1 **Glyphosate-based herbicide (GBH) challenged thermoregulation in lizards (*Eremias argus*),**

2 **but warming could compensate for it.**

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17   **Abstract**

18   The impacts of global climate change and its associated stressors on biodiversity are of mounting

19   concern. Glyphosate have attracted worldwide attention due to their ubiquitous occurrence, yet their

20   impact on reptiles remains unknown. Glyphosate-based herbicide (GBH) exposure in reptiles is

21   currently hampering efforts to predict how individual reptiles can buffer climate change. We

22   designed a simulated environmental exposure experiment over 60 days on the lizard (*Eremias*

23   *argus*). Preferred body temperature and active body temperature data were collected to calculate the

24   accuracy of thermoregulation, while liver detoxification metabolic enzymes, oxidative stress system

25   function, and the non-targeted metabolome of the brain tissue were assessed. Warmer-treated lizards

26   adjusted their physiological levels and behavioral strategies in response to increased ambient

27   temperatures and maintained body temperature homeostasis at moderate thermal perturbations.

28   GBH-treated lizards suffered from oxidative damage to the brain tissue and abnormal histidine

29   metabolism, thus their thermoregulatory accuracy reduced. Interestingly, at elevated ambient

30   temperatures, GBH treatment did not affect on their thermoregulatory, possibly through several

31   temperature-dependent detoxification mechanisms. Importantly, this data suggested that the subtle

32   toxicological effects of GBH may threaten increasingly thermoregulation behavior of *E. argus* with

33   species-wide repercussions, as climate change and exposure time extension.

34   **Keywords:** herbicide, global climate change, reptile, thermoregulation, temperature-dependent

35   toxicity, plastic response

36

1.Introduction

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38   Human activities have a profound impact on the natural world, from climate change and habitat

39   destruction to overfishing and the introduction of invasive species. According to reports from the

40   Intergovernmental Panel on Climate Change (IPCC), global warming is no longer in doubt and is

41   characterized by a rise in global average temperatures, with temperatures projected to rise by 2.6-

42   4.8°C by 2100 (RCP4.5 scenarios)(Stocker and D., 2013). Unfortunately, a large number of species

43   live in highly fragile environments, and many are reaching the limits of their ability to adapt to the

44   changing climate, which leads to local and global extinctions and a serious threat to biodiversity

45   (Sinervo et al., 2010a).

46   As ectotherms, reptiles are strongly affected by global warming, where environmental conditions

47   strongly influence their life history through critical behaviors and physiological processes. As there

48   is an optimum temperature range for physiological and biochemical reactions, too high or too low

49   body temperatures can disrupt an individual's metabolism and ultimately lead to death. For whole

50   populations or species, adverse environmental temperatures negatively affect ectothermic animals'

51   temperature regulation time, reproductive strategy, gender ratio, and survival ability of offspring

52   (Liu et al., 2022), ultimately leading to the extinction or evolution of a species. In the face of

53   changing environmental temperatures, reptiles mainly use two thermoregulatory methods, namely

54   behavioral and physiological thermoregulation (Bels, 2019). Behavioral thermoregulation is the

55   primary temperature regulation strategy adopted by most lizards and is an important strategy for

56   buffering climate fluctuations.

57   In addition to climate change, environmental contamination also threatens reptile populations,

58   resulting in the global decline of reptiles (Todd et al., 2010). Reptiles can be exposed to

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59   environmental pollutants through several routes, including ingestion of contaminated material,

60   dermal contact, maternal transfer to eggs and embryos, and incubating egg uptake from nest sites

61   (Rich and Talent, 2009). Exposure to toxic substances may impair an organism's ability to cope with

62   warming, while warming can also alter the effects of toxic substances on reptiles, as the physical

63   and bioactive properties of compounds change with temperature. This affects changes in their

64   environmental fate, bioavailability and degradation processes, and the sensitivity of organisms to

65   toxic substances will also change with body temperature.

66   Since the commercial product Roundup® with glyphosate (GLY) as the active ingredient was

67   produced in 1974 (Vijay K. Nandula, 2010), glyphosate-based herbicides (GBH) have become

68   popular due to their strong non-selective and high herbicidal activity. Especially with the large-scale

69   planting of genetically engineered glyphosate-tolerant crops around the world, glyphosate dominate

70   the global herbicide market (Benbrook, 2016). However, the application of large quantities of

71   glyphosate results in the accumulation of this chemical in farmland and surrounding soils and waters.

72   It may further enter the air, surface water, and groundwater through evaporation, leaching, and

73   surface runoff, thereby threatening biodiversity and ecological safety. Residues of glyphosate and

74   its metabolite aminomethylphosphonic acid (AMPA) have been detected in soils in different regions

75   all over the world (Laitinen et al., 2009; Maggi et al., 2020; Pelosi et al., 2022; Wee et al., 2021).

76   Glyphosate and its commercial formulations have adverse effects on environmental organisms such

77   as soil-living animals, aquatic organisms, amphibians, insects, and birds through oxidative stress,

78   genotoxicity, neurotoxicity and growth toxicity (Gill et al., 2018). In addition, commercial

79   formulations are actually thought to be more toxic than the glyphosate active ingredient alone. The

80   physiological stress caused by glyphosate herbicides is a new challenge for reptiles, which have

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81   evolved over hundreds of millions of years, and it is not known how reptiles will respond to this

82   emerging stress.

83   Chemical pollution and global warming are two major threats to reptiles, and these two factors can

84   interact with each other. This constitutes a key challenge for the realistic ecological risk assessment

85   and biodiversity research on the effects of global warming on reptile populations. Different studies

86   have demonstrated that reptiles can cope with global warming in various plastic ways, but exposure

87   to glyphosate herbicide pollution poses new challenges to the ability of lizards to resist climate

88   change-related stressors. We investigated thermoregulatory patterns in lizards in different thermal

89   and glyphosate-exposed environments and explored the underlying mechanisms behind their altered

90   thermoregulation. We hypothesized that: (1) Since reptiles, as the first truly terrestrial vertebrates

91   to adapt to terrestrial life, have various plastic abilities to adapt to complex and changeable land

92   habitats, small increases in ambient temperature would not pose a significant threat to these lizards,

93   and that they would alter their physiological state and behavioral activities to respond effectively to

94   thermal challenges; (2) within the GBH exposed habitats, the toxic effects of GBH will result in

95   impaired fitness and loss of the ability to precisely regulate body temperature in response to

96   increasing temperatures.

97   **2. Material and methods**

98   **2.1 Chemical reagents**

99   The Glyphosate-based herbicide, -Roundup used in this study, was purchased from local stores

100   (Beijing. China). The analytical-grade glyphosate (99.5%) was provided by Shenyang Research

101   Institute of chemical industry, AMPA (99%) was obtained from Macklin

102   Biochemical Technology Co., Ltd (Shanghai). 4-chloro-7-nitro-1,2,3-benzoxadiazole (NBD-Cl,

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103   98%) and other reagents were obtained from Sigma-Aldrich Co. (USA).

2.2 Experimental animals and husbandry

105   *Eremias argus* is a small live bearing lacertid lizard species inhabiting grasslands and has been

106   designated an endangered species by the Korean Ministry of Environment since 2005(Kim et al.,

107   2010). They are widely distributed, ranging from the Korean peninsula, Mongolia to certain areas

108   of Russia and China (Zhao et al., 1999). They commonly hibernate from October to March. Adult

109   female lizards were randomly selected from a semi-natural colony at China Agricultural University

110   after emergence and transferred to the laboratory. Lizards were housed in plastic incubators (58×

111   41×31 cm) with a layer of sand at the bottom. Water was supplied in glass containers situated

112   randomly throughout the incubator, and food (mealworms and crickets sprinkled with vitamin

113   powder) was provided daily. Each terrarium was divided into two equal-sized areas: one area with

114   a reflector bulb (25 W, Reptile Basking Spot Lamps;) hanging directly above it, is hereafter referred

115   to as the "warm" sector, while the other area supplied paper egg trays as shelters would be referred

116   to as the "cool" sector (Figure S1). The heating bulbs were sequentially on for 8 h/day between

117   09.00 and 17.00. Besides, LED lights provided a 12h light:12h dark cycle without heat. The lizards

118   were allowed to adapt to the experimental environment for one month before the start of the

119   experiment.

2.3 Experimental design

121   The experimental design had two treatment factors: GBH exposure (with or without GBH exposure)

122   × two temperatures (current climate treatment and warmer climate treatment). We altered the

123   ambient temperatures of the incubators by using reflector bulbs with two different intensities for the

124   two thermal treatments: one with a 25 W bulb above the terrariums, termed the current climate

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125 treatment (CT), and another treatment with a 50 W bulb above the terrariums, termed the warmer

126 climate treatment (WT). Electronic temperature data loggers (Apresys) were used to record the

127 temperature of the experimental terrarium every 20 minutes. Air temperatures were registered at 5

128 cm above the substrate in the middle of incubators. Soil temperatures mean the direct substrate

129 temperature in cool or warm sectors. Initial analyses showed that the mean air temperatures in CT

130 and WT terrariums were 26.99 and 30.18°C, respectively. We established a thermally heterogeneous

131 microhabitat within which the lizards can thermoregulate themselves in each treatment (Figure S2,

132 Table S1).

133 We contaminated the soil with 18 mg of glyphosate per kg soil. This method and concentration are

134 consistent with many ecotoxicology experiments on earthworms when these products are used as

135 directed. The commercial formulation of glyphosate (Roundup®) contains glyphosate (30%), The

136 recommended application of Roundup to field is 7.5L/ha, which equals 0.225ml a.i/m^2 of glyphosate.

137 We used twice as much as the label suggested to kill weeds in an area the size of our incubator, and

138 added the predetermined 0.36 mL of Roundup® to the appropriate incubators with 6kg soil sand,

139 mixing the soil thoroughly. This resulted in a nominal initial concentration of approximately 18 mg

140 of glyphosate per kg soil (twice the recommended dose). This amount is within the normal range of

141 soil glyphosate concentrations used in most published toxicological studies.

142 Thus, there were 4 treatments in the study: Control group (CT-CK), GBH polluted soil group (CT-

143 GBH), high temperature without GBH soil group (WT-CK), and high temperature with GBH

144 polluted soil group (WT-GBH) (Table S2). All lizards were randomly separated into four groups

145 (18 females per group) for 60 days (lizards were separated into 12 plastic incubators, three

146 incubators for each group with three replicates, and six females per incubators).

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147 After exposure for eight weeks, lizards were sacrificed by rapid decapitation after anesthesia. Their

148 brain and liver tissue were dissected, weighed, and collected in 2 mL tubes. Blood was collected in

149 an EDTA-2K tube, and plasma was collected by centrifugation. Samples for chemical or

150 biochemical assays were stored at -80 °C until further use. Animal experiments and protocols were

151 conducted under strict adherence to the principles for good laboratory animal care and were

152 followed the ethical guidelines set by China Agricultural University.

153 **2.4 Behavioral observation**

154 The thermal microhabitat preference (i.e., which sector lizards were observed in) and behavioral

155 patterns of lizards were evaluated at the end of the experiment (either after 59 or 60 days). A digital

156 camera (HERO8, GoPro Inc., capturing one frame every two seconds) was used to photograph the

157 lizard from above during their active period (from 09:00 to 17:00). The image sequences were

158 extracted every 5 min to examine the behavior of lizards. The lizard's location and behaviors were

159 classified into the following patterns: (1) cold sector: the lizard was hiding in the shelter (invisible)

160 or immobile on the ground; (2) moving: walking across the terrarium (noticeable ghosting and

161 blurry profiles of lizards can be observed because of fast-paced walking or running); (3) immobile

162 in the warm sector: An inactive lizard (not moving, scratching or basking) was defined as being

163 immobile; (4) basking in the warm sector: lizards were basking under the heat lamp (upright head

164 position and increased respiration). Finally, we counted the number of times each lizard exhibited a

165 given behavior.

166 **2.5 Thermal ecology parameters**

167 **2.5.1 Body temperature in the micro-environment**

168 To avoid disturbance caused by the repeated handling and cloacal body temperature measurements,

169   we measured the temperature of the body surface of the experimental lizards as body temperature

170   (T_b) using AR320 non-contact infrared thermometer (SMART SENSOR®) (on the 57th day). The

171   lizards were individually marked by paint codes before monitoring their active body temperature.

172   During the last days of experiment, we recorded the body temperature of all individuals from 10:00

173   to 16:00 hours at hourly intervals. If a lizard was found to be inactive, we gently lifted the refuge

174   and measured lizards' body temperature. If they were hidden in the substrate and we could not locate

175   them, body temperature measurements were left unscored.

176   **2.5.2 Preferred body temperature**

177   Ectotherms' preferred body temperature (T_{set}) is that one they attempt to achieve. It is calculated as

178   the mean of T_b selected by organisms free to choose where to thermoregulate in a laboratory gradient.

179   To determine T_{set}, we constructed a laboratory thermal gradient adapted from previous literature

180   (Wang et al., 2021). The device is shown in the Fig. S3, focused on providing an ecologically cost-

181   reduced environment aimed to lessen adverse effects of thermoregulation. On the 58th day, each

182   lizard was placed on a track for 2 h acclimation, and then the body temperature was measured every

183   10 min using AR320 non-contact infrared thermometer (SMART SENSOR®).

184   **2.5.3 The thermoregulatory accuracy**

185   We estimated the accuracy of thermoregulation (d_b) as the absolute value of the difference between

186   the T_{set} and the field-active T_b of animals ($d_b = |T_b - T_{set}|$). Low values of d_b represent high

187   thermoregulatory accuracy because animals can achieve their T_{set} or close to it in the field. In

188   contrast, high values of d_b represent low thermoregulatory accuracy because T_b is either much higher

189   or lower than T_{set}.

190   **2.6 GBH analysis**

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191 Soil (20 g) was collected at 1, 3, 7, 14, 28, and 60 days for soil degradation dynamics of GLY and

192 AMPA. The contents of GLY and AMPA were analyzed by high-performance liquid

193 chromatography (HPLC) using a modification of the method proposed by Wu et al. (2020) (Wu and

194 Lin, 2020). The recoveries of GLY and AMPA in the soil are listed in Table S3&S4.

195 **2.7 Biological analysis**

196 **2.7.1 Assay of enzyme activity**

197 Commercial test kits for Glutathione S-transferase (GST), superoxide dismutase (SOD),

198 Malondialdehyde (MDA) were purchased from Elabscience Biotechnology Co., Ltd (Wuhan,

199 China). Assay kits for glucuronosyltransferase (UDPGT) and cytochromes P450 (CYP450) were

200 purchased from Jiangsu Suke Biotechnology Co., Ltd (Jiangsu, China). The customized enzyme-

201 linked immunosorbent assay (ELISA) kits for reptilian heat shock protein 70 (HSP70) were

202 generated by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Tissue samples were

203 homogenized in ice-cold phosphate buffer (0.01 M, pH 7.4) at 4 °C, and a 10% (w/v) homogenate

204 was centrifuged for 4000 rpm, 15 min (4 °C). The obtained supernatant was used to measure GST

205 and SOD activities, as well as MDA, UDPGT, CYP450 and HSP70 content.

206 **2.7.2 Metabolomics**

207 Lizard brain sample extraction was performed as previously described with some

208 modifications(Chang et al., 2022; Zhang et al., 2018). Details are summarized in Supplementary

209 Material. The original result file obtained by instrument analysis was imported into Compound

210 Discoverer 3.2 software (Thermo Fisher Scientific, USA) for metabolites identified. Subsequently,

211 the on-line software MetaboAnalyst 5.0 was used to screen differentially expressed metabolites

212 (DEMs). DEMs was based on a VIP value > 1 for the first principal component of the OPLS-DA

10

213   model and a p-value < 0.05 for the T test. The SCMs were used for the pathway analysis via

214   MetaboAnalyst 5.0. The pathways with p value < 0.05 were considered as potentially affected.

215   **2.7.3 RT-qPCR**

216   Roughly 50mg liver samples and 50mg brain samples were homogenized in TRNzol Universal

217   reagent (TIANGEN Biotech Co., Ltd) for total RNA isolation, following the manufacturer's

218   instructions. Total RNA concentration was calculated from the absorbance at 260 nm using a

219   NanoDrop-1000 spectrophotometer (NanoDrop Technologies Inc.). To ensure RNA purity, quality

220   was verified by gel electrophoresis in 1% agarose gel, and only RNA samples with OD260/OD280

221   between 1.7 and 2.0 were used for cDNA synthesis. Complimentary DNA (cDNA) was generated

222   from 1 μg total RNA using FastKing RT Kit (with gDNase), as described by the manufacturer

223   (TIANGEN Biotech Co., Ltd). The quantitative real-time polymerase chain reaction (RT-qPCR)

224   were carried out using the TIANGEN RT-qPCR kits (Biotech, Beijing, China). The genes of

225   members of the thermosensitive transient receptor potential ion channels family (Thermo-TRPs)

226   were selected for gene expression analysis and their primers are listed in Table S5. The RT-qPCR

227   was carried out with Bio-Rad CFX (Bio-Rad, USA). The thermal cycling program was operated

228   according to the following protocol: 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s,

229   60 °C for 20 s and 72 °C for 20 s. The β-actin housekeeping gene was used for the internal

230   normalization of the target genes. The relative expression was calculated using b-actin and the

231   results were analyzed according to the delta-delta Ct method (ΔΔCt).

232   **2.8 Data analyses**

233   The results were expressed as mean ± standard deviation (mean ± SD) and analyzed with SPSS

234   v20.0 (IBM, USA). Graphical plots were realized with GraphPad Prism 9.5.0 (GraphPad Software,

235 Inc. USA). Data were tested for GBH and warming treatment interaction effects by two-way

236 analysis of variance (ANOVA) followed by the post-hoc Duncan test. Statistical differences

237 between different treatments were conducted by One-way ANOVA with a post-hoc Duncan test.

238 The assumptions of normality and homogeneity of variance were determined for analysis via

239 Kolmogorov-Smirnov and Levenes test.

240 **3.Results**

241 **3.1 Mortality**

242 No mortality was observed in the control or exposure groups during the entire experimental period.

243 **3.2 Thermoregulatory behavior**
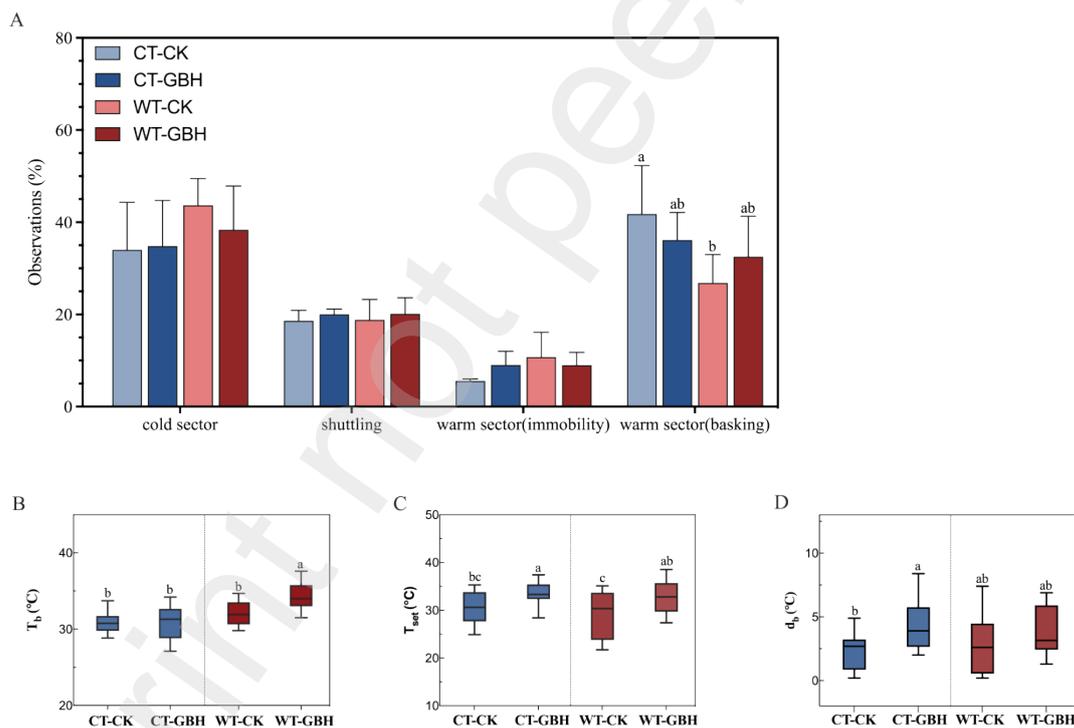
244

245 Fig. 1 Thermal performance parameters data of *Eremias argus* lizards. A: Partitioning of time

246 among the different behaviors in lizards ； B: The active body temperature of lizards; C: The

247 preferred body temperature of lizards; D: The thermoregulatory accuracy of lizards; bars indicate

248 standard deviation (SD). Boxplots provide the median (black horizontal line), the interquartile range

12

249   (upper and lower sides of the box) and minimum and maximum values (whiskers). Letters indicate

250   statistically significant differences between treatment groups.

251

252   Table 1 Two-way ANOVA analysis results of lizard thermal parameters and enzyme activity of

253   *E.argus.*

variables	GBH		Temperature		GBH×Temperature	
	F	P	F	P	F	P
The active body temperature (T_b)	6.488	**0.014**	27.710	**<0.001**	6.599	**0.013**
The preferred body temperature (T_{set})	13.081	**0.001**	1.430	0.237	0.048	0.828
The thermoregulatory accuracy (d_b)	7.775	**0.008**	0.026	0.873	0.781	0.381
GST	9.709	**0.005**	12.841	**0.002**	0.175	0.680
UDPGT	13.664	**0.001**	19.107	**<0.001**	0.010	0.919
CYP450	16.924	**0.001**	12.571	**0.002**	0.282	0.601
MDA	3.076	0.095	6.165	**0.022**	3.745	0.067
SOD	27.550	**<0.001**	65.615	**<0.001**	35.948	**<0.001**
HSP70 in liver	36.180	**<0.001**	5.909	**0.025**	1.020	0.325
HSP70 in plasma	1.719	0.205	5.585	**0.028**	1.181	0.290

254

255   Through the observation of the daily behavioral patterns of lizards, it was found that compared with

256   other groups of lizards, the proportion of basking in WT-CK group was significantly reduced (Fig.

257   1A). We found that both GBH and ambient temperature each had a significant effect on T_b in the

258   micro-environment ($F_{1,54}$=6.488, P=0.014 ; $F_{1,54}$=27.710, P<0.001), also with a significant

259   interaction effect ($F_{1,54}$=6.599, P=0.013) (Table 1). According to post-hoc tests, simultaneous

260   exposure to GBH and higher ambient temperature resulted in a significant increase in T_b in the

261   lizards, while there was no significant difference in the T_b of the other three groups of lizards (Fig.

13

262   1B). We also found that GBH had a significant effect on T_{set} ($F_{1,58}$=13.081, P=0.001), while

263   environmental temperature had no significant effect on T_{set} ($F_{1,58}$=1.430, P>0.05) and there was no

264   interaction effect between the two variables ($F_{1,58}$=0.048, P>0.05) (Table 1). In the current climate

265   treatment groups, lizards which exposed to GBH preferred higher body temperatures relative to the

266   control group. GBH exposure also resulted in a significant increase in preferred body temperature

267   in the warmer climate treatment groups (Fig. 1C). As for the thermoregulatory accuracy of the

268   lizards: GBH had a significant effect on thermoregulatory accuracy ($F_{1,47}$=7.775, P=0.008), ambient

269   temperature had no significant effect on thermoregulatory accuracy ($F_{1,47}$=0.026, P>0.05) and there

270   was no significant interaction between the two variables ($F_{1,47}$=0.781, P>0.05) (Table 1). In the

271   current climate treatment, GBH exposure led to significantly higher in d_b values, suggesting that

272   GBH decreased in the lizard's ability to precisely regulate its body temperature. However, the same

273   results were not observed in the two warmer climate treatment groups (Fig. 1D).

274   **3.3 Degradation of glyphosate in soils**

The dynamic degradation of GLY in soil — The dynamic degradation of AMPA in soil
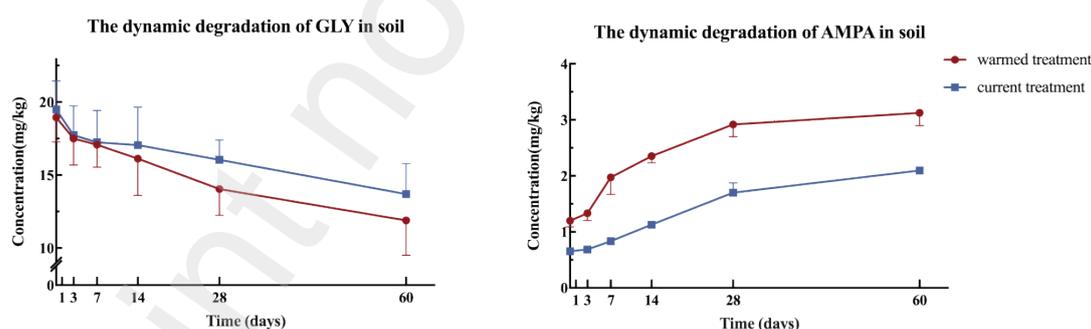
276   Fig. 2 Concentrations of glyphosate (GLY) and its metabolite AMPA in soil. Bars indicate standard

277   deviation (SD).

278   The soil degradation dynamics of GLY and AMPA over 60 days were determined and results are

279   shown in Fig. 2 and Table 2. The nominal exposure concentrations of GLY in the soil were 19.49

280   mg/kg and 18.95 mg/kg in the CT-GBH and WT-GBH groups, respectively. There was some

14

281 degradation of glyphosate in the soil during the 60-day exposure period, with a degradation rate of

282 29.8% in the CT-GBH groups, and more rapid degradation of 37.2% in the WT-GBH group. Its

283 metabolite AMPA was also detected in the soil at the concentrations shown in Fig. 2.

284 Table 2. The concentration and degradation rate of GLY and AMPA in the soil of incubators

Treatment group	GLY concentration in soil (mg/kg soil)		Degradation rate (%)	AMPA concentration in soil (mg/kg soil)	
	0 d	60 d		0 d	60 d
CT-CK	ND	ND	-	ND	ND
CT-GBH	19.49±1.95	13.69±2.10	29.8	0.65±0.031	2.10±0.05
WT-CK	ND	ND	-	ND	ND
WT-GBH	18.95±1.68	11.90±2.39	37.2	1.20±0.11	3.13±0.23

285

286 **3.4 Response of detoxification enzyme activities**
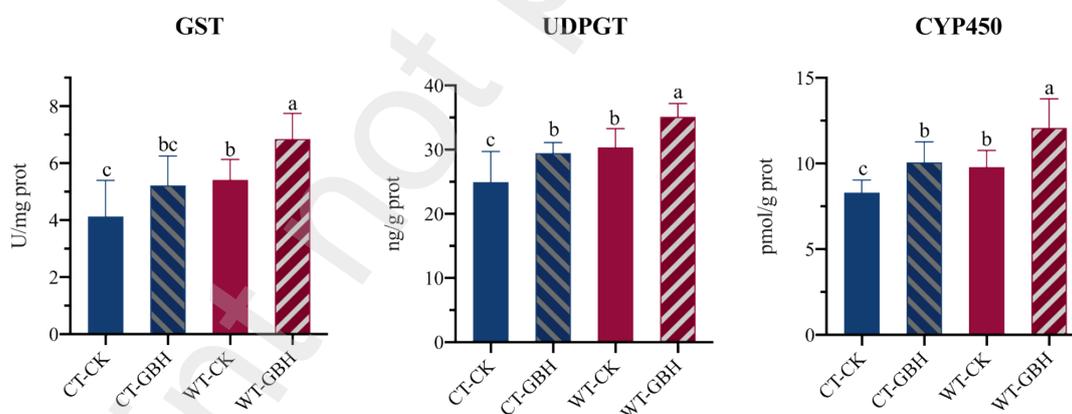
287

288 Fig. 3 Effects of GBH exposure and differential temperature treatments on the liver GST, UDPGT,

289 and CYP450 of lizards. Letters indicate statistically significant differences between treatment

290 groups.

291 The liver GST activity, UDPGT, and CYP450 contents of the lizards under each treatment are

292 shown in Fig. 3, and the results of the two-way ANOVA analysis are shown in Table 1. The three

15

293 biochemical parameters mentioned above did not interact significantly with GBH or ambient

294 temperature. Both temperature and GBH lead to a significant increase in these three enzymes.

295 Without glyphosate exposure, liver GST activity, UDPGT, and CYP450 content increased with the

296 increase of ambient temperature (CT-CK vs WT-CK). At the current climate treatment, GBH

297 exposure resulted in an increase in liver GST activity, UDPGT, and CYP450 by 26.62%, 18.14%,

298 and 21.31%, respectively (CT-GBH vs CT-GBH). In the warmer climate treatment groups, liver

299 GST activity, UDPGT, and CYP450 contents were 26.57%, 15.78%, and 23.42% higher in the

300 GBH-treated group than that of the control group, respectively (WT-GBH vs WT-GBH).

301 **3.4 Thermal stress and antioxidant system**
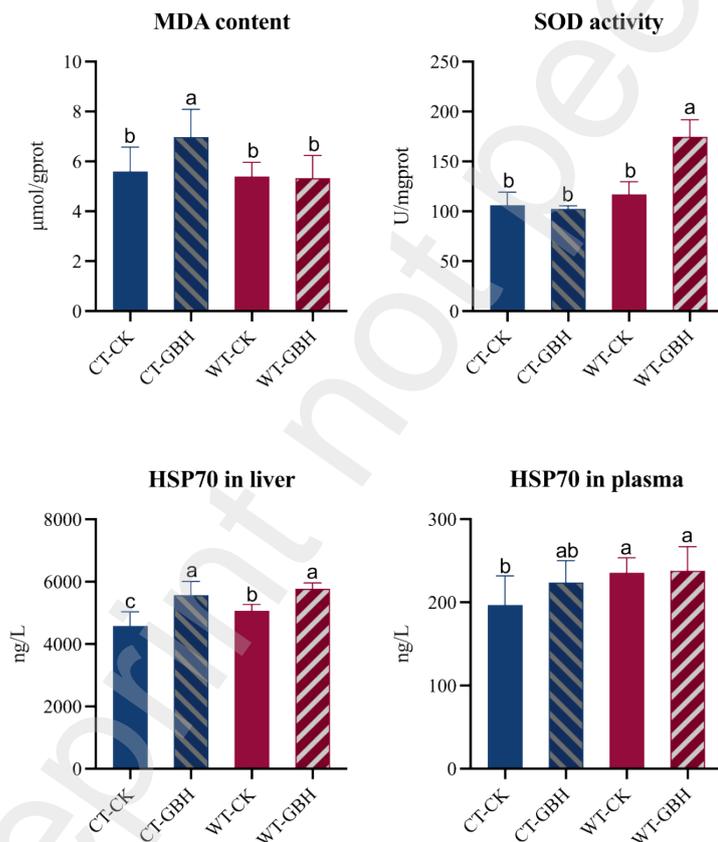
302

303 Fig. 4 Effects of GBH exposure and differential temperature treatments on brain MDA, SOD, and

304 HSP70 levels in the liver and plasma of lizards. Letters indicate statistically significant differences

16

305   between treatment groups.

306

307   Exposure to GBH significantly increased the MDA content in lizard brain tissue, independent of

308   changes in ambient temperature (GBH × Temperature: $F_{1,20}=3.745$, P=0.067; GBH: $F_{1,20}=3.076$,

309   P=0.095; Temperature: $F_{1,20}=6.165$, P=0.022). The level of MDA in the brain tissue of lizards in the

310   CT-GBH group was significantly higher than that of the other three groups, and there was no

311   significant difference between the other three groups. Both GBH and ambient temperature had

312   significant effects on brain SOD enzyme activity (GBH × Temperature： $F_{1,20}=35.948$, P<0.001;

313   GBH: $F_{1,20}=27.550$, P<0.001; Temperature: $F_{1,20}=65.615$, P<0.001). We also found that SOD

314   enzyme activity was significantly higher in the lizards in the WT-GBH group compared to the other

315   three groups, with no significant difference between the other three groups.

316   The expression of heat shock protein 70 (Hsp70) was used as a general stress response indicator to

317   monitor environmental stressors. According to the main effect model, temperature generally causes

318   liver (GBH × Temperature: $F_{1,20}=1.020$, P=0.325; GBH: $F_{1,20}=36.180$, P<0.001; Temperature:

319    $F_{1,20}=5.909$, P=0.025) and plasma Hsp70 (GBH × Temperature: $F_{1,20}=1.181$, P=0.290; GBH:

320    $F_{1,20}=1.719$, P=0.205; Temperature: $F_{1,20}=5.585$, P=0.028), and Hsp70 levels in lizard liver were

321   elevated by GBH exposure regardless of temperature.

322   **3.5 Metabolomics study in brain**

323   Non-targeted metabolomics analysis revealed that a total of 82 common metabolites were identified

324   in the upper extracts of the four sets of brain tissue samples, and a total of 131 common metabolites

325   were identified in the lower phase. The compounds extracted from the upper phase were mainly

326   non-polar metabolites, like lipids and lipid-like molecules, while the lower phaes were polar

17

327   metabolites, mainly nucleosides, nucleotides, and analogues, organic acids and their derivatives,

328   etc. Based on OPLS-DA model analysis and t-test analysis, there were 14 (upper) and 26 (lower)

329   differential metabolites in the lizard brains of the CT-GBH group compared to the CT-CK group

330   (Fig. 5A, D). The differential metabolites that were significantly altered in this group were used for

331   clustering analysis to generate heat maps, and these differential metabolites were used to examine

332   the changes in metabolic pathways via KEGG pathway analysis to further investigate the disruption

333   of metabolic pathways caused by glyphosate exposure. As shown in the Fig. 5J, exposure to GBH

334   can affect these metabolic pathways in the brain: histidine metabolism, beta-Alanine metabolism,

335   Aminoacyl-tRNA biosynthesis, Galactose metabolism, Amino sugar and nucleotide sugar

336   metabolism, Phenylalanine, tyrosine and tryptophan biosynthesis, Tyrosine metabolism. We

337   identified 15 (upper) and 16 (lower) metabolites in WT-CK group that changed significantly

338   compared with CT-CK group following an increase in ambient temperature (Fig. 5B, E). Compared

339   to the control, the warmer climate treatment had significant effects on some metabolic pathways:

340   Glutathione metabolism, Aminoacyl-tRNA biosynthesis, Sphingolipid metabolism, Alanine,

341   aspartate and glutamate metabolism, Pantothenate and CoA biosynthesis. Compared with CT-CK

342   group, the WT-GBH group had 14 (upper) and 18 (lower) differential metabolites in lizard brain

343   (Fig. 5C, F). Some pathways were significantly impacted between the WT-GBH and CT-CK groups,

344   including purine metabolism, Alanine, aspartate and glutamate metabolism, Pantothenate and CoA

345   biosynsis, Valine, leucine and isoleucine degradation, Arginine biosynsis, Aminoacyl-tRNA

346   biosynsis.
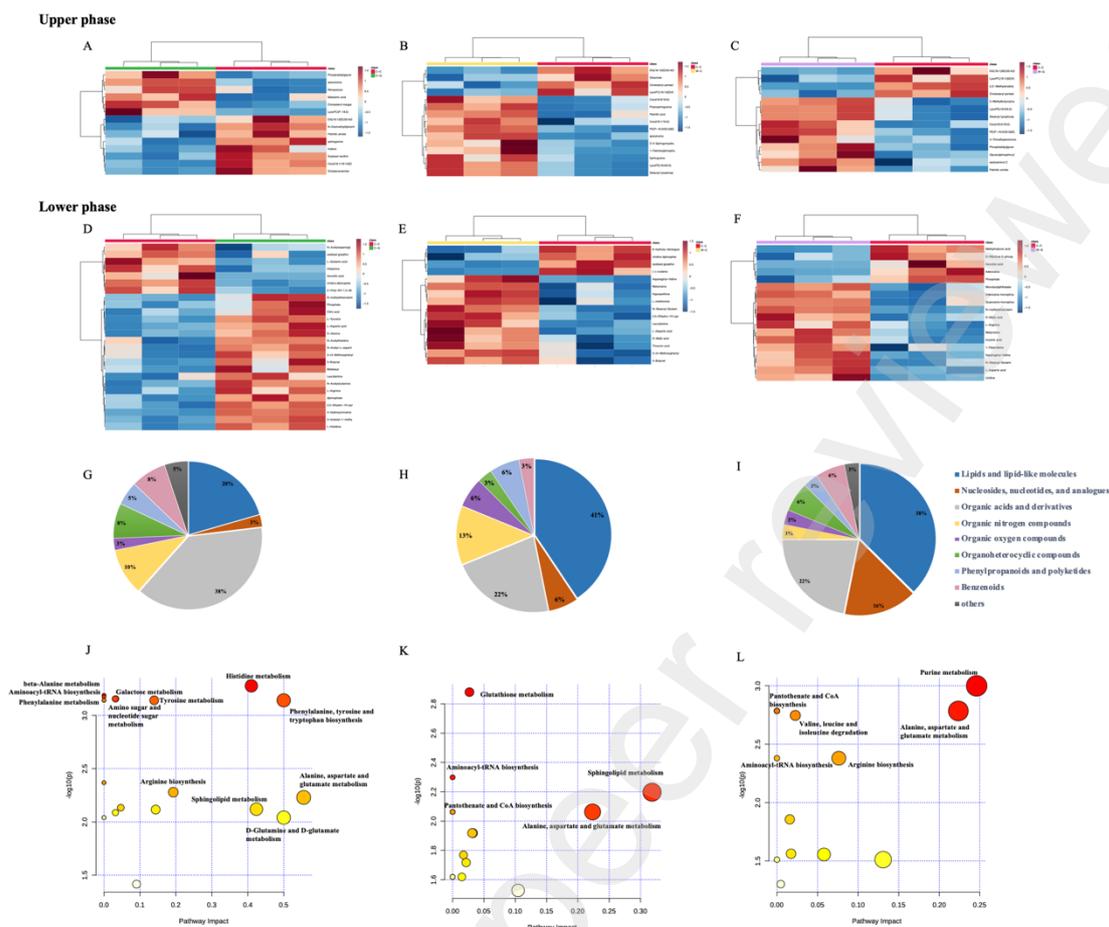
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Fig. 5 The heat maps, categorical proportions and KEGG pathway analysis of differential metabolites identified from lizard brains. (A, D, G and J) CT-GBH vs CT-CK; (B, E, H and K) WT-CK vs CT-CK; (C, F, I and L) WT-GBH vs CT-CK.

3.6 Gene expression of thermosensitive transient receptor potential ion channels (thermo-TRPs)

The gene expressions of the members of the thermosensitive transient receptor potential ion channels family in lizard brain and liver are shown in Fig.6. We found that compared with the CT-CK group, the expression of *TRPM7* in the liver of the lizards in the CT-GBH group was significantly decreased (p<0.05), while the expression of *TRPA1* was significantly increased, and the expression of *TRPM7*, *TRPA1*, and *TRPV2* in the brain tissue was down-regulated (p<0.05).

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358   Compared with the control group, the warmer climate treatment significantly increased *TRPM7*,

359   *TRPA1*, and *TRPV2* mRNA expression in the liver of the lizards in the WT-CK group, as well as

360   the expression of *TRPM7* and *TRPA1* in the brain tissue. In the WT-GBH group, the expression of

361   *TRPV2* was up-regulated in the liver and down-regulated in the brain.

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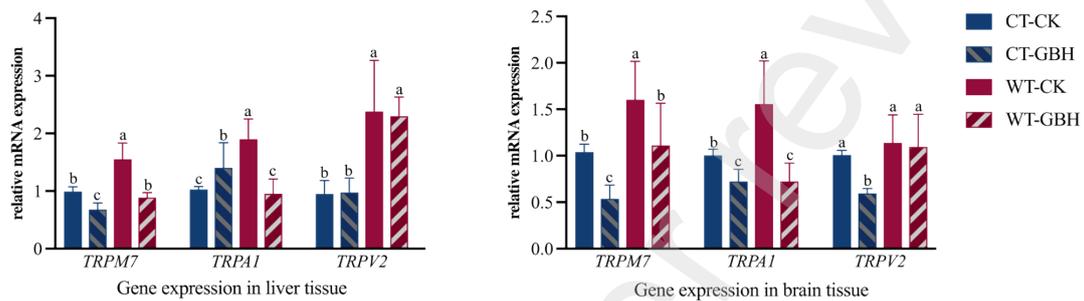
363

364   Fig. 6 Effects of temperature and GBH exposure on the expression of genes related to

365   thermosensitive transient receptor potential ion channels (thermo-TRPs) in the liver and brain

366   tissues of lizards.

367   **4. Discussion**

368   **4.1 The plastic response of lizards to warming**

369   If the ambient temperature is higher than the ectotherm's optimal physiological range, several

370   aspects of the behavior and physiology may be negatively affected, including locomotion, sensory

371   input, foraging ability, rates of feeding and growth and immune function (Angilletta et al., 2002).

372   Regardless of ambient temperature, endotherms can are gifted with an ability to regulate a steady

373   temperature inside their bodies through metabolic heat production and loss processes (Boyles et al.,

374   2011). Although ectotherms do not maintain body temperature homeostasis in a similar manner,

375   they can regulate body temperature through specific thermoregulatory strategies, including behavior

376   and physiological thermoregulation (Kearney et al., 2009; Seebacher et al., 2015).

20

377   In this study, when there was no GBH exposure treatment, the active body temperatures measured

378   in lizards did not increase, even though the ambient temperature increased, and their accuracy of

379   thermoregulation was not affected by warmer climate treatment. This suggests that *E.argues* lizards

380   can response to small increases in ambient temperature, and regulate their body temperature close

381   to their preferred body temperature and meets their heat needs. Adjustment of basking position and

382   posture, microhabitat selection and shuttling, and changes in basking frequency are considered to

383   be some important aspects of behavioral thermoregulation in these reptile species (Black et al., 2019;

384   Sinervo et al., 2010b; Spellerberg, 1972). Similar to these, it was observed in this study that the

385   frequency of basking behavior decreased significantly after the temperature increased, while time

386   spent on cold sector increased (although not statistically significant), which confirmed that they

387   compensated for the change of thermal environment by altering basking frequency and microhabitat

388   use. Furthermore, T_{set} of lizards was measured in our laboratory setup with a temperature gradient

389   without ecological constraints. The wide range of T_{set} in the CT-CK and WT-CK groups exemplifies

390   that lizards, as habitat generalists and ambush foragers, experience a complex and variable

391   environment, and exhibit more eurythermic behaviors and are adapted to a variety of environmental

392   temperatures, with a buffering capacity to cope with some degree of fluctuating environmental

393   temperatures (Crowell et al., 2021).

394   Heat shock proteins (HSPs) are a super-family of proteins in response to various biological and

395   abiotic stressors, also known as stress proteins (de Pomerai, 1996; Feder and Hofmann, 1999; Hamer

396   et al., 2004). The physiological response to hyperthermia exposure is often accompanied by the

397   expression of HSPs, which participates in protecting cells from heat damage by protecting cell

398   proteins from denaturation (Hassan et al., 2019). The level of Hsp70 in the liver and plasma of

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399   lizards in WT-CK group increased significantly with ambient temperature, which is considered a

400   protective mechanism against heat stress. For example, *rainbow trout* has a time- and tissue-specific

401   Hsp30 response to heat stress, which is expected to provide some protection in heat stress within a

402   certain range (Liu et al., 2019). Also, HSP70 can promote thermal tolerance of reptiles embryos and

403   plays an important role in coping with acute heat stress in koi (*Cyprinus carpio*) (Gao et al., 2014;

404   Yu et al., 2020). HSPs are ecologically relevant mechanisms used by animals to promote thermal

405   adaptation and provide protection to organisms in heat stress (Jin et al., 2020; Kaur et al., 2011).

406   It has been shown that the brain is the most sensitive organ to thermal stimuli in Cuban *Anolis*

407   lizards(Akashi et al., 2016). Several studies have suggested that physiological changes in the brain

408   may be related to Acclimatisation or acclimation responses to environmental temperatures. Lizards

409   (*Calotes versicolor*) adapt thermally by increasing the relative proportion of high melting point

410   phospholipids to alter the phase-transition temperature of hypothalamic cell membrane

411   phospholipids. In addition, the proportion of unsaturated fatty acids in the hypothalamus and the

412   brain as a whole increased with cold adaptation (Durairaj and Vijayakumar, 1984). These changes

413   in the fatty acid composition of the brain and hypothalamus would provide signals for temperature

414   selection or compensation (Seebacher, 2005). In this study, metabolomic analysis of lizard brain

415   tissue revealed that the majority of lipid metabolites up-regulated in brain tissue of the WT-CK

416   group following increased ambient temperature were of the phospholipid and sphingolipid classes

417   (Cer(d18:0/18:0), Phytosphingosine, Cer(d18:1/16:0), PE(P-16:0/20:3(8Z,11Z,14Z)), Sphingosine,

418   C14 Sphingomyelin, 1-Palmitoylphosphatidylcholine, LysoPE(18:0/0:0), Stearoyl

419   lysophosphatidylethanolamine). Phospholipids are one of the main components of cell membranes,

420   include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) etc.

22

421 Among them, PE and PC are the main components of neuronal cell membranes, and they are

422 involved in cellular signal transduction processes as precursors of second messengers (Gonzalez-

423 Riano et al., 2016; Joensuu et al., 2020). Studies have also proposed to adjust the fatty acid

424 composition of biological membranes to achieve temperature adaptation in ectotherms (Price et al.,

425 2017). The phospholipid content in lizards' brain increases in warmer environments in this study,

426 to allow temperature acclimation by maintenance of neuronal membrane stability and fluidity.

427 Sphingolipids are lipids that are closely associated with the nervous system and they play an

428 important role in maintaining the normal structure and function of the nervous system. In our study,

429 the changes in brain phospholipid and sphingolipid content in the WT-CK group might assist the

430 lizard in coping with the increased ambient temperature.

431 In ectotherm, thermo sensory organs receive thermal information, then send it to and integrate with

432 the central nervous system (CNS) which modulate behavioral outputs in a top-down fashion (Abram

433 et al., 2017). Various membrane ion channels and receptors act as the initial detectors of

434 environmental temperature cues. Among them, thermosensitive transient receptor potential ion

435 channels (thermo-TRPs) are arguably the most well-established molecular thermosensors in cross-

436 species studies (Xiao and Xu, 2021). For example, TRPV1 and the cool-sensing TRPM8 are

437 expressed in tissues throughout the body of the crocodile (*Crocodylus porosus*). Inhibition of TRP

438 proteins in *C. porosus* can significantly alter thermoregulatory behaviour, abolishes shuttling

439 behaviour, and leads to significantly altered body temperature patterns (Seebacher and Murray,

440 2007). In Cuban *Anolis* lizards, thermal tolerances are related to transient receptor potential ion

441 channel ankyrin 1 (TRPA1) heat sensitivities (Akashi et al., 2018). As TRPs are present in reptiles

442 and function to control thermoregulatory behaviour. We detected the change of mRNA expression

23

443   of *TRPV2*, *TRPM7* and *TRPA1* which are the subfamilies of this family. We found that all three

444   showed increased transcription and function in lizard brain tissue in response to higher ambient

445   temperatures. Taken together, the lizard can adjust its physiological status (Hsp70 content, brain

446   lipid changes, mRNA expression) along with behavioral strategies, to adapt to the rise of ambient

447   temperature.

448   **4.2 Interaction between temperature and glyphosate**

449   A previous study found that the fatal toxicity of Beta-cyfluthrin (BC) to lizards was negatively

450   associated with temperature. Therefore, after exposing to low BC dose, most lizards sought a

451   warming environment. The toxicokinetic process of toxicants (absorption, distribution, metabolism

452   and clearance) in animals is temperature-dependent (Brown et al., 2021). Among them, the

453   metabolism of exogenous pollutants is a process of enzyme-catalyzed biotransformation where the

454   rate of enzyme-catalyzed reaction is determined by the amount of enzyme and the temperature. It is

455   also known that relatively high temperatures are conducive to the biotransformation of toxicants.

456   Therefore, the thermoregulation behavior of seeking warmer environment after BC exposure is

457   considered as a "self- rescue" behavior in lizards (Wang et al., 2022). In this study, whether treated

458   with the current climate or warmer climate conditions, GBH lead to an increase in T_{set} of lizard (CT-

459   GBH, WT-GBH), meaning they actively seek warming. Such thermoregulation behavior is a

460   survival strategy in response to GBH exposure stress and increases the possibility of their survival.

461   Similarly, the dermal exposure of the diurnal lizard (*Oligosoma polychroma*) to Yates Roundup

462   Weedkiller (commercial glyphosate formulations) caused no change in body mass and a significant

463   increase in selected temperatures (Carpenter et al., 2016). The potential mechanism underlying this

464   heat-seeking behavior may be to increase the fever response of metabolism, thus offsetting

465 physiological stress.

466 Body temperature can aid reptiles to better cope with environmental pressure by increasing the

467 effectivity of physiological and biochemical systems because high body temperatures can activate

468 biological conversion enzymes which affect the metabolism and elimination of the poison. The T_b

469 of the WT-GBH lizard was significantly higher than other groups, indicating that they regulate the

470 body temperature so that it is closer to their prefered body temperature while improving the

471 metabolic rate to facilitate detoxification at the same time. On the contrary T_b in CT-GBH lizards

472 has not improved. The liver through biological metabolism and detoxifying exogenous compounds

473 (Kedderis, 2010). This process is known as Phase I and Phase II metabolism. The enzymes

474 participating in this process include the cytochrome P450 enzymes, UDG-PT and GST (Rourke and

475 Sinal, 2014). In our study, GBH and warmer temperature treatment could activate CYP450, UDGPT

476 and GST, and levels of the three enzymes in the WT-GBH lizards are significantly higher than in

477 the other three groups. High body temperature can protect liver tissue by stimulating the production

478 of liver metabolic enzymes, thus eliminating endogenous or exogenous harmful substances faster

479 and more efficiently.

480 In addition, temperature can not only change the physiological characteristics of animals, but also

481 change the behaviour and environmental fate of pesticides (Bento et al., 2016; Broznić and Milin,

482 2012; Daam and Van den Brink, 2010). Our results show that glyphosate degrades faster in soil

483 under warmer environment. Glyphosate is mainly decomposed by microorganisms, and microbial

484 activity may increase at higher temperatures (Sviridov et al., 2015; Zabaloy et al., 2022). An

485 earthworm's (*Eisenia fetida*) sensitivity to glyphosate varies with specific environmental conditions,

486 where glyphosate toxicity decreased with an increase in soil temperature (Pochron et al., 2019).

25

487   Developmental acclimation to higher temperatures reduces the sensitivity of *Ceriodaphnia silvetrii*

488   to glyphosate (Silva et al., 2020). In an aquatic environment, a 0.5-degree temperature increase

489   renders glyphosate toxicity more severe in zebrafish(Sulukan et al., 2023). During glyphosate

490   degradation, the metabolite aminomethyl phosphonic acid (AMPA) will be produced. Few studies

491   have shown that toxicological effect of AMPA to reptiles. The toxicity of glyphosate also depends

492   on other components in the commercial preparation of Roundup® (Gill et al., 2018), which was not

493   included in this study. We suggest that the toxicity effects of AMPA and other components in the

494   formulations on lizards should be taken into account further.

495   Higher ambient temperature can lower the overall risk of GBH as high temperature has been

496   associated with the greater dissipation of GLY and the increase of biochemical detoxification

497   mechanism by lizards. All in all, the lizards in WT-GBH group were not subject to GBH toxicity

498   stress, and the ability to control body temperature is not affected by GBH. The degradation of

499   glyphosate in the environment is accelerated with the increase of temperature, the activity of

500   biotransformation enzyme is high, and the metabolism and elimination of toxicants are fast.

501   **4.3 The thermoregulation of lizards affected by GBH**

502   Metabolic functions are usually optimized within a narrow range of body temperatures (Tb's),

503   making thermoregulation essential for the survival and fitness of an animal. However,

504   thermoregulation behavior of reptiles has associated costs. The cost-benefit model predicts that only

505   when the related fitness costs (such as time or energy loss, and predation risk) do not exceed the

506   fitness benefits, can the ectotherms take the initiative to carry out beneficial thermoregulatory

507   adjustments (Huey and Slatkin, 1976). This strategy enhances fitness by allowing lizards to adapt

508   more flexibly to changing environmental conditions, while maximizing the benefits of maintaining

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509 high Tb and minimizing energy consumption. A study evaluated the thermoregulatory behavior of

510 inland bearded dragons (*Pogona vitticeps*) under various thermal quality conditions requiring

511 different locomotory investments, and it showed that thermoregulatory precision was decreased in

512 environments where the motor costs associated with thermoregulation were high (Cadena and

513 Tattersall, 2009). Another experimental study on the desert iguana (*Diposaurus dorsalis*) also

514 concluded that lizards that have to shuttle more frequently to regulate their body temperature will

515 allow their body temperature passively close to the ambient temperature (Withers and Campbell,

516 1985). When more metabolic costs are required for body temperature regulation (energy cost of

517 shuttle movement), lizards were less able to regulate their body temperature. In this study, when

518 lizards were exposed to GBH at the current climate treatment, the variance of the T_b in lizards was

519 great, indicating that the thermoregulation is negative and not precise (Brattstrom, 1965); They also

520 did not frequently shuttle through microhabitats and increase the frequency of basking, possibly due

521 to the high cost of accurate thermoregulation. Also, the lizards in the CT-GBH group may tend to

522 invest more energy in the detoxification metabolism of GBH, as it is a process that consumes a lot

523 of energy. When animals have a certain amount of energy, priority should be given to meeting the

524 life history characteristics of self-sustainment that require low level energy (such as oxidative stress,

525 detoxification metabolism, immune response) rather than higher-level energy-demanding life

526 history activities such as fat storage, locomotor performance, reproduction, etc. (Rutschmann et al.,

527 2021).

528 In vertebrates, neutral centers in the hypothalamus are responsible for the control of body

529 temperature. To investigate the effect of GBH on the thermoregulatory of these lizards, we

530 conducted a non-targeted metabolomic analysis of brain tissue and found abnormal histidine

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531 metabolism in the CT-GBH lizards. Histidine can be enzymatically decarboxylated to obtain

532 histamine, which acts as a neurotransmitter in the brain and is associated with a variety of brain

533 functions, such as sleep-wakefulness, circadian rhythm, appetite, memory and stress responses

534 (Brosnan and Brosnan, 2020; Wada et al., 1991). The regulation of body temperature is largely

535 dependent on the histaminergic and cholinergic neuronal systems in the brain (A et al., 2010). The

536 existence of histaminergic thermoregulatory mechanisms in mice has been verified (Itoh et al.,

537 1986). The specific mechanism of histidine metabolism in reptile thermoregulation still needs

538 further research to clarify. Even though the lizards of the CT-GBH group preferred higher body

539 temperatures in the laboratory gradient, they did not refine their thermoregulation to bring their T_b

540 closer to the more beneficial high temperatures. On the one hand, the energy cost of the shuttle and

541 basking period was high. On the other hand, GBH caused oxidative damage to the brain tissue and

542 a significant disturbance in histidine metabolism of the CT-GBH lizards, possibly resulting in a

543 decline in the accuracy of body temperature regulation. However, thermoregulation is a complex

544 process, and the mechanisms by which GBH affects this process deserve further investigation. Over

545 a 60-day exposure to GBH, no mortality has been observed in any group. However, oxidative stress

546 levels in GBH-treated lizards indicate the presence of physiological toxic effects caused by GBH,

547 and as exposure time increases and climate change progresses, subtle toxicological effects may

548 increasingly limit thermoregulatory behavior and have a greater impact on the population as a whole.

549 **5. Conclusion**

550 With the development of global climate change, how the interaction of multiple stressors affects

551 reptiles is an urgent research focus. In this study, the WT-CK lizards thermoregulated with high

552 accuracy, which suggested that this species adapted to moderate environmental temperature increase

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553 through thermoregulatory strategies. However, GBH exposure resulted in the lizard's

554 thermoregulatory accuracy decreased, but the lizards were protected from the adverse effects of

555 GBH through several temperature-dependent detoxification mechanisms facilitated by ambient

556 temperature rise. The subtle toxicological effects of GBH may increasingly limit the behavioral

557 response of this species more and more. Therefore, it is worth exploring further whether lizards may

558 be challenged to survive as the extreme climate situation increases sharply in the global warming

559 scenario, as well as the relationship between environmental pollution and animal thermal physiology.

560

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