BIOCHEMICAL RESPONSES OF TWO ERYTHRINIDAE FISH TO ENVIRONMENTAL AMMONIA

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ABSTRACT

The non-ionized form of ammonia is very toxic to many aquatic species. It is especially important in several aspects of fish biology. A large range of organismal strategies for coping with environmental stressors is usually observed in living organisms. Among those, the responses for managing chemical stressors are well studied. The present work compares biochemical responses of two evolutionarily close species, *Hoplias malabaricus* and *Hoplerythrinus unitaeniatus*, exposed to environmental ammonia. Adult fish were submitted to 1.0 mg/L of ammonium chloride for 24 hours, and plasma ammonia and urea levels were determined. The activities of OUC enzymes OCT and ARG, and the accessory enzyme GS, were quantified in liver extract and are expressed below in μmol/min/mg of wet tissue. Increases in OUC enzymes (GS from 1.14 to 2.43, OCT from 0.81 to 1.72, and ARG from 3.15 to 4.23), plasma ammonia (from 0.95 to 1.42 mmol/L), and plasma urea (from 0.82 to 1.53 mmol/L) were observed ($p < 0.05$) in *H. malabaricus* exposed to 1 mg/L of ammonia chloride. The GS in *H. unitaeniatus* increased from 1.43 to 1.84, however the OCT, ARG, and plasma urea from *H. unitaeniatus* did not change. These data indicate that each species responds differently to the same environmental stressor.

Key words: Hoplias malabaricus, *Hoplerythrinus unitaeniatus*, ammonia, biochemical adaptation, fish, Ornithine-urea cycle enzymes.

RESUMO

Respostas bioquímicas de duas espécies de Erythrinidae à amônia ambiental

A forma não ionizada da amônia é muito tóxica a vários organismos aquáticos, sendo particularmente importante em muitos aspectos da biologia dos peixes. Um amplo grupo de estratégias para enfrentar os estressores ambientais pode ser observado nos organismos vivos. Dentre estas, as respostas aos estressores químicos são bem estudadas. O presente trabalho compara respostas bioquímicas de duas espécies evolutivamente próximas *Hoplias malabaricus* e *Hoplerythrinus unitaeniatus*, expostas à amônia ambiental. Peixes adultos foram submetidos a 1.0 mg/L de cloreto de amônio por 24 horas e foram determinados os níveis plasmáticos de amônia e uréia. As atividades das enzimas do COU, OCT e ARG e a enzima acessória GS foram quantificadas em extrato de fígado e são expressas em μmol/min/mg de tecido úmido. Foi observado em *H. malabaricus*, exposto a 1,0 mg/L de cloreto de amônio, aumento ($p < 0,05$) nas enzimas: GS, de 1,14 para 2,43; OCT, de 0,81 para 1,72; ARG, de 3,15 para 4,23; na amônia plasmática, de 0,95 para 1,42 mmol/L, e na uréia plasmática, de 0,82 para 1,53. A GS de *H. malabaricus* aumentou de 1,43 para 1,84, todavia, OCT, ARG e uréia plasmática não variaram. Esses dados mostram que ambas as espécies, taxonomicamente próximas, respondem distintamente ao mesmo estressor ambiental.

Palavras-chave: Hoplias malabaricus, *Hoplerythrinus unitaeniatus*, amônia, adaptação bioquímica, peixe, enzimas do ciclo Ornitina-urea.

ABBREVIATIONS

ARG (arginino hydrolase), EDTA (ethylenediaminetetracetic acid), GS (glutamine synthetase), Ki (inhibition constant), K_m (Michaelis-Menten constant), MS222 (3-aminobemzoic acid ethyl ester), HEPES (N-2-hydroxymethyl-piperazine-N'-2-ethanesulfonic acid), National Center of Research on Tropical Fish CEPTA-IBAMA, OCT (ornithine carbamoyl transferase), OUC (ornithine urea cycle), PCA (perchloric acid), ppm (parts per million), TCA (trichloroacetic acid), TRIS (Tris[hydroxymethyl] aminomethane).

INTRODUCTION

Several physical and chemical components of freshwater environments often fluctuate, producing changes to which organisms within the water must accommodate. Ammonia is a chemical example. However, if high content of ammonia is present, as is usual in eutrophic water environments, it works as a stressor (Alabaster & Lloyd, 1980). Stressing components in the medium demand adaptive responses from the organism. A large group of responses constantly stands ready to face ambient oscillations in order to preserve organism homeostasis. Those responses consist of a range of morphological (Val & Almeida-Val, 1995) or physiological and molecular adjustments (Hochachka & Somero, 1973, 1984), and determine the required biochemical adaptations.

Environmental ammonia concentration is dependent on temperatures ranging from 0.0083 to 89 percent (Emerson *et al.*, 1975). Two chemical forms exist, NH_3 and NH_4^+ and the ratio of both is pH dependent. In spite of ammonia being a nutrient in eutrophic cells, most of vertebrates exhibit a low tolerance to this compound. High ammonia concentrations are a limiting factor in farm fishing because such concentrations cause growth rate decrease, plasma cortisol increase, gill damage, impairment of gas diffusion, and excessive mucus production (Smart, 1976; Tomasso *et al.*, 1981; Spotte & Anderson, 1989; Wright, 1995). Sensitivity of many freshwater teleosts to increasing external ammonia concentrations can culminate in death (Olson & Fromm, 1971; Dabrowska & Wlasow, 1986).

Toxicity through increase of external ammonia comes with corresponding plasma level enhancement. Among the ammonia detoxification strategies, glutamine formation from glutamate and the urea synthesis from glutamine and aspartate (Mommsen, & Walsh, 1989, 1992; Walsh, 1997; McKenzie *et al.*, 1999) are relevant. Glutamine synthesis occurs independently of urea formation. However, fish urea synthesis is dependent on glutamine as substrate. Regulation of these biochemical processes is done in many ways, such as gene control, enzyme compartmentalization, metabolite concentration, and kinetic characteristics of enzymes. In this study of OUC enzymes, characteristics correlated to environmental pressures provide a path to understanding relevant aspects of regulatory mechanisms in biochemical processes.

Hoplias malabaricus (traíra) and *Hoplerythrinus unitaeniatus* (jeju) inhabit warm and lentic waters exposed to several biological, physical, and chemical changes (Godoy, 1975; Nelson, 1984). It should be expected that closely related species are likely to use similar strategies to cope with environmental changes. Our question is if evolutionary closely species, usually sharing the same environment, display similar strategies in coping with environmental stressors. We exposed two closely related teleost species, *H. malabaricus* and *H. Unitaeniatus*, to increasing environmental ammonia and observed the nitrogenous excretory pattern.

MATERIAL AND METHODS

Fish collection and maintenance

Adult fish of the species *H. malabaricus* and *H. unitaeniatus* were collected from shallow ponds on the shore of Mogi-Guaçu River, São Paulo State, Brazil and brought to the aquaculture facilities of the CEPTA-IBAMA in Pirassununga. The experiments were performed in January and February. Twelve jeju, weighing 20 ± 5 g (means $\pm$ SD), and 12 traira weighing 90 ± 10 g (means $\pm$ SD), were kept unfed in 500 L boxes for 14 days before the experiments. Aerated water (7.5 ppm of oxygen) was pumped from the Mogi-Guaçu River into the laboratory, and the water quality was the same for all experiments. Water temperature was kept constant at 25 ± 2^oC, and the pH was monitored and kept at 7.0 ± 0.5.

Experimental design

The specimens of jeju and traíra were transferred to four aquaria of 60 L (six fish per aquarium). Two aquaria, one per species, were kept undisturbed (control). Ammonia chloride was added to the others to the final concentration of 1.0 mg/L. Water hardness was 40 mg $CaCO_3$/L, alkalinity was 21 mg/L, and pH was constantly monitored and kept at 7.0 ± 0.2 throughout the experiment. Concentrations of NO_2^- and Cl^- were inconspicuous. The fish were kept throughout the experiment in a static system (without water renewal). After 24 h they were transferred to a single 3.0 L box containing 0.27 g/L MS222, pH 7.0 ± 2. Right after the anesthetic effect, a blood sample was taken from the caudal vein in 1.0 ml syringes and placed in heparinized 2.0 ml polypropylene tubes. The blood samples were centrifuged at 9,000 g for 5 min. The plasma was stored at –20°C for posterior biochemical analysis. Following the blood collection, the fish were killed by pinching the spinal cord. The liver was excised and immediately frozen in liquid nitrogen for later biochemical analysis.

Tissue extract

The liver samples were homogenized under ice-cold conditions at the ratio of 1:6 (w/v) in homogenization buffer (0.01 M Tris, 0.02 M Na phosphate, 0.01 M glycine, EDTA 0.5 mM pH 7.0, into glycerol v/v) in a glass vessel with a motor-driven Teflon pestle. The homogenate was centrifuged at 3,000 x g at 4°C for 15 min and the pellet was discarded. The supernatant was centrifuged at 8,000 x g at 4°C for 20 min and used as a soluble enzyme source (crude homogenate).

Perchloric acid plasma extract

The plasma, obtained by blood centrifugation at 9,000 x G for 5 min, was treated with 0.6 N PCA 1:10 (v/v). The samples were neutralized with 6.0 N $KHCO_3$ and the precipitate was removed by centrifugation. Urea (Rahmatullah & Boyde, 1980), ammonia (Gentzkow & Mansen, 1942), and uric acid (Henry *et al*., 1957) were determined in the supernatant.

Ornithine carbamoyl transferase (OCT)

The OCT enzyme activity was assayed in 1.5 ml of reaction mixture containing, to final concentration, 50 mM of HEPES (pH 8.0), 10 mM of ornithine, 10 mM of carbamoyl phosphate, and suitable enzyme aliquot (Boyde & Rahmatullah, 1980). The samples were incubated at 25°C for 30 min and the reaction was stopped by 70% TCA addition. The reaction mixtures were centrifuged at 12,000 x G for 2 min. Citrulline, the enzyme end product, was colorimetrically determined at 464 nm.

Glutamine synthetase (GS)

The GS enzyme activity was assayed by γ-glutamyl hydroxamate formation modified (Vorhaben *et al*., 1973). The incubation mixture, containing to final concentration 50 mM HEPES pH 7.0, 60 mM glutamine, 15 mM hydroxyl amine, 0.4 mM ADP, 20 mM $NaAsO_4$, and 3 mM $MnCl_2$ was brought to a final volume of 1.5 ml with a suitable amount of enzyme. After incubation for 60 min at 25°C, the reaction was stopped with 300 µL of ferric-chloride-reagent and centrifuged at 7,000 x G for 1 min at 5°C. The reaction product γ-glutamyl hydroxamate was directly estimated in the supernatant at 560 nm.

Arginino hydrolase (ARG)

The ARG activity was assayed by urea determination from the arginine hydrolysis (Rahmatullah & Boyde, 1980). The reaction mixture containing 50mM glycine (pH 10.0 for traira assays or pH 9.5 for jeju), 278 mM arginine, and 10 mM $MnCl_2$ was brought to final volume of 1.5 ml with appropriate enzyme aliquot. The reaction was stopped with 70% TCA, centrifuged at 7,000 x G for 1 min, and urea was colorimetrically determined at 460 nm.

Enzyme optimization

The enzyme reaction of ARG and OCT were optimized for pH and temperature. The pH range assayed varied from 4.0 to 11.0 for both enzymes, and the buffer systems citrate, TRIS, HEPES, glycine, and glycil-glycine were assayed. The best buffer and the optimum pH are depicted above in the enzyme assay procedures. The optimum temperature assayed was from 20 to 85°C. The reactions were carried out under a water-bath, observing temperature intervals of approximately 10°C, with the best buffer system at the optimum pH. Considering the elevated optimal temperature, the values of K_m for ARG and OCT were determined at room temperature (25°C).

Chemicals

All chemicals were analytical grade purchased from Sigma Chemical Co., or Merck. The MS222 was from Sandoz.

Statistics

Statistical tests were performed using the Mann Whitney test. The significance level was set at $p \leq 0.05$. The Pearson correlation coefficient was used for some parameters and the critical values for (r) were set at 95%.

RESULTS

Traíra exposed to ammonia did not show any behavioral change. However, jeju exhibited elevated activity in response to ammonia exposure. Enzyme kinetics of ARG for both species were similar, depicting a Michaellis-Menten profile (Fig. 1A and B). The V_{max} were 0.023 µmol/min mg of wet tissue for traíra, and 0.039 µmol/min mg of wet tissue for jeju. The carbamoyl phosphate saturation curves for OCT of traira and jeju were similar and a typical Michaelis-Menten kinetic was observed for both. However, OCT from traira was inhibited with 3 mM ornithine. The same effect for jeju was not evident but a trend for inhibition was observed. The OCT from both species were inhibited by inorganic phosphate (Fig. 2A and B). The kinetic parameters of OCT and ARG from both species are shown in Table 1. Plasma ammonia of traíra and jeju exposed to environmental ammonia increased significantly but increase of urea was observed only in traíra (Table 2). All liver OUC enzymes from traíra increased under ammonia exposure whereas only GS increased in jeju (Table 2). Changes in plasma uric acid were detected in neither species and the concentration was very low (traces).

DISCUSSION

Both species, traíra and jeju, probably arose in the Jurassic (Godoy, 1975), and are presently living in similar environments (Nelson, 1984). Traíra and jeju usually feed on fish and insects respectively, which are both rich in protein. This kind of rich protein nourishment was proposed to induce changes in nitrogen catabolism (Cvancara, 1969). Among the OUC enzymes, ARG activity is supposed to increase under rich protein diets supporting nitrogen excretion (Cvancara, 1969). The compartimentalization of OUC enzymes of erythrinidae were previously studied by Polez *et al.* (1998) under usual environmental conditions. However, the ammonia exposure induced differential changes in the enzyme kinetic characteristics. This suggests distinct roles of OUC enzymes for the species in coping with ammonia.

The enzyme kinetics of ARG and OCT presented interesting features. The ARG characteristics for both species appear very similar. In spite of the hypothesis supporting a direct relationship between ARG activity and dietary protein level (Cvancara, 1969), a large range of enzyme activities with very similar values for distinct species could be observed. Jeju and traíra feeding high protein diets should result in increase of amino nitrogen wasting through ARG.

TABLE 1
Kinetic parameters of liver ornithine carbamoyl transferase (OCT) and arginine hydrolase (ARG) from *H. malabaricus* and *H. unitaeniatus* exposed to 1.0 mg/L of ammonia.

	OCT		ARG	
Parameter	***H. malabaricus***	***H. unitaeniatus***	***H. malabaricus***	***H. unitaeniatus***
pH	8.0	8.0	10.0	9.5
Temperature ^{o}C	>85	>85	35	50
K_m (mM)				
Ornithine	0.47	1.08	–	–
Carbamoyl phosphate	0.39	0.83	–	–
Ornithine + Pi	0.55	1.16	–	–
Arginine	–	–	4.35	3.84

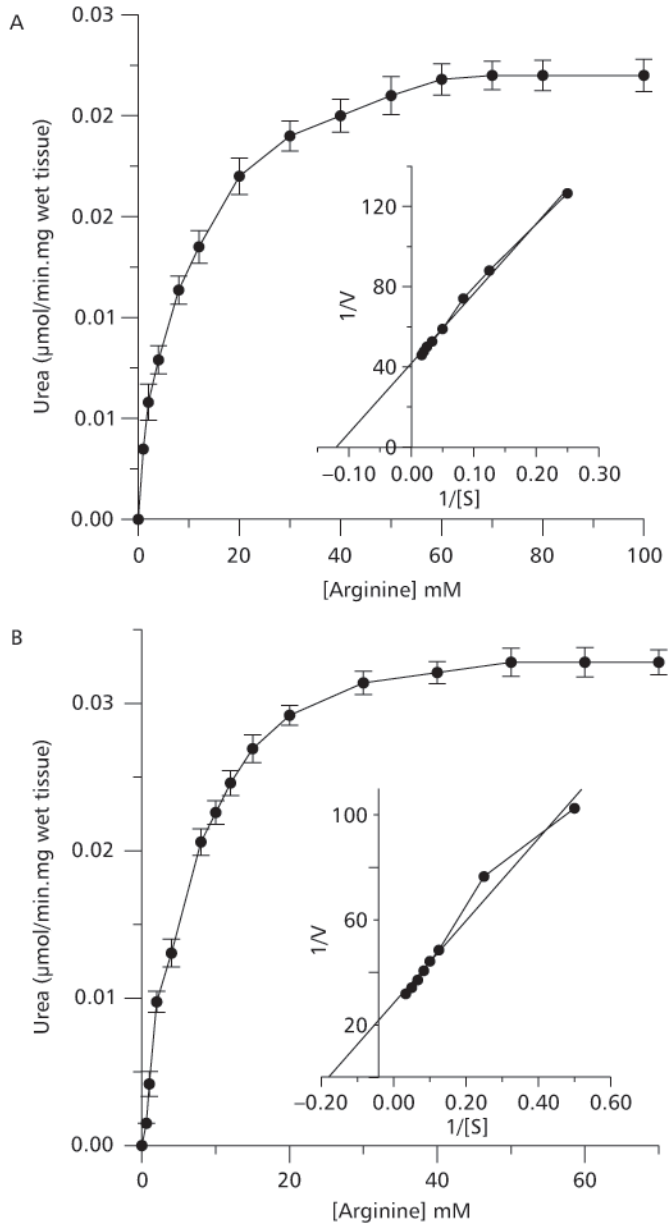

Fig. 1 — Substrate saturation curve of hepatic ARG of *H. malabaricus* (A) and *H. unitaeniatus* (B). The K_m values ware 4.35 mM (A) and 3.84 mM (B). The data were derived from hepatic homogenate of fish from environmental pH 7.0 (control). The pH reaction was 10.0 (A) and 9.5 (B) as previously optimized, and the incubation temperature was 25°C.

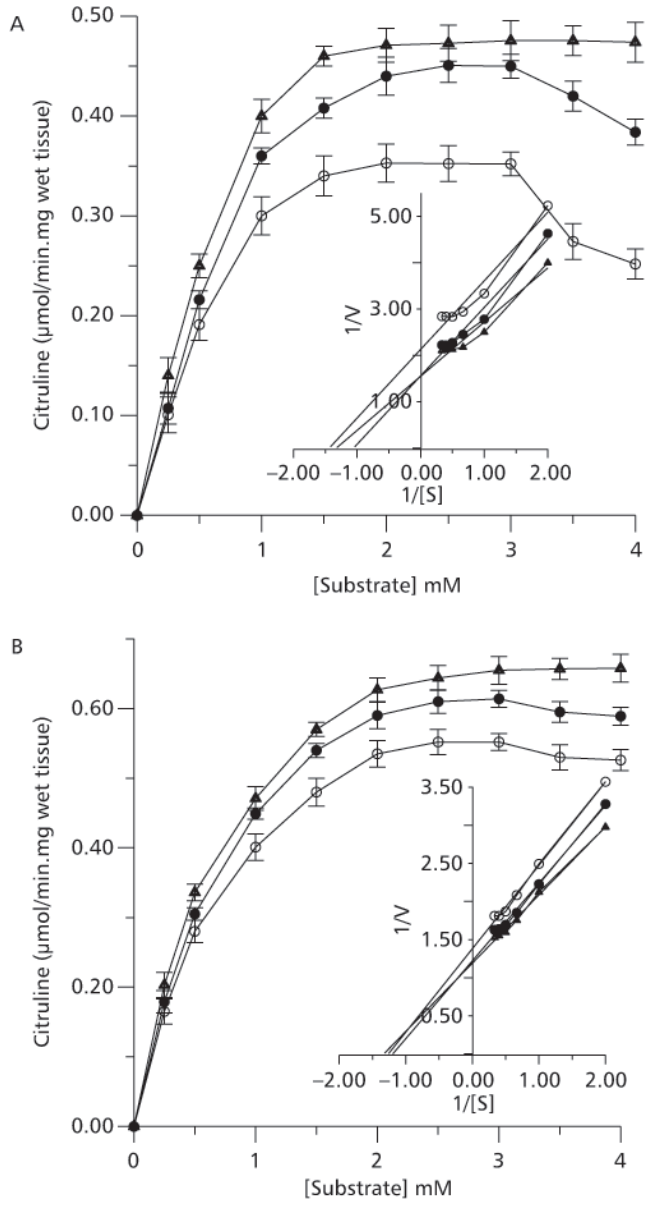

Fig. 2 — Substrate saturation curve of hepatic OCT from *H. malabaricus* (A) and *H. unitaeniatus* (B) and its phosphate inhibition curves. The K_m values were: **Fig. A**, Δ carbamoyl phosphate 0.39 mM, • ornithine 0.47 mM; **Fig. B**, Δ carbamoyl phosphate 0.83 mM, • ornithine 1.08 mM. The OCT Ki_s for inorganic phosphate (Pi) were O 0.55 mM (A) and O 1.16 mM (B). The data were derived from hepatic homogenate of fish from environmental pH 7.0 (control). The pH reaction was 8.0 for both assays as previously optimized, and the incubation temperature was 25ºC.

TABLE 2
Specific activities of liver glutamine synthetase (GS), ornithine carbamoyl transferase (OCT), and arginine hydrolase (ARG) from *H. malabaricus* and *H. unitaeniatus* exposed to 1.0 mg/L ammonia. All values are expressed as means ± SD.

	H. malabaricus		***H. unitaeniatus***	
OUC enzyme (μmol/min/mg of wet tissue)	Control	NH_4^+ exposed	Control	NH_4^+ exposed
GS	1.14 ± 0.04	2.43* ± 0.05	1.43 ± 0.05	1.84* ± 0.04
OCT	0.81 ± 0.02	1.72* ± 0.03	1.22 ± 0.03	1.27 ± 0.02
ARG	3.15 ± 0.12	4.23* ± 0.23	16.41 ± 1.22	17.11 ± 1.53
Plasma catabolite (mmol/L)				
Ammonia	0.95 ± 0.03	1.42* ± 0.02	1.19 ± 0.04	1.98* ± 0.03
Urea	0.82 ± 0.02	1.53* ± 0.04	0.50 ± 0.03	0.59 ± 0.02

Values were compared by Mann Whitney Test and significant difference ($p < 0.05$) between control *versus* exposed are marked by (*).

However, in the presence of high ammonia levels, OCT activity may improve nitrogen excretion with efficiency being inversely related to K_m. The apparent K_m obtained for jeju's OCT was twice that of traíra considering both substrates, ornithine and carbamoyl phosphate. These values, indicating different substrate affinities, suggest that ornithine catalysis toward urea was twice as fast in traíra if cellular concentration of ornithine was around 0.5 mM. The cell ornithine concentration was not determined, however, considering the inhibitory effect of 3.5 mM ornithine on both OCT, it is reasonable to suspect that the enzyme had a regulatory effect on urea synthesis. Inhibition of OCT by 10 mM ornithine has been reported for teleost fish (De Gregório *et al.*, 1993). The inhibitory effect of ornithine on OCT of jeju was less evident. These results agree with the distinct strategies we propose for ammonia detoxification in both species. Regulation of OCT by phosphate, the enzyme reaction product, is also indicative of adjustments in urea production in both species. However, traíra is more responsive to [Pi] than jeju.

Exposure of jeju and traíra to environmental ammonia resulted in different responses. Plasma ammonia in both species increased under ammonia exposure. This increase was slightly larger for jeju in which urea remained constant. The OUC enzymes OCT and ARG were unchanged but GS increased significantly. These results suggest that this species does not use urea synthesis strategy to reduce plasma ammonia. The tolerance concentration of blood ammonia ranges between 0.5-1.0 mmol/L for teleost (Wright, 1993). Compared to other fish species, jeju and traíra showed high blood ammonia and the exposure to 1.0 mg/L of environmental ammonia increased those values. The arctic char (*Salvelinus alpinus*) shows flaccid paralysis as plasma ammonia reaches 2.0 mmol/L (Lumsden *et al.*, 1993), nevertheless the air-breathing teleost *Heteropneustes fossilis* can tolerate the unusually high concentration of 4.0 mmol/L. The experimental ammonia concentration (1.0 mg/L) used in the present work did not cause death or behavioral disorders in traíra. However, a remarkable activity level was observed for jeju. The exposure time could have been insufficient to observe further effects, but biochemical changes were observed. The significant increase of the inducible enzyme GS seems to be a way to minimize the ammonia toxicity in jeju other than urea synthesis. The synthesis of glutamine as a chemical compound to hide the free ammonia for posterior excretion by glutaminase activity should be the strategy used by jeju. A distinct one was clearly observed for traíra. The increase of OUC enzymes ($p < 0.05$) and the plasma urea concentration (60 percent higher than jeju) are solid evidence that urea synthesis is the choice to detoxify the blood ammonia. The metabolic outlines of jeju and traíra in response to external ammonia increase permit one to assume that both species, although they are very evolutionary close and living in the same environment, present

different biochemical responses to the same aggressor. These different biochemical aptitudes should partially explain why traíra is able to survive for long periods in mud during dry seasons, while jeju snakes quickly over the grass looking for new water ponds.

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