Environment International 172 (2023) 107742

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Full length article

Consolidation of temperature-dependent toxicity and thermoregulatory behavior into risk assessments of insecticides under thermal scenarios: A prospective study on *Eremias argus*

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ARTICLE INFO

Handling Editor: Adrian Covaci

Keywords:
Eremias argus
Thermoregulatory behavior
Insecticide
Temperature-dependent chemical toxicity
Risk assessment

ABSTRACT

In this study, the temperature-dependent chemical toxicity of three insecticides and the resulting thermoregulatory (TR) behavior of the lizard *Eremias argus* have been consolidated into the current risk assessment framework. According to acute dermal toxicity assays, an increase of ambient temperature from 15 °C to 35 °C decreased the acute dermal toxicity of beta-cyfluthrin (BC) but increased the toxicity of chlorpyrifos (CPF). The toxicity of avermectin (AVM) did not show significant temperature-dependent responses. Based on thermal preference trials, lizards changed their body temperature via TR behavior to adaptively reduce toxicity under sub-lethal doses, which can be understood as a "self-rescue" behavior attenuating lethal effects. However, the risk quotient indicated that the effectiveness of this "self-rescue" behavior is limited. Metabolomics analysis showed that six different metabolites (i.e., creatine, glutamate, succinate, *N*-acetylaspartate, acetylcholine, and lactate) contributed to TR behavior changes. Biochemical assays and insecticide residue results demonstrated that the temperature-dependent toxicity of BC, CPF, and AVM affected lizards in the three aspects of biotransformation, oxidative stress, and neurometabolic interference. This work clarifies the ecotoxicological impacts of representative insecticides on reptiles from toxicological understanding to risk relevance. This knowledge may improve ecological predictions of agrochemical applications in the context of global climate change.

1. Introduction

Over the past several decades, global climate change (GCC) has altered phenological seasons, geographic distributions, and population scales of many species around the world (Kingsolver et al., 2013; Wan et al., 2022). Rapid shifts in ambient temperature change thermal characteristics of entire habitats, which may strongly influence the thermal-dependent life-history traits of inhabiting organisms (Glatz et al., 2017; Huang and Chou, 2017; Jager et al., 2017). This phenomenon is most pronounced in ectotherms, whose body temperature (T_b) is determined by the environment and therefore fluctuates according to environmental influences (Ljungstrom et al., 2015). Temperature is a

Abbreviations: 3-MT, 3-methoxytyramine; 5-HIAA, 5-hydroxyindole-3-acetic acid; 5-HT, 5-hydroxytryptamine; αKG, α-ketoglutaric acid; AAA, aromatic amino acid; ACh, acetylcholine; AChE, acetylcholinesterase; Ala, alanine; Asp, aspartate; AVM, avermectin; BC, beta-cyfluthrin; BCAA, branched-chain amino acid; CbE, carboxylesterases; Ch, choline; Cit, citric acid; CNS, central nervous system; CPF, chlorpyrifos; CPF-oxon, chlorpyrifos-oxon; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; E, epinephrine; GABA, γ-aminobutyric acid; GCC, global climate change; Glc, glucose; Gln, glutamine; Glu, glutamate; Gly, glycine; GST, glutathione S-transferase; HVA, homovanillic acid; Ile, isoleucine; Lac, lactate; Leu, leucine; MDA, malondialdehyde; MFO, mixed function oxidases; MHPG, 3-methoxy-4-hydroxy phenylglycol; MOA, mode of action; NAA, *N*-acetylaspartate; NE, norepinephrine; OAA, oxaloacetic acid; OSI, oxidative stress index; Phe, phenylalanine; PTDT/NTDT, positive/negative-coefficient temperature toxicant; Pyr, pyruvic acid; RQ, risk quotient; Succ, succinate; T_{acc}, accumulated temperature; TAS, total antioxidant status; Tau, taurine; T_b, body temperature; TCA cycle, tricarboxylic acid cycle; TDCT, temperature-dependent chemical toxicity; TOS, total oxidant status; T_{pref}, preferred temperature; TR, thermoregulatory; Trp, tryptophan; Tyr, tyrosine; UDPGT, uridine 5′-diphospho-glucuronosyltransferase; Val, valine; VMA, vanillylmandelic acid.

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https://doi.org/10.1016/j.envint.2023.107742
Received 11 October 2022; Received in revised form 10 December 2022; Accepted 6 January 2023
Available online 7 January 2023

key environmental factor controlling the physiological and metabolic characteristics of ectothermic animals. These changes can manifest in the response of animals to toxic contaminants in the environment (Henry et al., 2017; Li et al., 2014). Therefore, toxic responses of ectotherms to chemicals may vary greatly under different temperatures. In recent years, temperature-dependent chemical toxicity (TDCT) has gradually become a research focus of environmental toxicology under GCC scenarios (Silva et al., 2020). The physical and bioactive properties of compounds can also change with temperature, which can consequently affect modifications on environmental fate, bioavailability, and degradation processes of pollutants (Daam and Van den Brink, 2010; Majedi et al., 2014; Zhang et al., 2020).

Although ectothermic animals cannot freely moderate their T_b through metabolic heat-produce/loss processes, they can adjust T_b via specific thermoregulatory (TR) behavior. This behavior allows them to choose microhabitats, thus reaching the heat requirement in heterogeneous thermal environments (Parlin et al., 2017). It has been shown that the direction (i.e., seeking warmth or cold) and extent of TR behavioral strategies are determined by physiological tradeoffs under different temperatures (Hey, 1974). However, whether ectotherms can indeed benefit from thermal landscapes depends on the difficulty associated with accessing optimal temperature levels under changes of climatic conditions resulting from GCC scenarios. Therefore, in addition to metabolic and physiological changes, prompt and synchronic shifts of TR behavioral plasticity are the crux of adaptation to unpredictable thermal environments. According to the prediction by Fey et al. (2019), the African lizard *Agama atra* shapes its TR behavior to achieve a trade-off between metabolic costs and running performance benefits (Fey et al., 2019). Ortega et al. (2016) found that alpine lizards may buffer potential detrimental impacts of increasing environmental temperatures by TR behavior (Ortega et al., 2016). These findings are interesting and seem common for ectotherms, but whether the similar "behavioral rescue" for the amelioration of the impacts of TDCT exists remains unclear. Pioneering studies on TDCT conducted on aquatic organisms and testing various chemicals (Cairns et al., 1975; Wang et al., 2019) demonstrated that the relationship between the toxicity of several chemicals and temperature can be characterized as either negative or positive. These have been referred to as positive-coefficient temperature-dependent toxicant (PTDT) and negative-coefficient temperature-dependent toxicant (NTDT), respectively. Hence, changing T_b to mitigate the toxicity of environmental contaminants became possible in ectotherms based on toxicity-temperature relationships: ectotherms seek warmth when exposed to a NTDT or seek cold when exposed to a PTDT. Both are considered rescue behaviors. This potential interaction has inspired toxicologists to explore TR responses of ectotherms to TDCT (Peterson, 1976; Wang et al., 2022b). These studies have suggested that TR behavior has the capacity to attenuate the impacts of TDCT and its effects may be mediated by the central nervous system (CNS), although the mechanism underlying TR changes remains unclear.

As a class of environmental contaminants, insecticides are widely used in agriculture for killing pests (Costa et al., 2008). However, non-target organisms also inhabiting agricultural ecosystems are frequently exposed to insecticides via agrochemical applications. A plethora of insecticides have a mode of action (MOA) that classifies them as neurotoxins (Rajashekar et al., 2014). The physiological basis for TR behavioral strategies originates from the perception and evaluation of temperature information by the CNS, followed by conducting locomotion via efferent nerves (Abram et al., 2017; Angilletta et al., 2019). Hence, the interference of insecticides on the CNS could theoretically affect the trade-off decision and execution of TR behavior. The fluctuating metabolic equilibrium of neurofunctional chemicals can change the response of neurons to cold or heat signals, which plays an important role in maintaining the normal thermoregulatory function (Bicego and Branco, 2002; Gordon, 2005; Sanhueza et al., 2019; Seebacher and Franklin, 2001; Sengupta et al., 2014). In addition, fundamental metabolic imbalance and cellular damages following poisoning can also limit the execution of TR behavior (Fields, 2001; Rezende and Bacigalupe, 2015). Therefore, it can be assumed that shifts of thermal preference are related to toxicant-induced changes on neurochemical contents and metabolic profiles.

Once insecticide exposure has changed the thermotaxis of ectotherms, the physiological processes relevant for toxicological effects will also vary with T_b. The deleterious impacts of insecticides at the cellular levels, such as oxidative damage (Baag et al., 2021; Shayan-Nasr et al., 2021), toxicant accumulation (Camp and Buchwalter, 2016; Naqvi and Vaishnavi, 1993), metabolic collapse (Besson et al., 2020), and respiratory inhibition (Braguini et al., 2004), are key events leading to the acute mortality of organisms. These impacts can also be influenced by temperature. Because of the absence of data on temperature-dependent toxicity and quantitative TR behavior, predicting the TCDT risk for ectotherms under different temperature scenarios is difficult (Baag et al., 2021). In this study, it was hypothesized that the thermal preference of ectotherms can exhibit adaptive changes in response to TCDT to achieve "behavioral rescue". If this behavioral rescue generally reduces the risk of acute exposure to insecticides, the interaction between TCDTs and TR behavior should have profound ecological effects.

Here, *E. argus*, a lizard species native to China, was chosen as experimental ectothermic species. This lizard shows a rapid response to ambient thermal stimuli on both metabolic and behavioral levels (Chang et al., 2022; Zhang et al., 2019). The aims of this study were to: (i) determine the TCDT properties of three typical insecticides (BC, chlorpyrifos (CPF), and avermectin (AVM)) for *E. argus*, (ii) explore the mechanisms that trigger TR behaviors in *E. argus*, and (iii) identify whether the plasticity of TR behavior is adaptative in *E. argus*. For (i), the acute toxicity of the three selected insecticides to *E. argus* was assayed at different temperatures, and the relationships between lethality and temperatures were established for each insecticide. Because of TR behavior, the weighted critical threshold dose for each test compound was generated considering different thermal exposure scenarios. Risk quotitients (RQs) were calculated for risk assessments. For purpose (ii), neurochemical profiling was conducted to identify metabolite species and changes in the lizard brain associated with its TR behavior under sublethal insecticide exposure. For purpose (iii), the effects of temperature on toxic-related toxicokinetic and toxicodynamic processes were assessed.

2. Methods

2.1. Reagents

BC (97.3 %), CPF (99.0 %), and AVM (97.0 %) were purchased from Qinchengyixin Technology Company (Beijing, China). Stock solutions of each chemical (at a concentration of 10.00 mg/mL) were prepared using acetone vehicle. Test solutions with different concentrations were prepared by adding different volumes of stock solutions to acetone. In each test, the volume of vehicle dosing for lizards was$<$100 μL.

2.2. Lizard husbandry and acclimation

Adult male lizards (with an initial body mass of 2.85–3.35 g) were obtained from the captive breeding colony of China Agricultural University. After transfer to the laboratory, each lizard was caged individually for a two-week acclimation period in a plastic container (22 × 15 × 10 cm), the bottom of which was covered with filter paper that was renewed daily. Cultures were kept at a thermal gradient of approximately 25–35 °C with heat lamps (25 W) during the day, and at 20–22 °C overnight, with a 12/12 h light–dark regime. All experiments and protocols followed ethical guidelines set by the China Agricultural University for the care and use of laboratory animals.

2.3. Experiment 1: Acute toxicity assessment under different thermal conditions

Lizard toxicity tests were conducted under the temperatures of 15 °C, 20 °C, 25 °C, 30 °C, and 35 °C. The dose–response relationships of each compound under specific thermal conditions were assumed to obey a normal distribution of mortality probit to logarithmic doses. Hence, the model was established by two estimated parameters: the mean value (μ; i.e., LD50) and the standard deviation (σ). Meanwhile, the slope (1/σ) also reflects the interaction of temperature on compound toxicity.

To reduce the number of experimental animals while not compromising results, the up-and-down procedure, based on maximum likelihood methods, was performed to estimate LD50s. A method modified from Weir (2015) was used (Weir et al., 2015). Briefly, all individual lizards surviving the first 96 h were observed for 14 d, and LD50 calculations are based on mortality at 14 d. If the lizard survived for 96 h, the dose for the next lizard was increased to 1.26 times of the original dose; if it had died, the dose for the next lizard was decreased to 0.79 times the original dose. The dosage of 2000 μg/g was selected as the limit. The starting dose for BC and AVM was 175 μg/g according to the recommendation of the OECD (OECD, 2022). In CPF experiments, the starting dose was 17.5 μg/g because CPF has been found to have particularly high toxicity in several vertebrate species (Paracampo et al., 2015). Each toxicity assessment was executed independently at five ambient temperatures (15 °C, 20 °C, 25 °C, 30 °C, and 35 °C) in temperature-controlled climate rooms. After acclimation, lizards were dosed dermally (Wang et al., 2022a; Weir et al., 2014) and fasted until the 96-h observation window had finished. Food was available for an additional 10-day period.

To estimate the parameter σ, toxicity tests, consisting of three dosages of each test substance, were conducted and the mortality of lizards was recorded for 14 days (n = 6). The estimated LD50 from the up-and-down procedure was selected as medium dosage, and higher and lower dosages were first chosen in a geometric series at a common ratio of 3.16. The parameter σ was calculated by the probit method where at least two groups of experimental animals neither all survived nor all died; otherwise, the common ratio was changed into 2.15 or 5, and the assay was reconducted.

Based on the dose–response curves at each temperature for these three chemicals, the relationships of the death probability of lizards for different temperatures and doses were fitted using the curve fitting tool of MATLAB R2014b (MathWorks Inc). Data matrixes of the probability of death, logarithmic dose, and temperature were generated from the probit models using Monte-Carlo sampling. These datasets were then interpolated and denoised by a thin-plate spline method. For each insecticide, a curve of the relationship between LD50s and temperature was drawn by the intersection of the fitting surface with a plane where the probability of death is 50 %.

2.4. Experiment 2: Thermoregulatory behavioral and neurochemical changes

To evaluate changes of thermal preference induced by insecticide exposure, this experiment was carried out in shuttle boxes with a thermal gradient from ~ 10 °C to 45 °C. Similar to a previous study (Dominguez-Guerrero et al., 2019), lizards could select the ambient temperature that best meets their heat requirements. A period of 96 h after insecticide exposure was observed. Lizards were treated with two sub-lethal doses of each insecticide, and lizards in the control group were treated with acetone. A total of 42 lizards was divided evenly into seven groups (n = 6 per group): control, BC-L (0.5 μg/g), BC-H (5.0 μg/g), CPF-L (0.2 μg/g), CPF-H (2.0 μg/g), AVM-L (2.0 μg/g), and AVM-H (20.0 μg/g). To allow for acclimation, lizards were placed in the experimental facility 72 h before dosing. The T_b was recorded every hour during their active period using an AR320 infrared thermometer (Smart Sensor®, Dongguan, China), starting 24 h prior to dosing and continuing to the end of the 96-h exposure. Following the exposure period after dosing, all lizards were euthanized. The brains were collected and stored at −80 °C until further analysis.

The definition of TR behaviors followed previously described criteria (Wang et al., 2022a). Briefly, the behavior was deemed "unchanged" if an insecticide-treated lizard whose TR parameters (including preferred temperature (T_{pref}) and accumulated temperature (T_{acc})) remained within twice the standard deviation of the mean (SD) compared with the control group. If either T_{pref} or T_{acc} was found to be 2 SD above or below their means, the response was deemed "warmth seeking" or "cold seeking", respectively (Table S5).

A ^{1}H nuclear magnetic resonance (NMR)-based untargeted metabolomic approach and liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis of targeted neurochemicals were used to describe metabolic changes in the brains of lizards. Details are presented in the supplementary materials.

2.5. Experiment 3: Hepatic biotransformation and insecticide burden

In this experiment, the effects of temperature and insecticides on detoxification and biotransformation in lizards was investigated. The experimental design was a two-factor crossover experiment: ambient temperature (15 °C, 20 °C, 25 °C, 30 °C, and 35 °C) and insecticides (BC, CPF, ACM, and acetone control). A total of 100 lizards were divided evenly into 20 groups (n = 5 per group). The same dosage (10.0 μg/g) was given to experimental lizards among different insecticide treatments. Following dosing, lizards were incubated at a constant temperature (15 °C, 20 °C, 25 °C, 30 °C, and 35 °C) for 96 h. Food and water was provided *ad libitum*. All lizards were humanely sacrificed 96 h after dosing. The livers, brains, and serum were collected and stored at −80 °C.

The activities of glutathione S-transferase (GST) (Habig et al., 1974), carboxylesterases (CbE) (Vejares et al., 2010), uridine 5′-diphosphoglucuronosyltransferase (UDPGT) (Tephly et al., 1988), and mixed function oxidases (MFO) (Jones et al., 1997; Sai et al., 1999) for the same specimen were measured at both uniform temperatures and the ambient temperature where the lizard had been incubated ($T_{ambient}$). Horizontal comparison of enzymatic activity at uniform temperatures indirectly represents the amount of enzyme from cellular expression and synthesis. The paired comparison of the same specimen indicates a link between temperature and enzyme activity. The activity measured at $T_{ambient}$ suggests the actual performance of biotransformation under specific thermal conditions. Here, the serum AChE activity of the control and CPF-treated lizards was also studied using kits obtained from Nanjing Jiancheng Bioengineering institute. The serum total oxidant status (TOS), total antioxidant status (TAS), oxidative stress index (OSI), and malondialdehyde (MDA) levels were measured according to previous methods (Erel, 2004, 2005; Landi, 2017). Matrix solid phase dispersion was used to extract the insecticide residues, and the insecticide burdens were quantified using LC-MS/MS or gas chromatography/tandem mass spectrometry (GC–MS/MS). Details are presented in the supplementary material.

2.6. Risk assessments

The main route of acute insecticide exposure for lizards is the spraying of agricultural fields. Under this pesticide application scenario, it was assumed that the back side of lizards are exposed to the spray without consideration of crop interception. Therefore, the area a lizard occupies in the field was calculated as 50 % of its total of surface area (SA). The estimated acute dermal exposure dose (AD_{dermal}, μg/g bw) was calculated according to Formula (1):

$$AD_{dermal} = 50\% \times SA \times DR/BW \quad (1)$$

where SA (cm^2) was derived from the relationship between dermal area and body weight (BW) of the salamander as a proxy [= 8.42 ×

$BW^{0.694}$ (g)] (USEPA, 1993). The dose rate (DR, mg/m^2) was obtained from the China Pesticide Information Network (Table S6) (ICAMA, 2022).

According to the potential TR scenario, the relationships between temperature and LD50 were used to assume a weighting for temperature based on three modes: (1) the ambient thermal condition (gradient temperature with uniform distribution from 15 °C to 35 °C), (2) the baseline T_b selection (distribution according to the thermal preference without insecticide treatment), and (3) altered T_b selection (distribution according to the thermal preference following insecticide treatment). The three related types of $\overline{LD_{50}}$ values were calculated according to Formula (2) as the threshold of toxicity endpoints for risk assessment. The $\overline{LD_{50}}$ values were generated from the results of Experiments 1 and 2.

$$\overline{LD_{50}} = \frac{1}{35°C - 15°C} \cdot \int_{15°C}^{35°C} LD_{50}(T)dT \quad (2)$$

The RQ was obtained from Formula (3).

$$RQ = AD_{dermal}/\overline{LD_{50}} \quad (3)$$

2.7. Statistical analyses

One- and two-way ANOVA (fixed factors: "insecticide", "temperature", and "insecticide × temperature") followed by Tukey HSD test were performed in SPSS 23.0 (IBM Inc., USA). A paired-*t* test was used for comparisons between enzyme activities under $T_{ambient}$ and uniform temperature. The results were plotted by Prism 8 (GraphPad, USA). The raw results were log transformed to improve normality and homogeneity of variance as necessary, and data were determined adequately for analysis via Kolmogorov-Smirnov and Levene's tests.

The metabolites in lizard brains were analyzed via multivariate statistical analysis sorted by treatments and types of behavioral responses (i.e., cold seeking, unchanged, and warmth seeking). Pearson correlation as well as cluster and multivariate analyses in Experiment 2 were performed by MetaboAnalyst 5.0. Data were subjected to Pareto scaling, and the partial least squares discriminant analysis (PLS-DA) was employed as a supervisory method for segregating the specimen of different TR types. The variable importance for projection (VIP) values were ranked according to their contribution to behavioral divergence. Metabolites with a VIP > 1 and P < 0.05 (one-way ANOVA) were considered to indicate different variables for thermoregulations.

3. Results

3.1. Lethal toxicity variations with temperature

The results of Experiment 1 (shown in Fig. 1) indicate a gradual decline of BC lethal toxicity with increasing temperature, and dermal LD50s increased from 110 to 756.2 μg/g bw under experimental temperatures. A higher temperature may lead to a higher lethal toxicity of CPF to lizards, and the LD50 values decreased from 88 μg/g bw at 15 °C to 17.5 μg/g bw at 35 °C. The LD50s of AVM at experimental temperatures ranged from 440 to 735.6 μg/g bw, and there was no apparent difference between temperature treatments.

The slope parameter (1/σ; Fig. 1) of the models increased with increasing temperature following BC and CPF treatments, i.e., curves were steeper for lizards at a higher temperature (35 °C compared with 25 °C) and were less inclined for lizards at a relatively low temperature (15 °C compared with 25 °C). The toxicity results of AVM demonstrated that the slope parameter reached a low point at 25 °C, while the slopes of models were steeper at both a higher and lower temperature.

3.2. Thermoregulatory behavioral and neurochemical changes

In Experiment 2, no significant difference was found in the baseline T_{pref} (24 h prior to insecticide treatments) among groups ($F_{6,35} = 1.815$, $P = 0.125$). In general, compared with the baseline thermal preference of experimental lizards, BC exposure caused lizards to choose higher temperatures, while CPF exposure caused lizards to choose lower temperatures (Fig. 1). However, there were no statistically significant effects of AVM on the thermal preference parameters compared with the control group. For all three insecticides, there was no difference in T_{pref} between the two doses tested. The criteria of behavior types were implemented for each specimen (Fig. 2 B). The results showed that 83.3 % of BC-treated lizards exhibited warmth-seeking behavior, and 75.0 % of the CPF-treated lizards exhibited cold-seeking behavior. The thermoregulation of AVM-treated lizards was irregular, and 33.3 % of those lizards were classified as unchanged, 50 % as cold seeking, and 16.7 % as warmth seeking.

The results of ^{1}H NMR and targeted LC-MS/MS analysis indicated changes of metabolic profiles in lizard brains following insecticide treatments. A total of 25 metabolites could be identified in ^{1}H NMR spectrums by their chemical shifts, coupling, and splitting patterns of each peak (Table S3 and Figure S1). The metabolites of glutamate (Glu), glutamine (Gln), γ-aminobutyric acid (GABA), choline (Ch), taurine (Tau), and glycine (Gly) could be quantified by both ^{1}H NMR and LC-MS/MS analysis. The normalized bins of compound-specific chemical shifts and the absolute content of those metabolites are consistent (all correlation coefficients > 0.87; Table S2).

As shown in Fig. 2 A, in response to insecticide exposure, the contents of valine (Val) and isoleucine (Ile) decreased to varying degrees, except in the BC-H group. Compared with control, glucose (Glc) had been lost following the three insecticide treatments. Lactate (Lac) increased in the two CPF-treated groups, whereas succinate (Succ) decreased significantly in the CPF-H group. The creatine level was

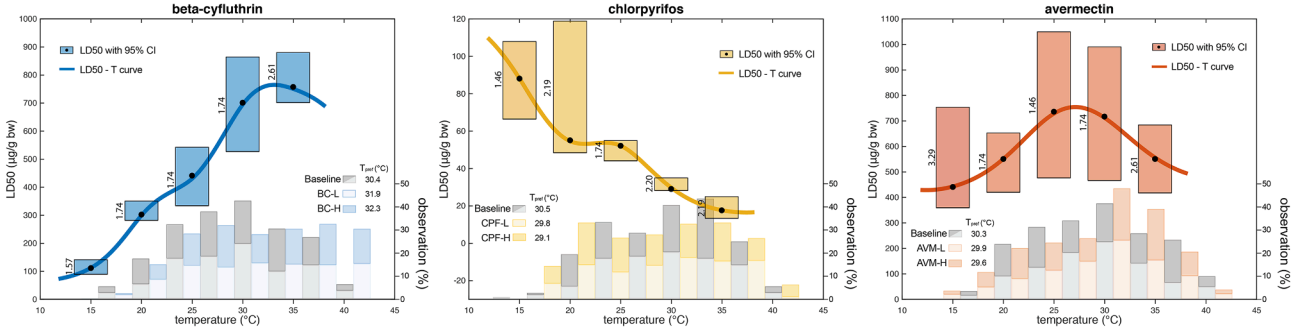

Fig. 1. Acute dermal toxicity of three insecticides to *Eremias argus* (Experiment 1) and characteristics of T_b selections (Experiment 2). Box plots indicate the experimental LD50s at five temperatures for each insecticide. Values alongside boxes indicate the experimental slopes (1/σ) in acute toxicity tests. The curves depict the relationships between LD50s and temperature via interpolation methods. Frequency histograms show all observed T_bs of lizards prior to insecticide treatments for 24 h (the baselines) and after dosing for 96 h.

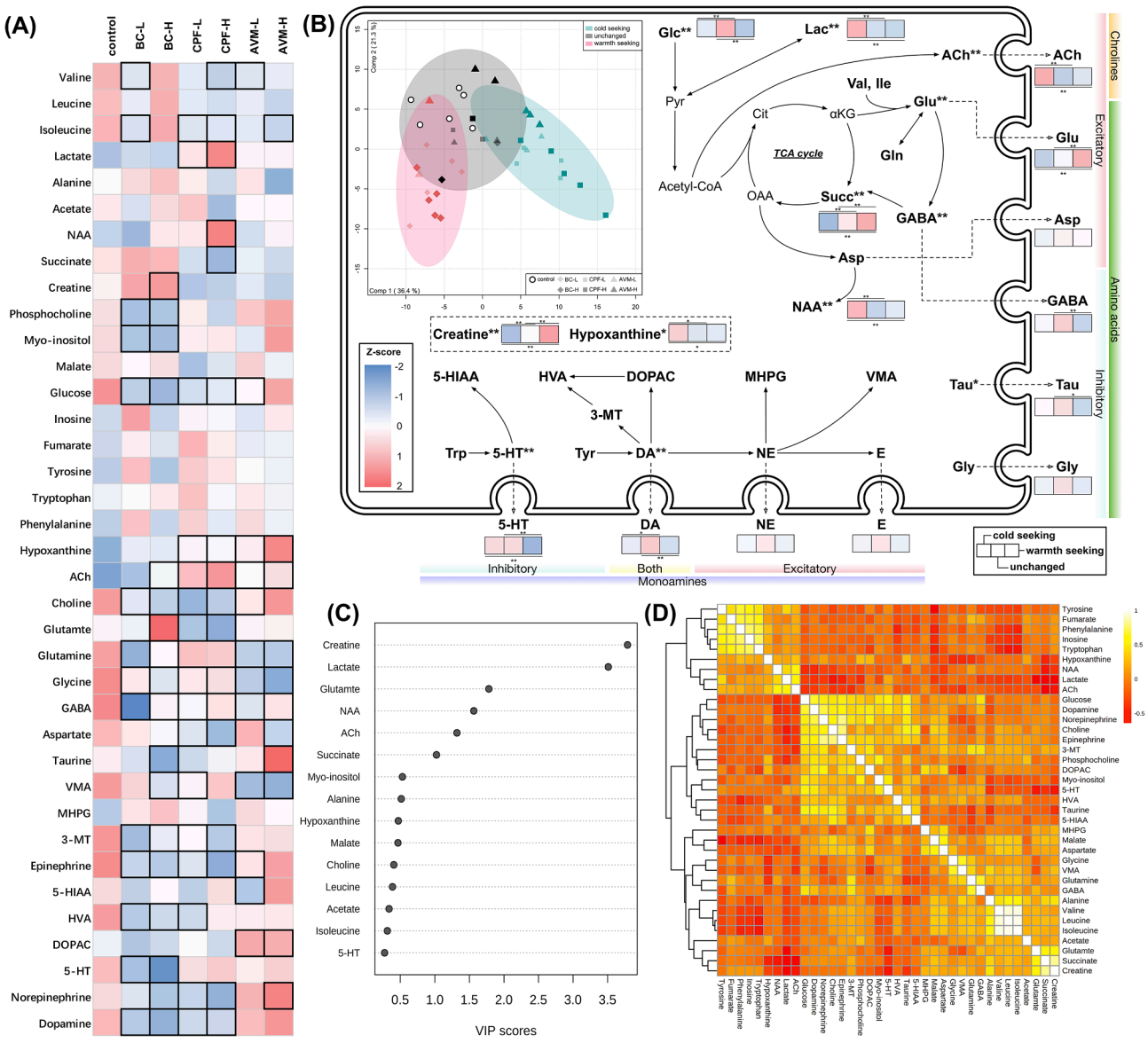

Fig. 2. Neurochemical metabolites in lizard brains subjected to Experiment 2 under three insecticide treatments. (A) Heatmap visualization of Z-scores of 37 metabolites. The black bold borders indicate significant differences ($P < 0.05$) compared with the control group. (B) PLS-DA score scatter plot sorted by thermoregulator types and schematic pathways of the neurotransmitter metabolism. Asterisks indicate the significance levels (*$P < 0.05$; **$P < 0.01$). (C) Variable importance for projection (VIP) values of metabolite variables in partial least squares discriminant analysis (PLS-DA) models. (D) Correlation and clustering results of metabolites.

increased in the BC-H group compared with the control, while the contents of phosphocholine and myo-inositol decreased following BC treatment. Hypoxanthine increased significantly following CPF and AVM treatments, whereas the level in BC-treated groups remained unchanged. Moreover, the levels of neurotransmitters and their metabolites in lizard brains were significantly different after exposure to all three insecticides. The level of ACh tended to increase after exposure to insecticides, while the levels of Ch, Gly, and GABA decreased. The levels of dopamine (DA) and its metabolites 3-MT, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), norepinephrine (NE), and epinephrine (E) were all decreased after exposure to BC and CPF. The decrease of 5-HT could only be found following BC treatments. After exposure to AVM, the levels of norepinephrine and DOPAC increased, but the level of vanillylmandelic acid (VMA) decreased in lizard brains.

The PLS-DA results (accounting for 57.7 % of variations; Fig. 2 B) demonstrated a clear separation between cold-seeking and warmth-seeking groups, while the unchanged group partly overlapped with the other two groups. Six compounds were characterized as differential metabolites (Fig. 2 B and C) contributing to the TR behavioral divergence: higher levels of creatine, Glu, and Succ were related to a warmer preference, whereas *N*-acetylaspartate (NAA), acetylcholine (ACh), and Lac were related to a cooler preference.

The results of pair-wise correlations of metabolites and clustering are shown in Fig. 2 D. Significant positive correlations were found among tyrosine (Tyr), fumarate, phenylalanine (Phe), inosine, and tryptophan (Trp). Positive correlations were also found within the cluster including ACh, NAA, and Lac, the cluster including Glc, DA, NE, Ch, and E, and the cluster including Glu, Succ, and creatine. Moreover, the contents of branched-chain amino acids (BCAAs; Val, leucine (Leu), and Ile) and aromatic amino acids (AAAs; Tyr, Phe, and Trp) were negatively correlated.

3.3. Biotransformation and insecticide burden

In Experiment 3, two-way ANOVA found that “insecticide” and “temperature” factors strongly affected the activities of GST, CbE, and

UDPGT where enzymatic reactions were incubated at either the same temperature (25 °C) or at $T_{ambient}$ (Fig. 3 A). The significant interaction between both factors implied that the changing pattern of enzyme activity with temperature was different under different insecticide treatments. The MFO activity at 37 °C could be similarly affected by "insecticide" and "temperature" treatments (Figure S3). However, because of the low activity of MFO below 25 °C, not all samples could be measured at $T_{ambient}$ (Figure S3). Differences in enzyme activity of the same liver specimen incubated under different thermal conditions were analyzed by Student's paired T test. The results indicate that a higher temperature could up-regulate the activities of all three biotransform enzymes (all $P \leq 0.013$), except for the GST activity incubated at 30 °C

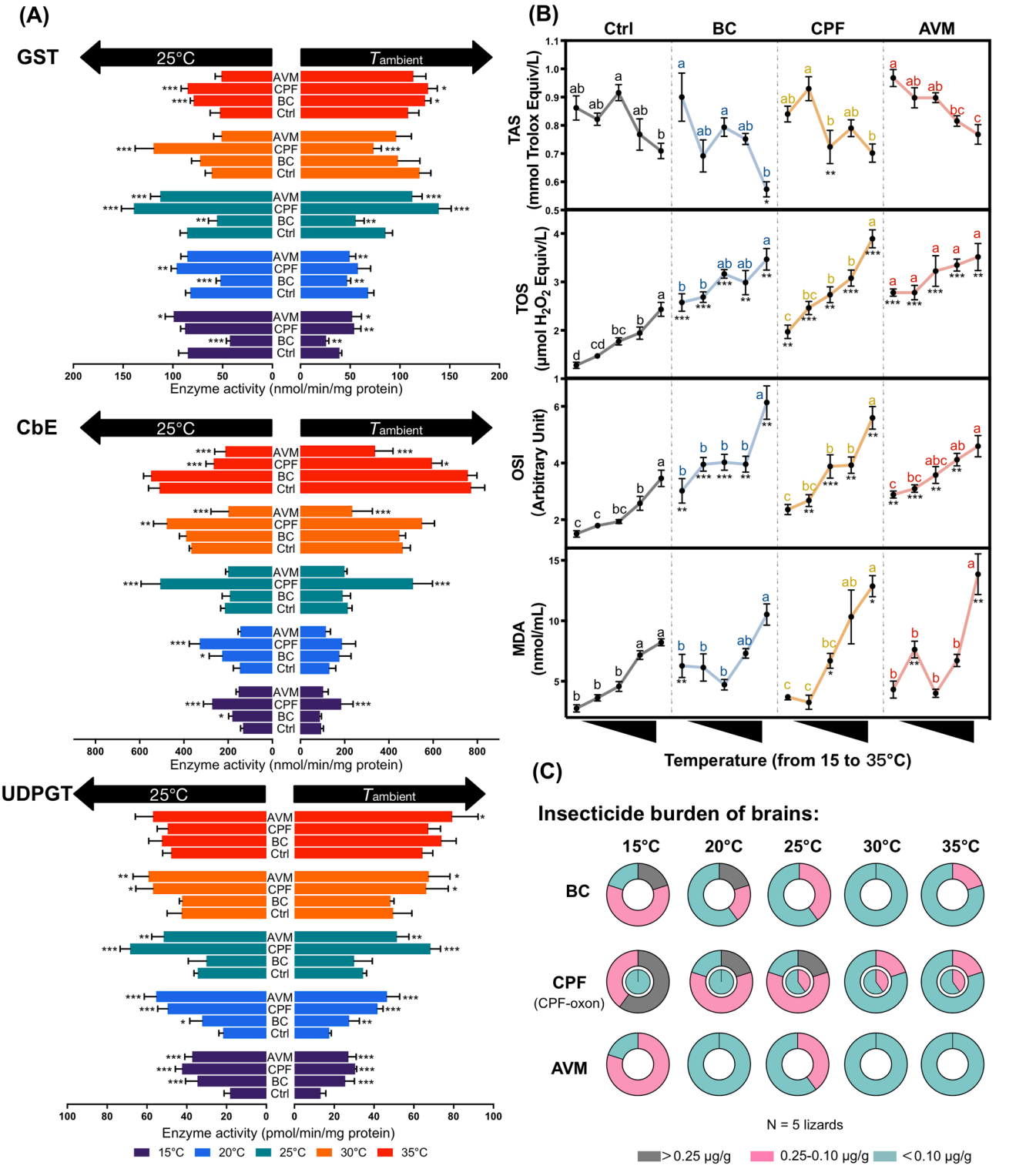

Fig. 3. Biotransformation and oxidative status of lizards subjected to Experiment 3. (A) Detoxifying enzyme activity in lizard livers exposed to insecticides under different temperatures and biotransform performance under different incubating temperatures ($T_{ambient}$). Asterisks indicate significant differences compared with the control group in the same thermal condition (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). (B) Nonenzymic oxidant/antioxidant parameters in lizard serum. Different letters (a, b, and c) indicate significant differences between groups ($P < 0.05$). (C) Insecticide residues in lizard brains following 96 h of exposure at given doses.

following CPF treatment (P = 0.015).

Both insecticide and temperature treatments strongly affected nonenzymatic antioxidative parameters (Fig. 3 B). While a higher temperature could lead to a higher TOS, the TAS of lizards decreased. Therefore, OSI increased with increasing temperature. Compared with the control at each thermal condition, TAS increased in response to all insecticides, while TAS was not suppressed except in lizards treated with BC at 35 °C and those treated with CPF at 25 °C. Nevertheless, insecticide treatments significantly increased OSI levels in lizards. A clear MDA increase occurred following CPF and AVM treatments at 35 °C compared with the control group at the same temperature. At a relatively lower temperature (15 °C), there was no statistical increase of the MDA level in CPF- and AVM-treated lizards, but the MDA content increased at 15 °C following BC treatment.

After 96 h of insecticide exposure, the residues of insecticides in brains were negatively related to the temperature treatments, but the content of the metabolite chlorpyrifos-oxon (CPF-oxon) increased at higher temperatures (Fig. 3 C). The highest concentrations of each compound in brains were 0.42 μg/g of BC at 15 °C, 0.32 μg/g of CPF at 15 °C, 0.27 μg/g of CPF-oxon at 35 °C, and 0.24 μg/g of AVM at 15 °C.

3.4. Risk assessment

In this experiment, the average body weight of adult male lizards is 3.15 g, and the calculated cover area is 9.34 cm^2. Based on the highest recommended application rates of BC, CPF, and AVM, the values of AD_{dermal} should be 0.52, 19.20, and 2.96 μg/g bw, respectively. The weighted LD50s ($\overline{LD_{50}}$) and RQs are summarized in Table 1.

4. Discussion

4.1. Effects of temperature on insecticide poisoning of lizards

In the acute toxicity tests, BC behaved like a NTDT to lizards, while CPF behaved like a PTDT under 15–35 ℃. This study showed that the toxicity of CPF to lizards increased 2.17-fold with a 10 °C increase in temperature. This is consistent with the findings that the LC50 value of CPF for predatory mites increased by 2.28 times at 28 °C compared with 20 °C (Jegede et al., 2017). In contrast, in the current study, a 10 °C increase in temperature was associated with an average 2.06-fold reduction in BC toxicity to lizards. Similarly, a negative temperature coefficient of pyrethroids (lambda-cyhalothrin and permethrin) has also been observed in several other pest species with 2- to 13-fold increases in toxicity noted for a decrease in temperature of approximately 10 °C (Brown, 1987; Grafius, 1986; Harwood et al., 2009; Valles et al., 1998). The trends in dose-dependent mortality with $T_{ambient}$ for these two insecticides were similar to the trends found in toxicological tests for other ectothermic species. The difference in toxicity of BC and CPF with temperature may be related to MOAs and toxicokinetics explained in Section 4.2. Although there was no clear temperature-dependent trend in the toxicity of AVMs, the LD50 values at 15 °C and 35 °C were lower than at other temperatures. This suggests that heat and cold stress may lead to a severe state of AVM exposure in lizards.

Steeper curves suggest that small changes in dose can have large response effects. The slope parameters (1/σ) of the three insecticides were larger in relatively warm environments (35 °C) than at the normal temperature (25 °C), indicating that at higher temperatures, a narrower variation in lethal responses within the lizard population leads to a shrinkage of the sensitivity distribution (Figure S5) (Silva et al., 2020). One possible reason is that heat stress can reduce individual fitness because of increasing basic metabolic requirements; therefore, a relatively small change in exposure dose may result in a significant change in mortality. In this case, the tolerance to insecticides tends to be uniform within the lizard population, and the impact of lethal-dose exposure may completely wipe out an entire lizard population. Conversely, regarding the toxicities of BC and CPF, the smoother curves at 15 °C suggest that the inequalities in individual lethal response expand within the population below normal temperatures. More individuals would be able to survive insecticide application under potential lethal rates.

4.2. Effects of temperatures on key events causing lethality

4.2.1. Thermal effects on toxicokinetics

Insecticides are rapidly metabolized by biotransformation enzymes. These include phase I and (MFO and CbE) and phase II metabolic enzymes (GST and UDPGT), as determined in this experiment. The enzyme-catalyzed reaction rate is determined by the amount of enzyme and the temperature under the same substrate concentration and chemical environment (Warshel and Bora, 2016). Thus, the activity measured at the same temperature can reflect the relative enzyme amount, which is representative for the induction of enzyme expression and synthetic plasticity in the liver. In groups with temperature treatments only (i.e., control groups at different temperatures), a relatively higher temperature generally induced the activities of MFO, CbE, and UDPGT, but not that of GST (measured at uniformed temperature of each enzyme). Changed GST activities may be related to the weakened antioxidant defense capacity at high temperature. The decrease of TAS at high temperature indicates a lack of non-enzymatic antioxidants (such as GSH). For both antioxidant and biotransformation via GST, it is futile to up-regulate GST in the absence of substrates.

The activated activity of detoxification enzymes at higher temperature reduces the burden insecticides pose on organisms. Biotransformation of BC to CPF usually occurs through the hydrolysis of carboxylates/phosphates, resulting in the formation of metabolites with lower toxicity (Chanda et al., 2002; Crow et al., 2007). These metabolites are mainly related to the function of carboxylesterases. CYP450 3A can metabolize AVM to 3-O-desmethyl, 24-hydroxymethyl, and 26-hydroxymethyl derivatives (Zeng et al., 1996), which correlate with MFO. However, CPF would undergo oxidative desulfurization under MFO catalysis to form the more toxic CPF-oxon (Harwood et al., 2009). Lower temperature exacerbates the toxicities of BC and AVM with accumulating level of insecticides. However, the limitation in producing highly toxic byproducts CPF-oxon at lower temperatures could be beneficial to lizard's survival following CPF exposure. For the same specimen, the catalysis capabilities of CbE, GST, UDPGT, and MFO were generally activated at higher temperature. The result suggests that enzyme activity increases with increasing temperatures in the range from 15 °C to 35 °C. Therefore, the toxicokinetic process of toxicants was improved via enzyme kinetics induced by enzyme amounts and thermal activities at higher temperature. In this situation, the insecticides in lizards could be eliminated rapidly.

Table 1
The $\overline{LD_{50}}$ weightings on temperature and the risk quotients (RQs) of lizards to three insecticides. The values formatted in bold indicate that the RQ values exceed the level of concern (=0.5).

Insecticides	Ambient thermal condition		Baseline T_b selection		Altered T_b selection	
	$\overline{LD_{50}}$(μg/g bw)	RQ	$\overline{LD_{50}}$(μg/g bw)	RQ	$\overline{LD_{50}}$(μg/g bw)	RQ
BC	463.6	0.0011	584.9	0.0009	600.3	0.0009
CPF	38.60	0.4975	29.00	**0.6622**	30.93	**0.6209**
AVM	633.5	0.0047	623.3	0.0048	625.3	0.0047

Insecticide treatments similarly affect biotransformation in the liver, and further feed back into the insecticide content in lizards. The MFO activity increased following CPF exposure at 30 °C and 35 °C. The results indicate that both CPF and higher temperature promoted the formation of the highly toxic metabolite CPF-oxon. A relatively low temperature appears to suppress CPF-oxon production from CPF-induced MFO activation. In contrast, at higher temperature, the MFO activity was weakened by BC and AVM compared with the control at the same temperature treatments, thus impeding the oxidative biotransformation of both compounds.

Overall, the amount of enzyme (derived from both expression and synthesis) for up-regulated biotransformation is an adaptive manifestation of an organism's response to exogenous pollutant stress (Lu, 2009; Waring, 2020). The detoxification requirements after exposure to the same dose of pollutants are consistent, but the detoxification capacity is regulated by the amount and activity of enzymes. As the enzyme activity is positively correlated with experimental temperature, an economical strategy should appropriately down-regulate the enzyme amount at high temperature. Following CPF treatments, CbE activity measured at the same temperature (25 °C), representing the amount of enzymes, increased significantly from 15 °C to 30 °C. However, the activity decreased at 35 °C compared with the controls at each temperature. Still, the CbE activity measured at 35 °C remained at a high level to meet the detoxification requirement and therefore, the downregulation of CbE at 35 °C seems to be adaptive and economical. Similarly, because of the low catalytic ability of enzymes at low temperature, cells should synthesize more enzymes to effectively metabolize exogenous toxins. However, such an economical strategy at low temperature was not found in this experiment, indicating that the feasible range of the metabolic adaptability of lizards in biotransformation is limited.

4.2.2. Thermal effects on toxicodynamics

The three insecticides tested in this study have different neurotoxicity mechanisms. CPF belongs to organophosphate pesticides, which function by inhibiting AChE activity and resultant cholinergic hyperstimulation (Bebe and Panemangalore, 2003). The results demonstrated that AChE activity could be stimulated by higher temperature and CPF could significantly inhibit AChE in any temperature treatment (Figure S4). Compared with the controls at the same temperature, the inhibition rate at 35 °C (79.4 %) was clearly higher than that at 15 °C (56.8 %). The differences of inhibition at different temperatures may be due to the higher level of CPF-oxon at higher temperature, as CPF-oxon is an approximately 1,000 times more potent AChE inhibitor than CPF (Das and Barone, 1999). BC is a kind of pyrethroid, which are sodium channel modulators with axon demyelinating effects (Davies et al., 2007). Pyrethroids bind more strongly to Na^+ channels at low temperatures (Gupta, 2018), and the steady-state resting potential of pyrethroid-exposed neurons is also higher at low temperatures (Narahashi, 1971; Salgado et al., 1989). AVM acts by interacting with the chloride channel in nerve and muscle cell membranes, resulting in an inhibitory effect on the nerve impulse (Rugg et al., 2005). Previous studies pointed out that the GABA content increased during hibernation in the lizard *Agama stellio* (Michaelidis et al., 2002), and AVM and heat stresses could both increase the level of GABA in the same species. Those findings indicate that both cold and heat stresses may be synergistic with AVM to activate GABAergic systems, which could induce chloride channel opening and consequently cause neural dysfunctions. Thermal effects on toxic MOAs of the three insecticides is highly consistent with the change trend of LD50s.

In addition to neurotoxicity, oxidative stress is the most common factor causing cellular damage under insecticide stress (Wang et al., 2016). When incubating lizards between 15 °C and 35 °C, increasing temperature has been found to alter the prooxidant-antioxidant balance, leading to lipid peroxidation. Heat stress leads to overproduction of free radicals and reactive oxygen species and weakens antioxidant barriers (Slimen et al., 2014). Thus, as shown in this study, in control groups, TOS increased but TAS decreased with increasing temperature. The prooxidative action mechanism of these three insecticides has been widely reported (Huang et al., 2019; Verma et al., 2016; Weis et al., 2021). Heat stress combined with insecticide exposure could exacerbate oxidative damage in the serum of lizards. The elevated serum MDA content of lizards incubated at 30 °C and 35 °C demonstrated the occurrence of lipid peroxidation, and exposure to CPF and AVM further aggravated oxidative damage. Lower temperatures can suppress cell metabolism and production of radicals, consequently alleviating the oxidative damage induced by CPF pesticides to a certain extent. However, this alleviating effect was inadequate, and the MDA content after BC treatment at 15 °C and AVM treatment at 20 °C still showed significant increases compared with the control groups at the same temperature.

4.3. Thermoregulatory behavior and biochemical profiles

In the current study, the processes of action and reaction were observed between the divergence of thermoregulation and insecticide exposures with TDCT. On the one hand, the specific toxicological MOAs of insecticides can interfere with the thermoregulatory response of the CNS. On the other hand, the TR response of lizards alters T_b, which is detrimental to lizards on multiple levels (Fig. 4). A framework proposed by Abram et al. (2017) suggests that the plasticity of behavioral thermoregulation can be generalized into two main components: the CNS-integrated effect and the physiological kinetic effect (Abram et al., 2017). It has been demonstrated that thermal preference is correlated with neurochemicals. Based on metabolomics data mining, six candidate compounds, including neurotransmitters and cellular energy metabolites, were screened out (Fig. 2C) to elucidate the neurochemical basis of TR behavior. The following sections explain how insecticides interfere with those metabolites and effects on TR behavior via toxicological MOAs.

4.3.1. Central nervous system-integrated effects: Neurological pivot on thermoregulatory behavior

The CNS can delicately modulate TR behavior in ectothermic vertebrates, which depends on thermal information transferred via excitation/inhibition of temperature-sensitive neurons, and the integration of complex networks in the nervous system (Angilletta et al., 2019). The neurophysiological and neurochemical bases of thermal preferences have been identified previously (Frank et al., 2015; Liu et al., 2015), and insecticide exposure can strongly interfere with related metabolites via toxicological MOAs. In this study, the level of Glu (the most major excitatory neurotransmitter) increased in the brain of lizards at higher temperature, while Tau and GABA (two important inhibitory neurotransmitters) were depleted in their brains. This result suggests that seeking a warmer environment occurred simultaneously with the amino-acid-induced excitation of nerves. In studies on rodents, Glu and GABA play a crucial role in thermal afferent signaling. The neurons in the dorsomedial hypothalamic nucleus (DMH) are glutamatergic, which could derive cold-defensive responses such as metabolic heat production and shivering (Morrison and Nakamura, 2019). Glu can activate several heat productive responses via the circuit in the preoptic area (POA) (Nakamura and Morrison, 2010). Conversely, activation of GABAergic neurons could eventually inhibit the excitatory neurons in the DMH, and therefore lead to heat-loss responses (Zhao et al., 2017). The increase of Glu and the decrease of GABA are likely the reason for the selection of a higher T_b following BC treatments (Fig. 2 A). Glu can be catalyzed to GABA by glutamate decarboxylase, and while the enzyme activity can be stimulated by AVM (Abdallah et al., 2020), it is suppressed by both BC (Wang et al., 2022a) and CPF (Zurich et al., 2004). To a certain extent, this can explain the increase in Glu and the decrease in GABA following BC exposure. Nevertheless, the alteration of amino-acid neurotransmitters could not explain the observed changes in thermal preference after CPF treatment. The inhibitory effects of CPF on AChE may lead to the accumulation of ACh in brains, causing abnormal activation of

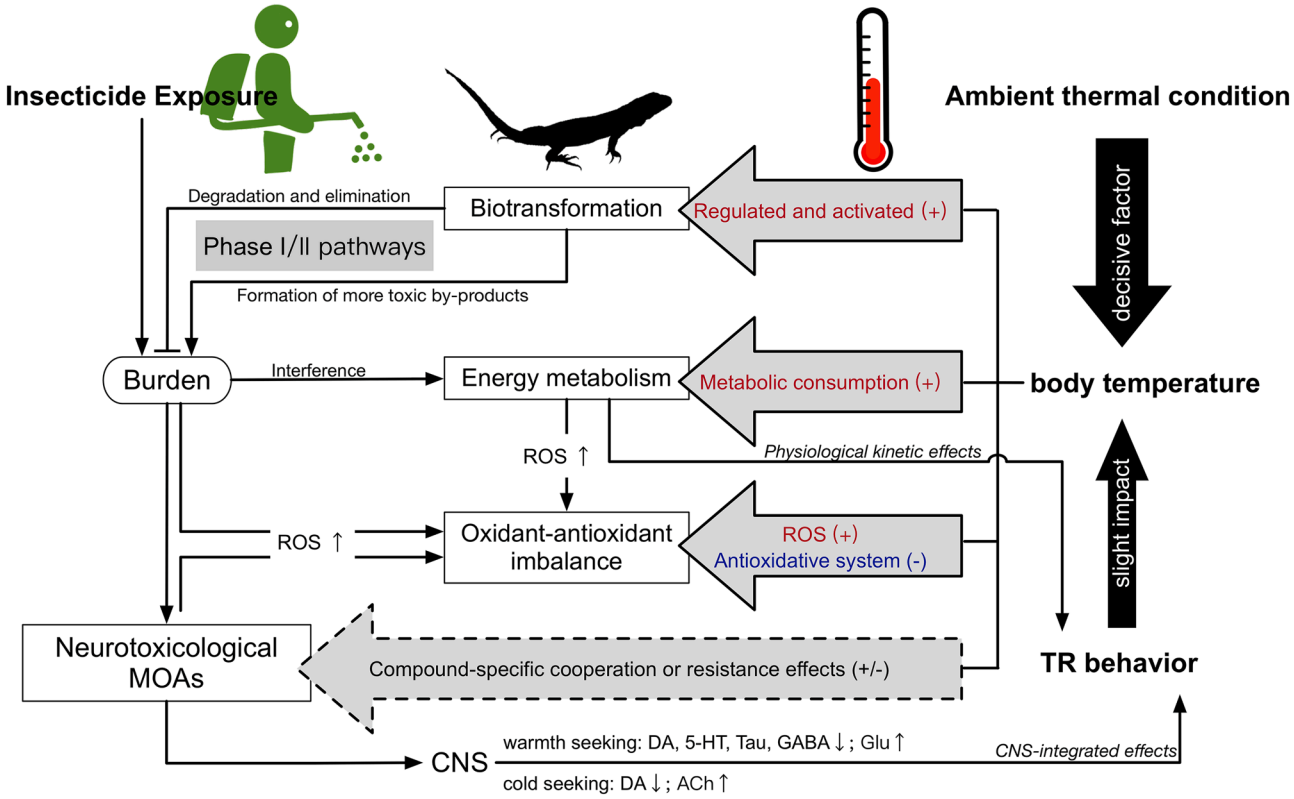

Fig. 4. Illustration of the interactions between insecticide exposure, thermal condition, and the effects on biotransformation, energy metabolism, and oxidative status of lizards.

cholinergic neurons. However, different from the Glu-excited POA, ACh, can directedly stimulate warm-sensitive neurons, especially at high levels (Chawla et al., 1975; Gordon, 2005; Meeter, 1972), and is integrated into the heat-defense response (i.e., seeking cooler environments in the current experiment). Cholinergic stimulation may dominate the thermotaxis center and overwhelm the excitation of Glu in POA. Consequently, CPF-treated lizards were more likely to select lower temperatures. Regarding AVM treatment, the increase of ACh and the decrease of GABA may reciprocally inhibit the warm and cold neural pathways. A noticeable thermoregulation change could not be produced from the combination of both effects.

In the CNS mechanisms involved in the control of T_b, the role of monoamines is complex and inconsistent. Previous studies in mammals proved that DA (Lee et al., 1985) and 5-HT (Mohamed and Rahman, 1982) can stimulate catecholaminergic synapses in POA that mediate heat dissipation-promoting and thermogenic-inhibiting pathways. NE may have increasing and hypothermic effects in CNS, depending on the species (Cooper et al., 1976; Osaka, 2009; Preston, 1975). However, the PLS-DA results did not support the contribution of changes in monoamine neurotransmitters to thermal preference. Still, ANOVA results showed that 5-HT levels were significantly lower in the heat-seeking lizard brain, and this reduction may stem from the fact that higher temperatures accelerate the decarboxylation of 5-HT, resulting in its depletion (Wilhoft and Quay, 1965). Therefore, catecholaminergic neural pathway activity is reduced in cold-seeking lizards, suggesting that monoamines play a secondary role in the thermoregulation of lizards with complex mechanisms. Moreover, the depletion of DA and its metabolites implies the occurrence of neurodegeneration following BC and CPF treatments (Betarbet et al., 2000; Xiao et al., 2021). This suggests that insecticide exposure adversely impacts the sensitivity and effectiveness of the strategic and TR behavior of lizards. Crossover excitation of thermoreceptors may occur simultaneously, and the interactions among neurochemicals are subtle, other than the thermoregulatory effects. The impaired CNS forms one of the functional bases of the maladaptive plastic behavior.

4.3.2. *Physiological kinetic effects: Behavioral compensation*

Adaptive changes in TR behavior can buffer the metabolic stress caused by toxicants, because changes in T_b caused by thermoregulation can partially compensate for temperature-dependent toxic damages. The membrane fluidity, a crucial aspect of cellular function, is enhanced at higher temperatures (Hazel, 1995). Myo-inositol is an important regulator of intracellular osmolarity (Lan et al., 2009). Phosphocholine and myo-inositol are precursors for the synthesis of the cell membrane, and they play an important role in the lipid metabolism (Paoletti et al., 2011). The results of the present study suggest that BC treatment reduced the levels of myo-inositol and phosphorylcholine in lizard brains, possibly leading to cell membrane disruption. Selecting a higher T_b, which can be helpful for chemicals to diffuse through the cellular membrane, may be a compensation for the functional impaired of the cell membrane following BC treatment.

Furthermore, temperature can strongly affect the rate of biochemical reactions in lizards, especially those active in cellular respiration (Piercy et al., 2015). Glucose is the main direct source of energy in the brain, and it is crucial for the synthesis of neurotransmitters by brain cells (Jequier, 1994). In particular, the monoamines DA, NE, and E, which were positively related to Glc in our experiments (Fig. 2 D), are known to be released in response to Glc (Koshimura et al., 2003; Sheridan, 1988). Exposure to all three insecticides decreased the glucose level in the lizard brain, which is a potential hazard for normal nervous function. CPF and most of AVM-treated lizards avoided choosing higher temperatures to exacerbate energy deficits, which can be understood as a compensatory behavioral strategy. However, selection of higher T_b under BC treatment appears to be contrary to this strategy for reducing glucose consumption. Lactic acid is produced through anaerobic respiration (Cohen, 1918), while succinate is an important metabolite of the

TCA cycle pathway in aerobic respiration, occurring in the mitochondrial matrix (Tretter et al., 2016). Following BC treatment, lizards sought a warmer environment; consequently, the level of Lac decreased, but the succinate level increased in their brains. The trends of these two metabolites suggest that mitochondrial function was fully mobilized with a higher T_b, which has been identified as an adaptative metabolic strategy to temperature changes in the lizard *Takydromus wolteri* (Sun et al., 2022). The increasing aerobic flux in cellular respiration at higher temperature induced by TR behavior could maintain a higher level of energy currency (e.g., ATP and creatine) while consuming the same amount of energy substances. The warmth-seeking strategy of BC-treated lizards originated from glutamatergic nervous excitation (neurotoxicological MOA of BC, as described in Section 4.3.1), which is also coupled with metabolic acceleration and increasing energy requirements (Fig. 2 D, clustering and positive correlations between Glu, Succ, and creatine). Energy requirements and metabolic costs are elevated in the toxic state, which involves the synthesis of more detoxifying enzymes, excretion of exogenous substances via active transport, reconstruction of damaged cell structures, and other defense- and repair-related processes (Wang et al., 2012). Selecting a higher T_b accelerates the generation of energy but depletes stored energy sources; therefore, it can also be understood as an adaptive consequence by which the energy supply meets neurophysiological demands. From the perspective of behavioral adaption, both warmth- and cold-seeking behaviors could compensate metabolic stresses caused by insecticide exposure, but different budget allocations derive two reasonable behavioral types. The first type is the conservative behavioral type, where cellular metabolism is suppressed to remit energy substance depletion. The second type is the progressive behavioral type, where more energy is produced to meet physiological requirements needed for countering toxicant stresses.

NAA, synthesized in neuronal mitochondria from aspartate and acetyl-CoA, provides energy for the conversion of amino acids to Glu (Zhang et al., 2009). The increase of NAA levels in CPF-treated groups indicate that deamination from amino acid into ketone bodies was enhanced. Combined with the elevated level of Lac, this shows that CPF exposure results in insufficient energy supply from aerobic respiration in lizards. Anaerobic respiration and ketone body pathways were stimulated to compensate for this energy depletion. Therefore, the cold-seeking behavior likely derives from the energy metabolic disorder to reduce energy consumption. In addition, the decrease in BCAAs caused by insecticide exposure may originate from increasing BCAA utilization in the TCA cycle (Maciejak et al., 2014). The initial step in the catabolism of BCAAs is reversible transamination with α-ketoglutaric acid (αKG) to produce branched-chain α-keto acid and Glu via BCAA transaminase (Sperringer et al., 2017). In light of our findings, it is conceivable that Glu accumulation in the BC-H group blocks BCAA deamidation, and the Val, Leu, and Ile contents remained at the control levels. Moreover, the effect of insecticide exposure on both the creatine cycle and purine metabolism (hypoxanthine increased with CPF and AVM treatments) was closely related to ATP synthesis. The changes in these two metabolites also implied the relationship between cellular energy flow and TR behavior.

In addition, the contents of BCAAs and AAAs were negatively correlated in the lizard brain (Fig. 2 D), which may be explained by the competitive transport of the two classes of substances. BCAAs and AAAs are transported to the brain by the same carrier system via large neutral amino acid (LNAA) transporters (Maciejak et al., 2014). Reduced BCAA levels may favor AAA entry into the brain, leading to changes in its concentration and metabolism as well as to altered monoamine neurotransmitter synthesis and release. Although the contributions of BCAAs and AAAs to TR behavioral variability were relatively small in this study after a short exposure period, insecticide-induced BCAA declines may have substantial effects on energy metabolism and monoamine neurotransmitters.

Briefly, the shortage of energy caused by insecticide treatments can restrict the allocation of energy for physiological processes, thus affecting lizards' TR strategies. The different neurochemicals discovered in this study provide a basis for further studies of the mechanisms underlying TR behavioral changes induced by toxicants.

4.4. *Thermoregulatory behavior is not the silver bullet for attenuating temperature-dependent chemical toxicity*

The coupling between TR behavior and TDCT may lead to two short-term outcomes (Fig. 5): adaptation (coupling ① and ②) and maladaptation (coupling ③ and ④). The results of this study support the adaptative changes of TR behaviors in lizards following BC and CPF exposure at sub-lethal levels. The toxicity of AVM did not show clear temperature dependence, and the variation of T_b selection would not result in notable changes of lethal effects. Although maladaptive conditions were not observed in this study, whether the reliance of lizards on TR strategies to mitigate toxicity is intentional is doubtful. Existing evidence cannot explain how lizards without a history of exposure to toxicants should judge if a chemical is PTDT or NTDT. The shift of thermal preference may only be the outcome of toxic action, and TR behavior is probably blind rather than proactive and cautious. Thus, the self-rescue behavior is likely an accidental coupling of insecticide-induced TR with a heat requirement that attenuates toxicity. In contrast to the performance of lizards exposed to sublethal doses of BC, lizards exposed to lethal doses of BC selected a cold environment (Wang et al., 2022a). This however is considered suicidal behavior from the perspective of acute intoxication (coupling ③). A possible reason for the inversion of TR behavior for the same chemical is that BC has a crossover stimulating ability to both glutamatergic (Mense et al., 2006) and cholinergic nerves (Rajawat et al., 2019). However, the activating potency on the former effect is much higher than that on the latter. Therefore, the sub-lethal dose of BC could only activate glutamatergic-related warmth seeking behavior, while the activation of cholinergic nerves is overwhelming at the lethal level leading to cold seeking behavior. Because of the mechanistic possibility, for lizards exposed to an assumed PTDT insecticide (with glutamatergic stimulating mechanisms on CNS, potentially resulting in warmth seeking), it would be hard to perform the self-rescue strategy, or even execute "suicidal" behavior (coupling ④). The chance that ectotherms employ TR behavior for toxic attenuation exists but it is slim.

TR behavior influences a variety of physiological functions; however, every coin has two sides. Firstly, selecting a higher T_b not only increases the biotransformation capacity to scavenge toxic compounds, but also induces oxidative stress and metabolic depletion (Fig. 4). The TR strategy cannot simultaneously reduce the damage caused by toxicants to all traits of life history, and the final performed TR behavior should depend on the optimal tradeoff on relative impaired strength. In our pervious study (Wang et al., 2021), simazine exposure prompted well-fed lizards to select a higher temperature for allocation to detoxification and physiological metabolism; however, starved lizards could not perform warmth-seeking behavior because saving energy seemed much more important than the stimulation of biotransformation under extremely severe conditions. Secondly, the thermal requirements for anti-toxicity action and natural metabolism may conflict. In the life history of lizards, variations of TR strategies may represent a short-term compensation for toxic stress, but the fitness for population sustainability would decrease abruptly. Acute exposure to sub-a lethal dose of AVM does not change a lizard's thermal preference (as shown in the current study), but chronic AVM exposure from soil has been shown to cause lizards to select higher T_b and induces long-term maladaptation (Nie et al., 2022). Avoiding the occurrence of adverse events can be understood as a "bargaining chip" in the decision of the TR strategy of organisms. However, while the trade-off is complex, it seems realizable.

Still, the ability of lizards to thermoregulate via their behavior enables the persistence of populations in environments that would otherwise exceed physiological and toxicological limits. The results of this study indicate the formation of self-rescue coupling. Therefore, a shift of thermotaxis could lead to northward or southward movements of lizard

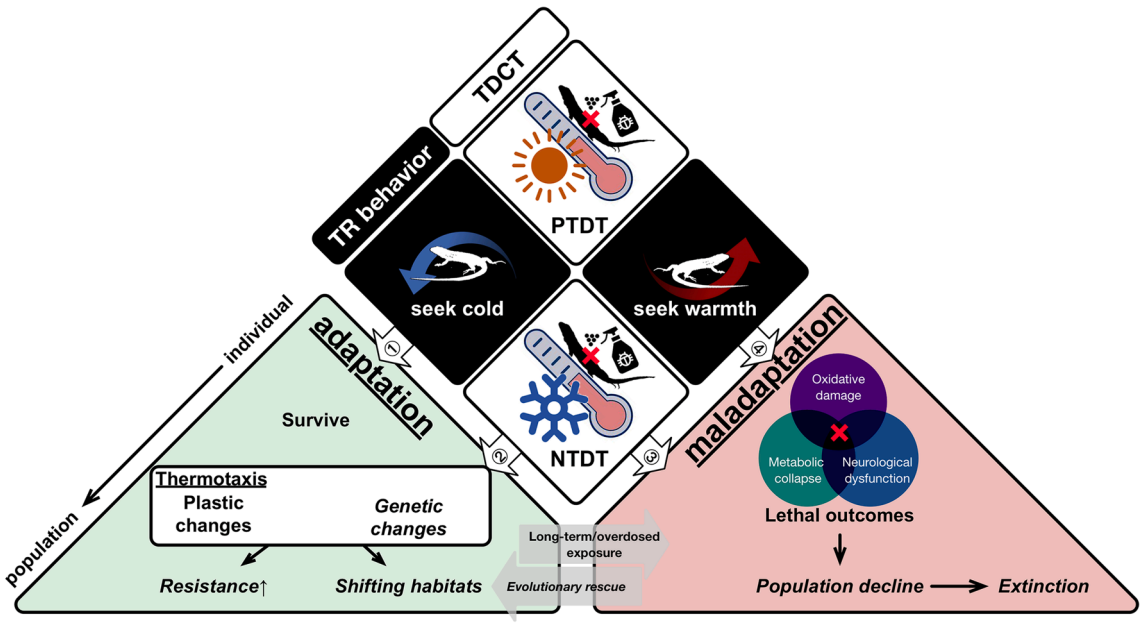

Fig. 5. Illustration of the couplings between temperature-dependent chemical toxicity (TDCT) and thermoregulatory (TR) behaviors, as well as two potential outcomes from the individual to the population levels. In this study, individual plasticity has been discovered, while the population and intergeneration changes (see italic text in the figure) require further research to be proved.

habitats, raising the fitness against toxic stresses, which has been proved in research on heat stress caused by global warming in populations (Auth et al., 2018; Krehenwinkel and Tautz, 2013). Furthermore, the sustained interaction between behavioral adaptations and pesticide exposure may be a driving force of population evolution. Insecticides are regularly introduced into the environment for crop protection, and consequently, lizards may be exposed periodically. The intergenerational conservation of self-rescue behavioral traits is particularly important for population fitness in response to insecticide stress. Insecticide-induced TR behavior may diverge because of different dosages and periods of exposure. Thus, insecticides could induce Darwin selection to preserve adaptative behavioral traits. A population in decline because of toxicant exposure would return to positive growth with propagation of resistant survivors. TR behavioral adaptation may allow "evolutionary rescue" for the persistence of lizard populations challenged by worldwide agrochemical applications, which results in opportunities for ectothermic species in environments with variable temperature. Whether behavioral traits are a result of genetic plasticity, allowing for individual acclimation, or local population adaptation, leading to inherited insecticide-tolerance, remains unclear and further research is required.

4.5. Risk assessments considering temperature-dependent chemical toxicity and thermoregulatory behaviors

A RQ provides a quantitative estimate for the environmental risk certain chemical substances pose (Nika et al., 2020). In general, insecticides with RQ $\geq$ 1 have been defined to carry significant environmental risk (Belew et al., 2021). The RQ value for the three insecticides investigated in this study under three thermal scenarios is<0.6622, indicating that the risk of single acute exposure under agrochemical application is acceptable for these lizards. However, according to USEPA (USEPA, 2022), the calculated RQ of wild animals should be less than the level of concern (LOC), a threshold set at 0.5 under acute high-risk presumption; if this threshold is exceeded, further management action should be employed. The RQ value of lizards exposed to CPF is<0.5 without considering TR behavior, but calculated values exceeded this threshold in both conditions considering thermal preference. Although self-rescue TR behavior results in a 0.04 reduction in the RQ value, this reduction ineffectively avoids the ecological risk of CPF for lizards. The inclusion of temperature-dependent endpoints and TR behavior in risk assessments would bridge the gap between traditional risk assessments and actual ecological effects caused by thermal-specific toxicity and behavioral plasticity. However, only male adult lizards were used in this study, and the generalizability of the results are therefore unclear. Lizards may have different susceptibilities to toxicants by age (egg, juvenile, yearling, and adult), sex (male and female), and physiological state (breeding season, post-breeding season, and hibernation) during exposure to insecticides (Peveling and Demba, 2003; Yu et al., 2022). Lizards inhabiting the north temperate zone experience prolonged hibernation annually in the winter, and the effects of TDCT would be strongly related to the environmental temperature in the absence of TR behaviors. Lizards in the egg stage are similarly unable to perform TR behavior, and the temperature conditions are determined by the location of spawning. Females usually choose laying locations with a suitable temperature and humidity (Huang and Pike, 2011). It will be very interesting to evaluate whether female lizards could detect and assess toxicants, temperatures, and TDCT when seeking nesting sites. In addition, the exposure analysis in this work used conservative estimates for simple agricultural application scenarios. Environmental factors (e.g., temperature, humidity, and light) can also affect the environmental fate of insecticides. Furthermore, insecticide formulations often contain surfactants, which may alter skin permeability. This may affect the absorption of toxicants and alter bioavailability. Changes in burden due to environmental factors may also introduce uncertainty in TR behavior, and these are worth considering in pesticide risk assessment.

The latitudes of China's territory cover nearly 50° in the northern hemisphere, making it difficult for unified risk management policies to achieve balanced consideration for agricultural areas with diverse thermal conditions. It is necessary for administering authorities to update appropriate strategies based on GCC and geographical climates. In addition, it may also be essential to consider physiological and behavioral compensation mechanisms of ectotherms to pesticide exposure in the sophisticated chemical management of agroecosystems. The authors propose that the adaptative changes in TR behavior of ectotherms leaves room in the protection of organisms in agroecosystems regarding the application of agrochemicals. Consequently, risk management policies would retain flexibility and resilience.

5. Conclusion

In this study, the temperature-dependent toxic effects of BC, CPF, and AVM on lizards were explored from the perspectives of biotransformation, oxidative stress, and neurometabolic disturbance. The causes of TR behavior and its role in alleviating acute exposure toxicity were elucidated. Generally, lizards have a higher biotransformation capacity at higher temperatures, which is not only conducive to the reduction of the concentration of toxicants, but also makes the generation of highly toxic metabolites more likely. Higher temperatures can systematically induce severe oxidative stress and oxidative damage. Changes in the levels of metabolites identified the association of TR behavior with neurochemicals related to neurotransmitters, energy supply, and membrane structure. Six metabolites were identified as possible causes of the observed changes in thermotaxis. Temperature could interact with insecticide toxicity resulting in insecticides exhibiting different effects at different temperatures, where insecticides cause lizards to actively change their T_b in an attempt to adaptively reduce toxicity. However, the effectiveness of this "self-rescue" is very limited because the RQ did not decrease noticeably with the insecticide-induced alteration of TR behavior. These results may guide the development of a more flexible ecological risk management strategy for insecticides at the population and evolutionary levels for reptiles.

CRediT authorship contribution statement

Zikang Wang: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Yufan Nie:** Investigation. **Simin Yu:** Investigation. **Li Chen:** Investigation. **Luyao Zhang:** Investigation. **Wentao Zhu:** Supervision. **Zhiqiang Zhou:** Supervision. **Jinling Diao:** Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Contract Grant number: 42277276) and the 2115 Talent Development Program of China Agricultural University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2023.107742.

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