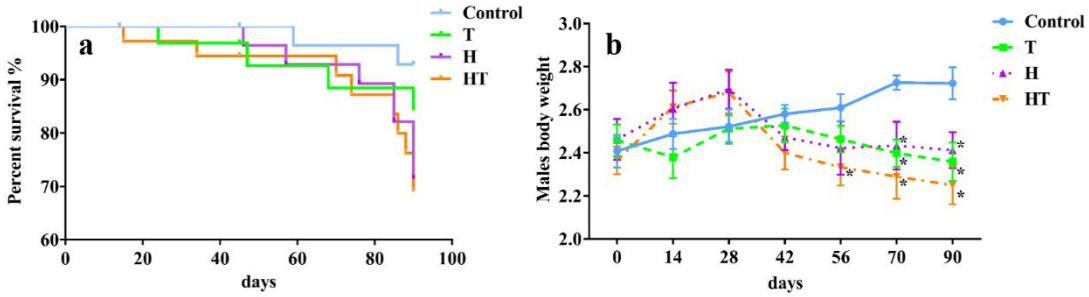
Fig. 3. The levels of hormones in plasma: testosterone (T) (a), estradiol (E2) (b), progesterone (Pg) (c) (T for males, E2 and Pg for females) and (LH) (d) luteinizing hormone (for both sex) during 60 days for control, T, H, and HT group. Bars indicate standard deviation (SD). * represents a significant difference compared to control. # represents a significant difference compared to the T group.

Fig. 4. The content of neurotransmitters in brain tissues: dopamine (DA) (a) and gamma-aminobutyric acid (GABA) (b) during 60 days for control, T, H, and HT group. Bars indicate standard deviation (SD). * represents a significant difference compared to control. # represents a

significant difference compared to the T group.

Fig. 5. The degradation of L-GLA in soil (a) and accumulation in testis, ovary and egg (b) during 90 days. Bars indicate standard deviation (SD). * represents a significant difference compared to the T group at each sampling time. Different uppercase letters indicate a statistically significant difference between different tissues in the HT group. Different lower case letters indicate a statistically significant difference between different tissues in the T group.

492 Figure 1



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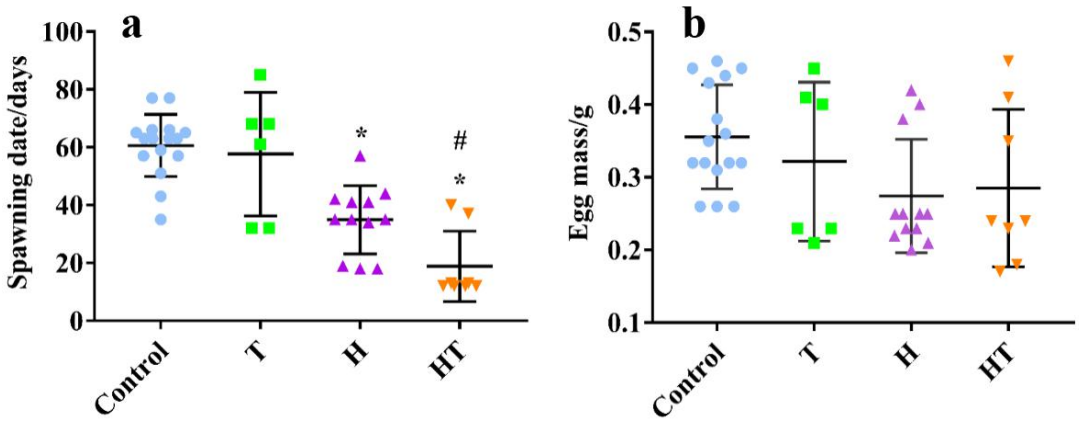
495 Figure 2



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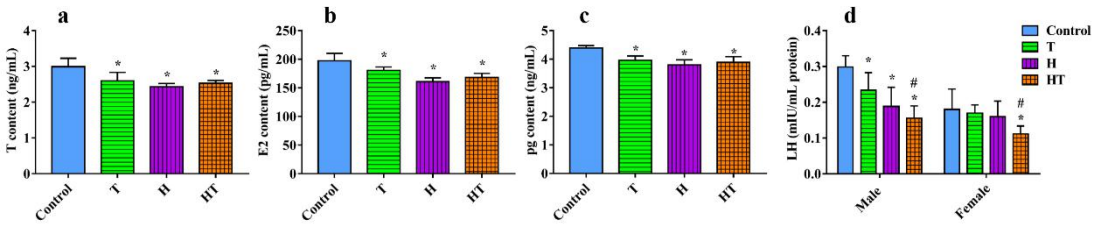
498 Figure 3



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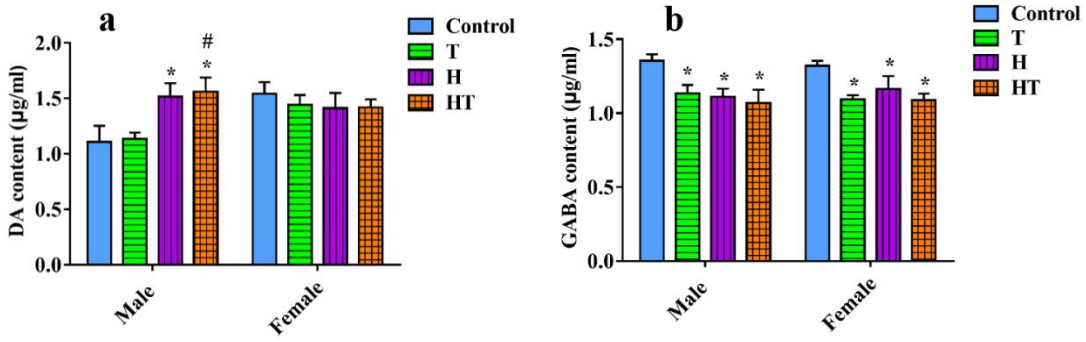
501 Figure 4



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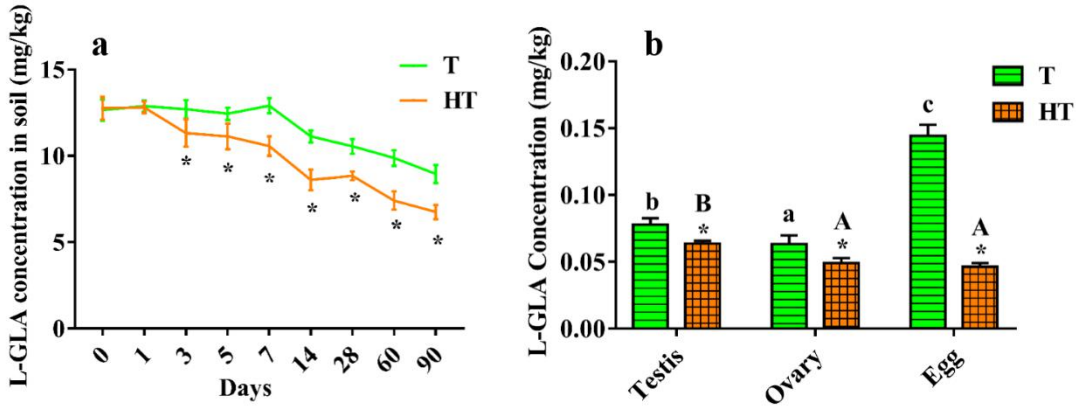
504 Figure 5



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Conflict of interest

I declare that this work does not create a conflict of interest with any other organization or individual.

Supporting Information

Effects of L-Glufosinate-ammonium and temperature on reproduction controlled by neuroendocrine system in lizard (*Eremias argus*)

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Table S1

Calculation of predicted environmental concentration (PEC) and determination of experimental concentration

Calculation parameter	
Soil depth (d)	0.05 m
Bulk density (D)	900 kg/m^3
Volume (V) = d × length (l) × breadth (b)	0.05 × 1 × 1 = 0.05 m^3
Mass (M) = D × V	0.05 × 900 = 45 kg
Application rate (a.r)	3000 g/ha
a.r converted to g/m^2 (1 ha = 10000 m^2)	3000/10000 = 0.3 g/m^3
PEC in mg/kg (Given 1m^2 = 45 kg of soil)	0.3/45 = 6.67 mg/kg
PEC	6.67 mg/kg
Experimental concentration (2×PEC : application more than once per season)	13.34 mg/kg

Table S2 The results of method recovery for L-GLA.

Matrix	Fortification	Recovery (%)	Matrix	Fortification	Recovery (%)
Soil	0.5	90.75±2.40	Ovary	0.04	91.40±1.88
	5	88.13±3.37		0.4	95.52±5.54
	15	94.22±2.62		4	84.14±1.93
Testis	0.04	84.06±2.19	Egg	0.04	92.21±0.82
	0.4	84.45±1.59		0.4	87.80±5.27
	4	96.03±0.72		4	87.36±6.10

Table S3 The results of method recovery for neurotransmitters (dopamine (DA) and gamma-aminobutyric acid (GABA)) in brains.

Neurotransmitter	Fortification	Recovery (%)	Neurotransmitter	Fortification	Recovery (%)
DA	0.015	89.42±1.19	GABA	0.015	88.33±2.00
	0.15	95.35±1.30		0.15	91.62±0.90
	1.5	95.82±0.96		1.5	93.62±1.41

Table S4 The characteristics of clutch including spawning date, number of eggs, egg mass, and fertilization rate are listed in the Table. T: L-GLA polluted soil group, H: high temperature without L-GLA soil group, HT: high temperature with L-GLA polluted soil group.

Treatment groups	Commence of egg-laying	Ending of egg-laying	Egg mass (g)
Control	20/05	01/07	0.35 ±0.06
T	17/05	09/07	0.32 ±0.09
H	03/05	11/06	0.27 ±0.07
HT	27/04	25/05	0.28 ±0.10

Semen analysis

1. The specific method of semen analysis

The quality assessment of the lizard's fresh semen is mainly to evaluate whether the testis and epididymis function is normal, and thus can predict the sperm fertilization ability. Firstly, lizards were sacrificed by freezing anesthesia and collected right testes and epididymides immediately. Then, cut an opening at the end of the epididymis, gently squeeze the epididymis with sterile tweezers, and make the semen flow into the 1.5 mL Eppendorf tube. Finally, 10μL semen were transferred to a tube containing 190μL 1×PBS Eppendorf tube, and mix well to make a 20-fold diluted semen dilution.

1.1 Semen physical properties

Normal semen should be a milky white viscous liquid. If the color of the semen is abnormal, it indicates that there is a lesion in the lizard's reproductive organs: semen with rare sperm is whey-clear, suggesting that it may have epididymitis or testicular dysplasia; if the genital tract has bleeding, the semen has a reddish color; if there is inflammation in the genital tract, the semen may contain lumps or flocculents.

1.2 Sperm concentration

Sperm concentration (number of sperm contained per ml of semen) was determined by blood cell counting chamber. Blood cell counting chamber was placed on the microscope stage and covered with the coverslip. The diluted semen was dripped by a sterile pipette to the edge of the coverslip. Microscopic examination was performed by an Olympus BX43 light microscope (at a 400×). The

number of spermatozoa in the five squares (N) (80 small squares) located at the four corners and the center of the counting room (the sperm within the small square and pressed on the left and upper lines) were recorded. The sperm concentration calculation formula is as follows:

Where 5 represent the total number of spermatozoa in the 25 squares; 10 represent the total number of sperm in 1mm3; 1000 represent the total number of sperm in 1ml diluted semen; 20 represent dilution factor.

The sperm quantity calculation formula is as follows:

Where 0.2 represent the total volume of diluted semen.

1.3 Sperm vitality

Sperm vitality (the number of sperm in a linear motion as a percentage of the total number of sperm) was also determined by blood cell counting chamber. The number of sperm in a linear motion (A) was counted based on the determination of sperm concentration. The sperm vitality calculation formula is as follows:

1.4 Sperm deformity rate

Sperm deformity rate (the number of sperm with abnormal morphology and structure as a percentage of the total number) were investigated by Giemsa staining. Ten μL of semen was dropped onto a glass slide to make a semen smear. Sperm morphology was observed by Olympus BX43 light microscope (×1000 magnification) after stained with Giemsa. Three semen smears

were made for each animal, and 300 sperm were recorded for each smear.

$$= \frac{\qquad\qquad}{\qquad\qquad} \times \%$$

2. The results of semen analysis

Thirteen semen samples were obtained during this study (Control = 4, T = 1, H = 4, and HT =4). Each sample was measured three times and the result was expressed as the average of three measurements. The three semen sample that are missed in the T group were due to problems in the availability of the semen. A possible explanation is that the semen secretion cycle of lizard become more dispersed after L-GLA exposure as same as the result of spawning date. Semen secretion could not be concentrated in the same period, which made it impossible to obtain four semen samples at the same time. Therefore, the result in the T group was expressed as three measurements of a semen sample.

As for the semen physical properties, animals' semen in all groups were normal milky white viscous liquid. Through the semen analysis, no obvious difference sperm concentration and quantity could be found in control and T (Fig. S1a and 1b). However, compared to control, great changes have taken place in H and HT ($F = 24.3$, $df = 11$, $P = 0.002$ and $P < 0.001$ for H and HT, respectively). Moreover, sperm concentration and quantity in HT was lower than them in T ($F = 24.3$, $df = 11$, $P < 0.001$). The results have shown that the concentration and quantity of sperm were greatly reduced under high temperature. In addition, compared with exposure to L-GLA at 25 ± 2 °C, high temperature enhanced the toxicity effect of L-GLA on sperm. Regarding changes in sperm vitality (Fig. S1c), all treatment groups comprised in T, H and HT were reduced significantly compared to control ($F = 64.47$, $df = 11$, $P < 0.001$, $P = 0.007$, and $P < 0.001$ for T, H,

and HT, respectively).

The sperm deformity rate is shown in Figure. S2 and the observed sperm are classified into 3 types: normal, abnormal head, and abnormal tail (Fig. S3). In all treatment groups, the sperm head deformity rate were higher than that in control (Fig. S2a) ($F = 31.11$, $df = 11$, $P = 0.006$, $P < 0.001$, and $P < 0.001$ for T, H, and HT, respectively). The result of total deformity rate (sperm head deformity rate + sperm tail deformity rate) (Fig. S2c) was consistent with the head deformity rate ($F = 34.88$, $df = 11$, $P = 0.020$, $P < 0.001$, and $P < 0.001$ for T, H, and HT, respectively). Compare to the T group, sperm deformity comprised in head, tail, and total deformity in HT were declined significantly ($F = 31.11$, $df = 11$ and $P < 0.001$ for head deformity; $F = 3.722$, $df = 11$ and $P = 0.030$ for tail deformity; $F = 34.88$, $df = 11$ and $P < 0.001$ for the total deformity) indicating that high temperature enhanced the ability of L-GLA deforming sperm.

Additionally, the results of testes histopathological analysis were listed in Fig. S4. Lizards exhibited normal histological features in the testes in control (Fig. S4a), with successive stages of spermatogenesis in seminiferous tubules and many normal seminiferous tubules with clear tubule wall were also observed. However, in the T, H, and HT groups (Fig. S4b, c and d), deformation of the seminiferous tubules, severe shedding of germ cells and thinning interstitial tissue were observed.

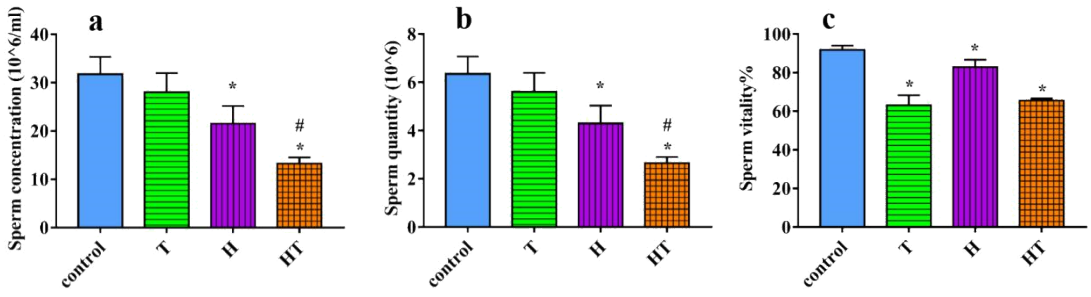

Fig. S1. Sperm concentration (a), sperm quantity (b), and sperm vitality (c) during 14 days for control, T (L-GLA polluted soil group), H (high temperature without L-GLA soil group), HT (high temperature with L-GLA polluted soil group). Bars indicate standard deviation (SD). * represents a significant difference compared to control. # represents a significant difference compared to the T group.

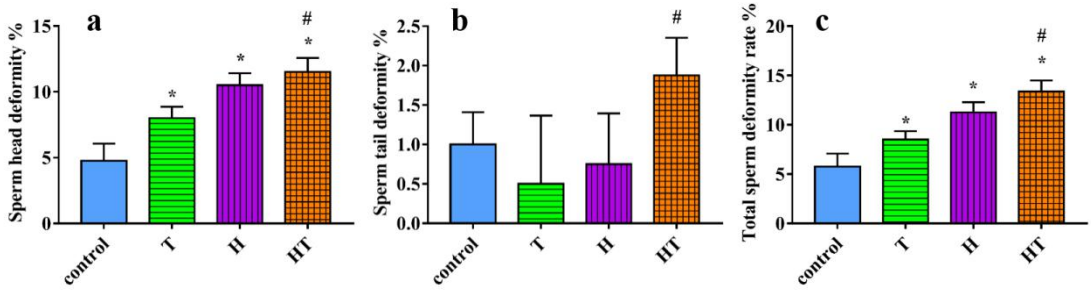

Fig. S2. Sperm head deformity (a), sperm tail deformity (b), and total sperm deformity (c) during 14 days for control, T (L-GLA polluted soil group), H (high temperature without L-GLA soil group), HT (high temperature with L-GLA polluted soil group). Bars indicate standard deviation (SD). * represents a significant difference compared to control. # represents a significant diffe rence compared to the T group.

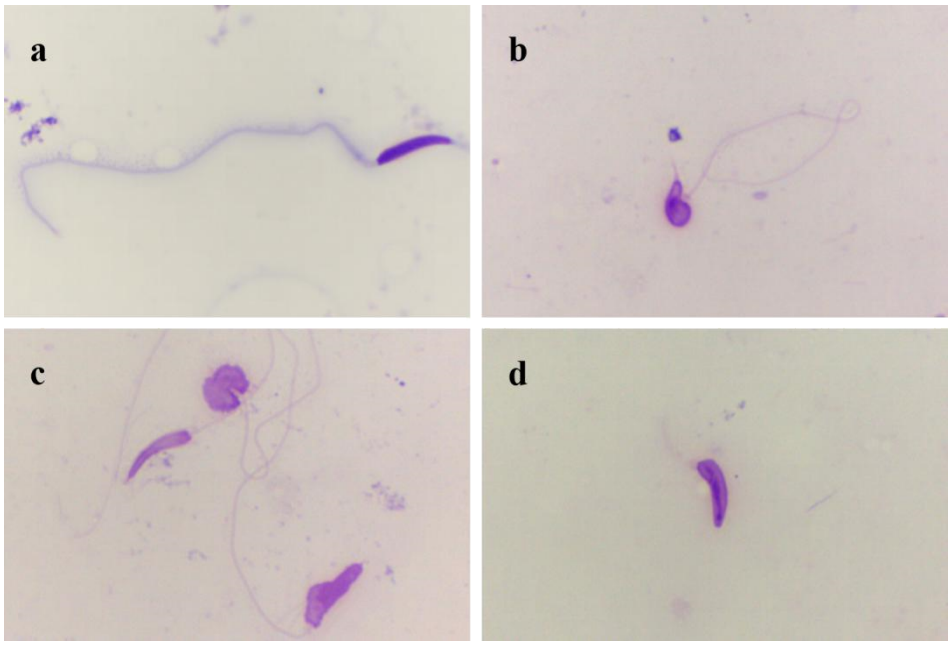

Fig. S3. Sperm classified into 3 types: normal (a), abnormal head (b and c), and abnormal tail (loss-tail) (d)

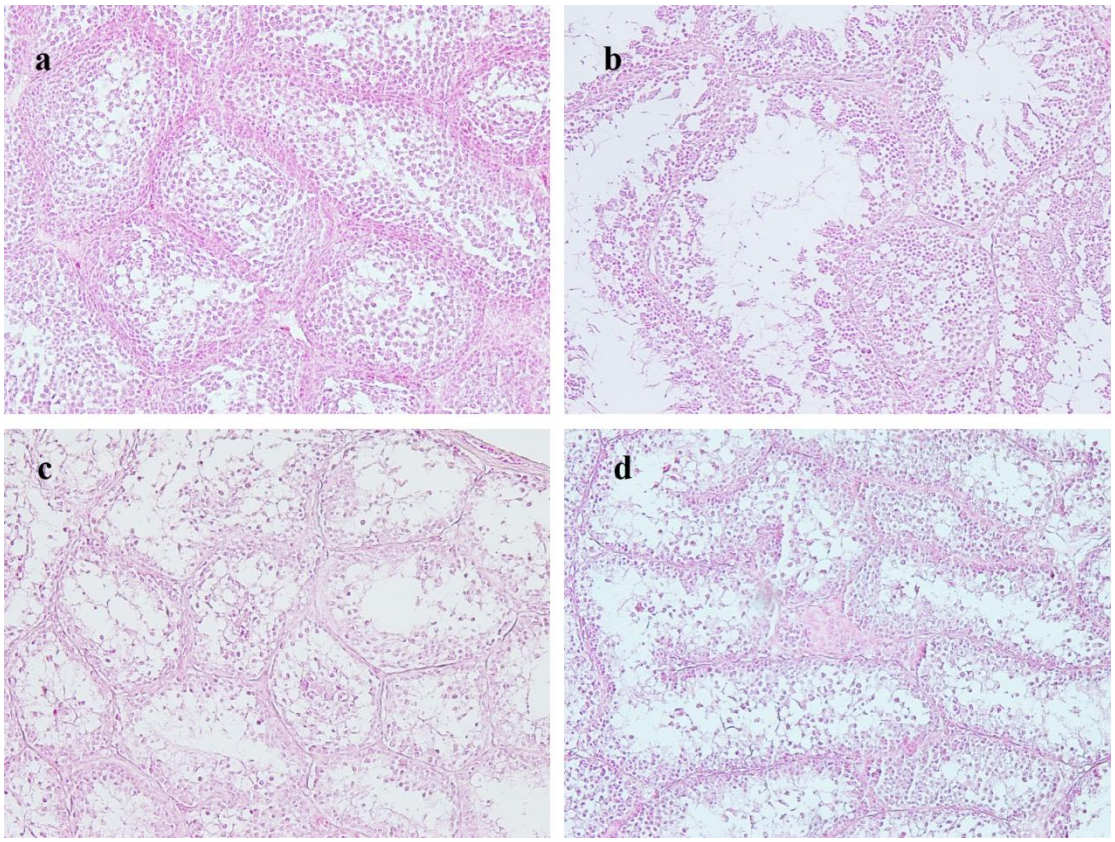

Fig. S4. Histopathological sections of testes with hematoxylin and eosin staining for control (a), T (L-GLA polluted soil group) (b), H (high temperature without L-GLA soil group) (c), and HT (high temperature with L-GLA polluted soil group) (d), at 200× magnification. Only representative photos are shown.

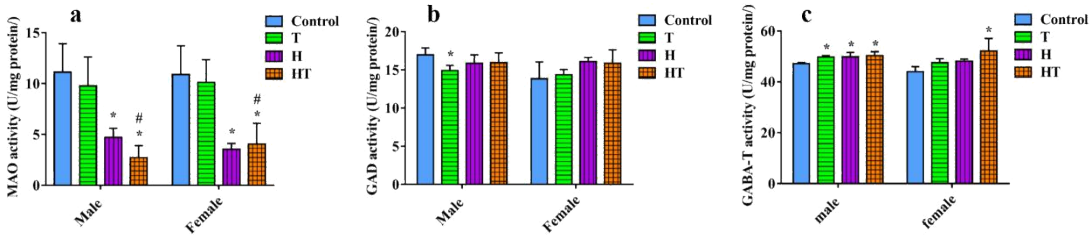

Fig. S5. The activity of neurotransmitters related enzymes involving Monoamine oxidase (MAO, Fig. a), Glutamate decarboxylase (GAD, Fig. b), and Gamma-aminobutyric acid transaminase (GABA-T, Fig. c). * indicate a statistically significant difference ($P < 0.05$) compared to control; # indicate a statistically significant difference ($P < 0.05$) compared to the T group.

Heat shock protein 70

Physiological responses to thermal stress begin at the molecular level, frequently with the expression of heat-shock protein (HSP) that mitigate damage to membranes, proteins and DNA [1]. Moreover, exposure to different exogenous pollutants leads to the synthesis of HSP [2]. HSP 70, investigated in this study, is used as general stress response for monitoring environmental stressors (Fig. S6). The content of HSP protein had a similar trend in gonads (Fig. S6a) and plasma (Fig. S6b): rose after exposure to L-GLA and increased further under high temperature condition, whereas decreased exposure to combination of L-GLA and high temperature (In testis, $F = 18.33$, $df = 12$, $P = 0.012$, 0.024, and 0.046 for T, H, and HT groups, respectively; In ovary, $F = 26.64$, $df = 12$, $P < 0.001$, and $P = 0.028$ for H and HT groups, respectively. For plasma, in males, $F = 23.19$, $df = 8$, $P = 0.004$, and < 0.001 for T and H groups; in females, $F = 4.226$, $df = 8$, $P= 0.020$, 0.027 for H group). In addition, Hsp 70 protein expression in the HT group was lower than that in the T group in gonads and males' plasma ($F = 18.33$, $df = 12$, $P < 0.001$ for gonads; $F = 23.19$, $df = 8$, $P = 0.010$ for males' plasma). These founding demonstrated that Hsp 70 protein expression could be induced by L-GLA and high temperature and this is likely an adaptive response to the damage caused by different environmental stressors. However, exposure to L-GLA under high temperature condition resulted in reduction of Hsp 70 expression. This may be due to the severe environmental stress beyond the defense capability of Hsp 70.

According to previous reports, oxygen radicals could cause hsp70 expression, and this increase is followed by oxidative stress [2-4]. Thus, reactive oxygen species (ROS) and 8-

hydroxydeoxyguanosine (8-OHdG) as oxidative stress biomarkers were measured (Fig. S7). Normally, the generation and scavenging of reactive oxygen species (ROS) are always in a dynamic balance. However, it leads to the accumulation of ROS if an exogenous stimulus breaks this balance [5]. In this study, distinctly increase of ROS levels were observed in high temperature groups (In males, $F = 9.61$, $df = 12$, $P = 0.010$ and $P = 0.002$ for H and HT, respectively; In females, $F = 17.06$, $df = 12$, $P = 0.002$ and $P < 0.001$ for H and HT, respectively). Moreover, in both sexes, ROS content in HT was higher than that in T ($F = 9.61$, $df = 12$, $P = 0.005$ and $F = 17.06$, $df = 12$, $P < 0.001$ for male and female respectively).

8-OHdG, which is one of the most vital products of DNA peroxidation, can aggravate DNA damage. The result of 8-OHdG content were similar to ROS: they showed an elevation in the high temperature groups (In males, $F = 2.487$, $df = 12$, $P = 0.025$ for HT; In females, $F = 11.43$, $df = 12$, $P = 0.003$ and $P = 0.002$ for H and HT, respectively). In females, 8-OHdG content in HT was higher than that in T ($F = 11.43$, $df = 12$ and $P = 0.004$). Taken together, high temperature broke the dynamic balance of ROS, inducing excessive ROS production, which led to increased 8-OHdG level.

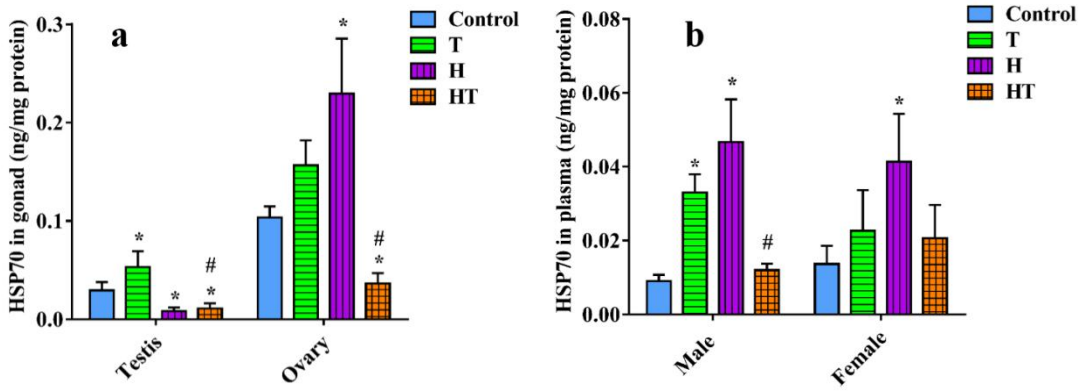

Fig. S6. The content of heat shock protein (HSP 70) in gonad (a) and plasma (b) during 30 days

for control, T (L-GLA polluted soil group), H (high temperature without L-GLA soil group), and HT (high temperature with L-GLA polluted soil group). Bars indicate standard deviation (SD). * represents a significant difference compared to control. # represents a significant difference compared to the T group.

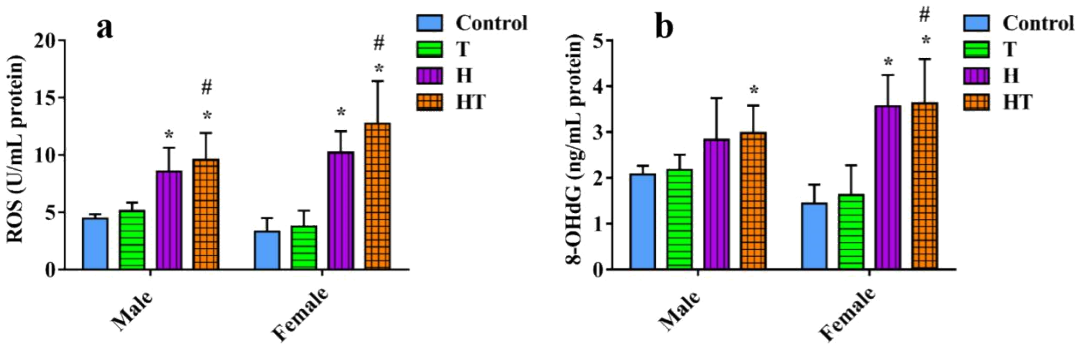

Fig. S7. The content of reactive oxygen species (ROS) (a) and 8-hydroxydeoxyguanosine (8-OHdG) (b). * indicate a statistically significant difference ($P < 0.05$) compared to control; # indicate a statistically significant difference ($P < 0.05$) compared to the T group.

Integrated Biomarker Response (IBR)

1. The detailed method of IBR calculation

The calculation is based on five major steps: (1) Biomarker data for all treatment groups were normalized with the control. (2) Calculate the standardisation of the mean value of each biomarker obtained for a condition, called X, using the mean value for all conditions (m) and the standard deviation for all conditions (s) to produce a value called Y: Y=(X-m)/s. (3) After this standardisation, we computed the Z value; Z = Y or Z = -Y whether an activation or an inhibition of the biomarker was expected in response to a contamination. (4) The value S was finally computed, with S = Z+|Min|, where Min is the minimal value observed for all exposure conditions for each biomarker, and finally plotted on a radar diagram. These S values thus represent the gradient of values for each biomarker in the different exposure conditions, with highest values corresponding to the highest biological effects. (5) The IBR corresponds the total area displayed by the radar diagram. Larger area indicates stronger biomarker responses and more serious the impact of chemicals on the organism.

2. The result 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 neurotransmitters-related enzyme (MAO, GAD, and GABA-T), gonadotropin and sex steroids (T, E_2, and Pg), and antioxidant system (ROS and 8-OHdG) were calculated and shown in Figure. S8 and the IBR score are listed in Table S5. Whether in the male or female, IBR value (radar diagram area) in the

HT group is much larger than that in the T group. Larger area indicates stronger biomarker responses and more serious the impact of chemicals on the organism [6]. Thus, our founding suggest that thermal stress aggravated the reproductive toxicity of L-GLA to lizards.

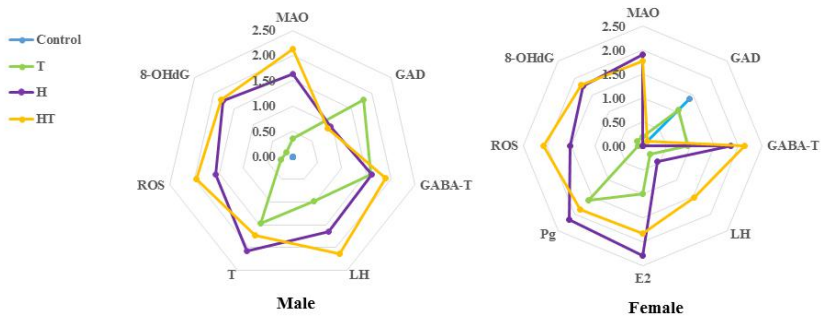

Fig. S8. Star plots for biomarker responses in male and female lizard. (MAO = monoamine oxidase; GAD = glutamic acid decarboxylase; GABA-T = gamma-aminobutyric acid transaminase; T = testosterone; E2 = estradiol; Pg = progesterone; ROS = reactive oxygen species; 8-OHdG = 8-hydroxy-2 deoxyguanosine).

Table S5 The scores of the integrated biomarker response (IBR). (MAO = monoamine oxidase; GAD = glutamic acid decarboxylase; GABA-T = gamma-aminobutyric acid transaminase; T = testosterone; E2 = estradiol; Pg = progesterone; ROS = reactive oxygen species; 8-OHdG = 8-hydroxy-2 deoxyguanosine).

	MAO	GAD	GABA-T	T	E_2	Pg	LH	ROS	8-OHdG
					Control				
Male	0	0	0	0			0	0	0
Female	0	1.39	0		0	0	0	0	0
					T				
Male	0.35	1.79	1.59	1.47			0.97	0.24	0.17
Female	0.2	1.07	0.94		1	1.59	0.23	0.10	0.15
					H				
Male	1.63	0.96	1.62	2.08			1.64	1.56	1.18
Female	1.91	0	1.84		2.28	2.18	0.45	1.51	1.76
					HT				
Male	2.13	0.98	1.90	1.72			2.14	1.96	1.41
Female	1.78	0.15	2.14		1.82	1.86	1.53	2.07	1.81

Precised description of extraction method of L-GLA

The tissue samples were placed into a 1.5 mL EP tubes with 0.5ml sodium borate buffer, 0.5ml acetonitrile, and grinding ball, homogenized for 3 minutes, and then centrifuged for 3min at 10000 rpm. The supernatant (0.5ml) was treated with 0.5ml sodium borate buffer and 0.5ml 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl). The mixture was derived in 40℃ water for 1h. Finally, the supernatant was filtered through a 0.22 μm filter before liquid chromatography–mass spectrometry (HPLC–MS/MS) analysis. GLA was detected by ThermoFisher TSQ Quantum Access MAX system (Tewksbury, Massachusetts, USA). The separation was achieved with A Hypersil GOLD C18 column (100 mm × 2.1 mm, 3 mm) at room temperature with the flow rate of 0.2 mL/min. The mobile phase was made up of 5 mM ammonium acetate, and acetonitrile (95/5, v/v) and the injection volume was 5μL. Mass spectrometry conditions refer to previous reports. Glufosinate-FMOC, the product derived from glufosinate was detected by multiple reaction monitoring (MRM), and 404 (m/z) was selected as the precursor ion. Product ion 182 (m/z) was used for quantification, and 208 (m/z) was used for qualitation. The collision energies of two product ions of 15 V and 10 V, respectively.

Precised description of extraction method of neurotransmitters (DA and GABA)

The fresh brain tissue samples were frozen with liquid nitrogen immediately and placed into 2 mL eppendorf tubes with 0.4ml methanol-water (V/V = 1/1), and grinding ball, homogenized for 3 minutes, and then centrifuged for 3min at 8000 rpm. The supernatant (100 μL) was treated with 200 μL acetonitrile, vortex mixed for 1 minute and then centrifuged again. The supernatant was

filtered through a 0.22 μm filter before liquid chromatography–mass spectrometry (HPLC–MS/MS) analysis. DA and GABA were detected by ThermoFisher TSQ Quantum Access MAX system (Tewksbury, Massachusetts, USA). The separation was achieved with A Hypersil GOLD C18 column (100 mm × 2.1 mm, 3 mm) with the flow rate of 0.2 mL/min. The mobile phase was made up of 0.1% formic acid - water, and acetonitrile (20/80, v/v) and the injection volume was 5μL. Mass spectrometry conditions refer to previous reports. For DA, 154 (m/z) was selected as the precursor ion. Product ion 137 (m/z) was used for quantification, and 119 (m/z) was used for qualitation. For GABA, 104 (m/z) was selected as the precursor ion. Product ion 87 (m/z) was used for quantification, and 69 (m/z) was used for qualitation. The collision energies of two product ions of 10 V, 18V and 8 V, 10V for DA and GABA, respectively.

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