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Acute Toxicity of Nitrite to Various Life Stages of the Amazon River Prawn, *Macrobrachium amazonicum*, Heller, 1862

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Abstract This study determined the effects of nitrite on different life stages of the Amazon river prawn *Macrobrachium amazonicum*. Prawns of each life stage (postlarvae, juveniles and adults) were stocked in 24 experimental units (n = 10 prawns), under a complete randomized design. Individuals were exposed to nitrite (0, 1, 2, 4, 8 and 16 mg L^{-1}). The median lethal concentration after 96 h (96 h LC_{50}) was calculated through the Weibull I. The mortality results showed that *M. amazonicum* is slightly less tolerant to nitrite than other species of *Macrobrachium*. The 96 h LC_{50} for postlarvae, juveniles and adults of *M. amazonicum* were of 1.49, 2.36 and 2.34 mg nitrite/L, respectively. Nitrite intoxication risk quotient suggest moderated risk to low risk to the species. Usually in production systems nitrite values are lower than safe levels suggested in this study (0.1 mg L^{-1} to postlarvae and 0.2 mg L^{-1} nitrite to juvenile and adults), which makes our results appropriate for the production of this species.

Keywords Freshwater prawn · Nitrogen compound · *Macrobrachium* · Toxicity

Freshwater prawn farming has shown great increase worldwide in the last two decades (FAO 2014; Hayd et al. 2014). Although the main reared prawn in Brazil is still the exotic species *Macrobrachium rosenbergii*, the indigenous species *M. amazonicum* has demonstrated similar production potential (Moraes-Valenti et al. 2010). The production of native species is essential for the conservational aspect of good aquacultural practices. Production of native species of prawns has become a trend, as the production of *Macrobrachium nipponense* in China (Kutty and Weimin 2010) and *Macrobrachium malcolmsonii* in India (Kutty 2005; Kutty and Valenti 2010). This practice prevents possible problems caused by the introduction of exotic species in the environment (Bridger and Garber 2002), besides reducing the uncontrolled exploitation caused by fishing (Silva et al. 2007).

Physiological processes of aquatic organisms, decomposition of organic matter, and food leftovers are the main sources of nitrogen compounds in aquaculture production systems (Campos et al. 2012). Nitrite is the intermediate compound in the bacterial nitrification of ammonia to nitrate. It may present high toxicity, depending on its concentration in the environment and organism developmental stage (larvae to adult) (Miranda-Filho et al. 1995). Several studies with *M. amazonicum* were already carried out to better understand its biology (Boudour-Boucheker et al. 2013; Meireles et al. 2013), ecology (Moreira and Collart 1993; Dutra et al. 2014), and production features (Anger and Hayd 2010; Moraes-Valenti et al. 2010). However, studies on nitrite effects available in the literature are limited to larvae (Hayd et al. 2014), since the studies to cultured shrimps are mainly focused on penaeids (e.g., Cheng and Chen 2002;

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Chen and Cheng 2000). Mallasen and Valenti (2006) recommend nitrite values below 2.0 mg L^{-1} in the production of freshwater prawns.

Therefore, based on the hypothesis that the early stages of development of *M. amazonicum* are more sensitive to nitrite than later stages, the aim of the study was to assess the effect of nitrite on different life stages of the Amazon river prawn—*Macrobrachium amazonicum*, Heller, 1862, through the determination of 96 h LC_{50} concentrations of nitrite. To determine LC_{50} values, the prawns were submitted to six different nitrite concentrations (0, 1, 2, 4, 8 and 16 mg nitrite/L) in four replicates, during 96 h. Afterwards, the mortalities were assessed for each life stage (postlarvae, juveniles and adults).

Materials and Methods

The experimental work described here was conducted at the Prawn Culture Laboratory, located at the Federal University of Paraná—Sector Palotina, with animals provided by the Laboratory of Prawn Culture of the CA-UNESP (Universidade Estadual Paulista "Julio de Mesquita Filho"). The experiments used 240 prawns of each life stage (postlarvae, juveniles and adults). The prawns reach the post-larvae stage after they go through all larval development (nine different stages from zoea I to zoea IX), the juvenile stage is reached approximately 30 days after they reach the post-larvae stage and the prawns are considered adults when they reach the reproductive stage and can be identified through the gonadal development (Vega-Villasante and Carrillo 2006; New et al. 2010). The prawns were randomly divided into 24 experimental units, with 10 prawns in each experimental unit. The wet weight and the total length (rostrum to telson) were measured in postlarvae (0.088 ± 0.022 g; 2.250 ± 0.170 cm, mean $\pm$ SD), juveniles (2.023 ± 0.271 g; 6.729 ± 0.296 cm) and adults (6.240 ± 1.827 g; 9.084 ± 0.793 cm); n = 50 out of the 240 used, randomly chosen. As experimental units we chose beakers with a volume of 1 L to post larvae, or glass aquaria containing 10 L of useful volume to juveniles and adults. Experimental units were equipped with aeration systems and light sources for a light:dark rhythm of 12:12 h, in a climate-controlled room (25–27 °C). The design was completely randomized, with six different nitrite concentrations (0, 1, 2, 4, 8, and 16 mg nitrite/L), and four replicates per nitrite concentration. The nitrite levels resulted from the addition of appropriate volumes of the stock solution of $NaNO_2$ PA—Synth® (stock solution of 250 mg nitrite/L) to produce the desired final nitrite concentrations. The concentrations used were based on work done with *M. rosenbergii* (Mallasen and Valenti 2006). Mortality was assessed by total absence of movement or reaction to mechanical stimuli using a glass rod. Prawns were observed every 1 h for the first 8 h. Between 8 h and 96 h, observations were performed every 12 h (Armstrong et al. 1976).

The following variables of water quality were daily evaluated: dissolved oxygen (Oxymeter, Hanna HI 9146), temperature (digital thermometer Incoterm), and pH (Phmeter, Tekna T-100). At the beginning and at the end of the experiment, alkalinity and hardness of water samples were assayed by titration. Total ammonia levels were determined according to Koroleff (1976), where the sample containing ammonia reacts with phenol and sodium hypochlorite in alkaline solution to form a blue color solution which is catalyzed by sodium nitroprusside. The resulting absorbance was measured in a spectrophotometer (BEL photonics 2000UV, Brazil) at 630 nm. Nitrite and nitrate levels were determined according to Mackereth et al. (1978). The nitrite concentration was determined through the formation of a purple-red solution formed by diazotization of sulfanilic acid with N-(1-naphthyl)-ethylenediamine dihydrochloride. The resulting absorbance was measured at 540 nm. Nitrate concentration was determined through the reduction of nitrate to nitrite by amalgamated cadmium. The resulting concentration (nitrite originally present in the sample plus reduced nitrate) was measured according to the methodology described above for nitrite. The concentration of nitrite originally present was subtracted from the total concentration of nitrite obtained. The nitrogenous compounds were detected and measured in a spectrophotometer, at detection limit of 0.02 mg L^{-1} (lower limit), 0.005 mg L^{-1}, and 0.05 mg L^{-1} to ammonia, nitrite and nitrate, respectively. Afterwards, absorbance data were read against a standard curve to calculate the concentrations of the different nitrogenous compounds in the samples using the software "winspec" (version 2.3.1). As standard compounds to determine the standard curve of ammonia, nitrite and nitrate were used ammonium chloride, sodium nitrite and potassium nitrate, respectively. Water quality variables were determined independently for each treatment in order to establish whether they remained at appropriate levels for the species (Moraes-Valenti and Valenti 2010).

The determination of the median lethal concentration (96 h LC_{50}) was based on the manual of the Environmental Protection Agency USA (Peltier and Weber 1985). The safe level (SL) was determined by multiplying the value obtained in the test of toxicity by an application factor of 0.1, as recommended by Sprague (1971).

Scatter plots were built between the nitrite concentration (mg L^{-1}) and the cumulative mortality for post larvae, juveniles and adults separately. Various dose–response models were tested and the best fit was achieved with the Weibull I function (Ritz et al. 2015). The choice of the "best fit" was based on the values of standard error (S) and of the coefficient of determination (r^2), on the residual analysis (Sokal and Rholf 1995) and of the biological adequacy.

Table 1 Water quality parameters (mean ± SD; n = 4) for experiments carried out for the different life stages of *M. amazonicum*

Variables	0 mg L^{-1}	1 mg L^{-1}	2 mg L^{-1}	4 mg L^{-1}	8 mg L^{-1}	16 mg L^{-1}	Recommended values for prawn culture
Postlarvae in experiment with nitrite							
Dissolved oxygen (mg L^{-1})	6.60 ± 0.41	6.67 ± 0.35	6.50 ± 0.30	6.60 ± 0.39	6.50 ± 0.29	6.34 ± 0.33	3–7
Temperature H_2O (ºC)	25.01 ± 0.28	25.12 ± 0.33	24.99 ± 0.22	24.83 ± 0.05	24.90 ± 0.14	24.85 ± 0.06	24–30
pH	8.32 ± 0.10	8.26 ± 0.09	8.24 ± 0.05	8.24 ± 0.05	8.15 ± 0.05	8.16 ± 0.06	7–8.5
Alkalinity (mg L^{-1} $CaCO_2$)	26.35 ± 0.73	28.50 ± 0.89	24.08 ± 0.97	23.13 ± 0.66	23.88 ± 1.07	24.03 ± 1.12	20–60
Hardness (mg L^{-1})	37.08 ± 2.10	34.78 ± 1.50	29.38 ± 2.98	28.70 ± 2.92	22.00 ± 1.27	24.25 ± 6.18	20–150
Total ammonia (mg L^{-1})	0.623 ± 0.009	0.568 ± 0.059	0.619 ± 0.042	0.625 ± 0.02	0.494 ± 0.034	0.435 ± 0.123	<1
Nitrate (mg L^{-1})	0.159 ± 0.027	$4\times10^{-5}\pm2\times10^{-5}$	$9\times10^{-5}\pm4\times10^{-5}$	$2\times10^{-5}\pm3\times10^{-6}$	$4\times10^{-4}\pm1\times10^{-4}$	$7\times10^{-4}\pm1\times10^{-4}$	80
Nitrite (mg L^{-1})	0.019 ± 0.002	1.087 ± 0.036	2.244 ± 0.1	4.108 ± 0.675	8.337 ± 0.239	16.547 ± 0.325	<2
Juveniles in experiment with nitrite							
Dissolved oxygen (mg L^{-1})	6.84 ± 0.49	6.71 ± 0.56	6.62 ± 0.46	7.16 ± 0.31	7.02 ± 0.10	7.02 ± 0.26	3–7
Temperature H_2O (ºC)	25.04 ± 0.16	25.06 ± 0.15	25.11 ± 0.18	25.27 ± 0.09	25.37 ± 0.05	25.02 ± 0.05	24–30
pH	8.02 ± 0.11	8.01 ± 0.12	8.02 ± 0.12	8.00 ± 0.05	7.69 ± 0.02	8.05 ± 0.03	7–8.5
Alkalinity (mg L^{-1} $CaCO_2$)	46.40 ± 5.75	51.98 ± 4.70	50.20 ± 2.51	43.07 ± 0.40	41.05 ± 0.83	37.87 ± 1.42	20–60
Hardness (mg L^{-1})	31.85 ± 8.74	42.20 ± 9.63	37.12 ± 6.32	25.27 ± 0.99	24.80 ± 0.59	22.42 ± 0.66	20–150
Total ammonia (mg L^{-1})	$3\times10^{-4}\pm4\times10^{-6}$	$2\times10^{-4}\pm2\times10^{-5}$	$3\times10^{-4}\pm4\times10^{-6}$	$3\times10^{-4}\pm5\times10^{-6}$	$3\times10^{-4}\pm1\times10^{-6}$	$3\times10^{-4}\pm1\times10^{-6}$	<1
Nitrate (mg L^{-1})	$1\times10^{-4}\pm4\times10^{-5}$	$4\times10^{-5}\pm2\times10^{-5}$	$9\times10^{-5}\pm5\times10^{-6}$	$1\times10^{-4}\pm3\times10^{-5}$	$3\times10^{-4}\pm1\times10^{-4}$	$7\times10^{-4}\pm1\times10^{-4}$	80
Nitrite (mg L^{-1})	0.040 ± 0.006	0.955 ± 0.041	2.170 ± 0.11	4.026 ± 0.085	8.176 ± 0.31	16.088 ± 0.307	<2
Adults in experiment with nitrite							
Dissolved oxygen (mg L^{-1})	6.43 ± 0.27	6.40 ± 0.33	6.51 ± 0.29	6.44 ± 0.19	6.67 ± 0.17	6.21 ± 0.45	3–7
Temperature H_2O (ºC)	25.98 ± 0.48	25.88 ± 0.38	26.14 ± 0.43	25.63 ± 0.41	25.78 ± 0.37	25.60 ± 0.36	24–30
pH	7.73 ± 0.09	7.70 ± 0.08	7.75 ± 0.12	7.69 ± 0.14	7.64 ± 0.03	7.71 ± 0.06	7–8.5
Alkalinity (mg L^{-1} $CaCO_2$)	20.65 ± 1.66	19.25 ± 2.73	23.83 ± 1.12	23.03 ± 1.16	19.60 ± 1.51	20.53 ± 1.75	20–60
Hardness (mg L^{-1})	29.43 ± 4.88	24.25 ± 1.35	34.68 ± 5.68	34.28 ± 3.44	23.48 ± 1.03	30.50 ± 2.43	20–150
Total ammonia (mg L^{-1})	0.042 ± 0.083	0.033 ± 0.066	0.028 ± 0.057	0.447 ± 0.253	$1\times10^{-4}\pm3\times10^{-5}$	$3\times10^{-4}\pm2\times10^{-5}$	<1
Nitrate (mg L^{-1})	$1\times10^{-5}\pm3\times10^{-6}$	$4\times10^{-4}\pm1\times10^{-5}$	$1\times10^{-5}\pm2\times10^{-6}$	$1\times10^{-4}\pm1\times10^{-5}$	$3\times10^{-4}\pm1\times10^{-5}$	$7\times10^{-4}\pm6\times10^{-5}$	80
Nitrite (mg L^{-1})	0.048 ± 0.02	1.103 ± 0.009	2.385 ± 0.444	4.117 ± 0.255	8.012 ± 0.036	16.074 ± 0.139	<2

The intoxication risk classification by nitrite was performed by quotient method, adapted of Urban and Cook (1986). The quotient was calculated by ratio between the recommended concentration in literature to the genus *Macrobrachium* and the 96 h LC_{50} calculated value to species in study. We used the following intoxication risk classes: without risk ($Q \leq 0.1$); low risk ($0.1 < Q \leq 1$); moderated risk ($1 < Q \leq 10$) and high risk ($Q > 10$).

Table 2 LC_{50} calculated and their 95 % confidence intervals of nitrite to 96 h, safe level of nitrite, standard error (S) and r^2 for the different life stages of *M. amazonicum*

Life stages	96 h LC_{50} calculated of nitrite (mg L^{-1})	Confidence interval (95 %)	Safe level to nitrite (mg L^{-1})	S	r^2
Post larvae	1.49	1.30–1.72	0.14	0.67	0.98
Juvenile	2.36	2.11–2.63	0.23	0.78	0.94
Adult	2.34	2.00–2.67	0.23	0.73	0.96

Results and Discussion

During the experiment, temperature, pH, water hardness and alkalinity remained within suitable ranges for freshwater prawns culture (Coler et al. 1999; Sampaio et al. 2007; Vasquez et al. 2007). Ammonia concentrations measured here were lower than those levels suggested by Timmons et al. (2002), who recommended a threshold value for ammonia of 2 mg L^{-1} in warm water aquaculture. In the same way, the nitrate concentrations were lower than those levels recommended by Moraes-Valenti and Valenti (2010), who suggested that nitrate values should not reach levels above 80 mg L^{-1} (Table 1). Therefore, none of the water quality parameters measured in this study, except nitrite, were limiting or stressful for the prawns. Thus, it can be argued that

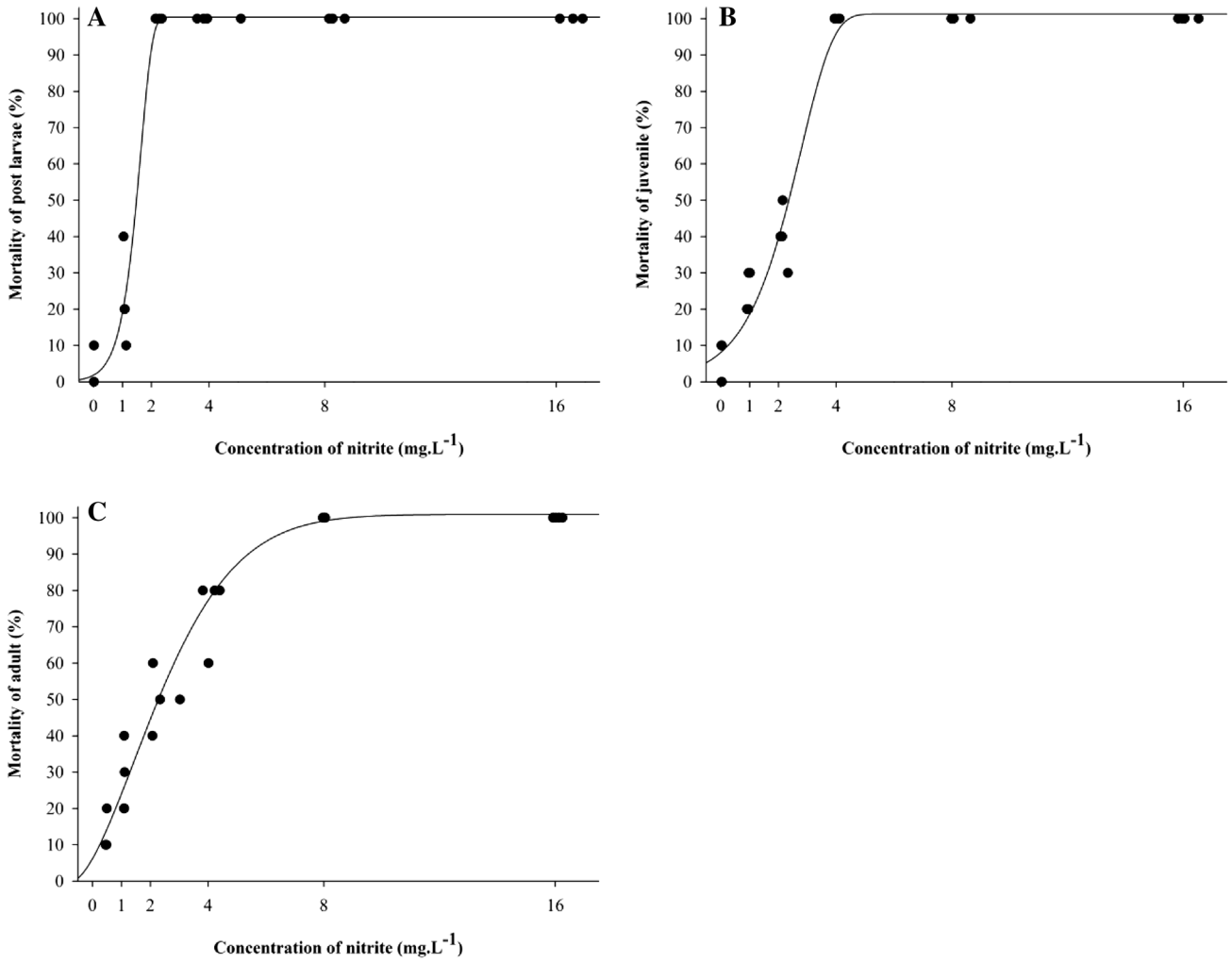

Fig. 1 Mortality curves for each life stages of *M. amazonicum* after 96 h of exposure to nitrite. **a** Post larvae. **b** Juveniles. **c** Adults

the lethal effects observed were due to the nitrite concentrations evaluated.

Mortality observed for the different life stages at the control treatment (not exposed to nitrite) during the trial period was below 10 %, which may be related to the behavior of animals in competition for area (territorialism), as demonstrated in previous research (Armstrong et al. 1976; Ostrensky and Wasielesky Jr. 1995).

Independently of the life stage of prawns, it is possible to verify that mortality increased with increasing nitrite concentration. Postlarvae of *M. amazonicum* exposed to nitrite showed 48 ± 2 % (mean ± SD) mortality after 96 h of exposure to 1 mg nitrite/L. The concentration of 2 mg L^{-1} yields mortality of 90 ± 2 % in 96 h. At the concentrations of 4–16 mg nitrite/L, mortality was of 100 % in 24 h. When evaluating mortality caused by nitrite by the Weibull I function, the lethal concentration for 50 % of the prawns (LC_{50}) after 96 h is 1.49 mg nitrite/L (Table 2; Fig. 1a).

Juvenile *M. amazonicum* exposed to concentrations of 2 mg L^{-1} and 4 mg L^{-1} showed average mortality of 55 ± 3 and of 95 ± 1 % after 96 h, respectively. Concentrations of 8–16 mg nitrite/L exhibited 100 % mortality after 24 h. Lethal concentration for 50 % of the juveniles in 96 h was 2.36 mg nitrite/L (Table 2; Fig. 1b).

For adult *M. amazonicum*, the concentrations of 1 and 2 mg L^{-1} produced average mortality of 27 ± 8 and 50 ± 8 % in 96 h, respectively. Concentration of 4 mg L^{-1} caused average mortality of 80 ± 3 % in 96 h. 100 % mortality was also observed at 8–16 mg nitrite/L in 24 h. Average mortality for 50 % of the adults after 96 h is obtained with 2.34 mg nitrite/L (Table 2; Fig. 1c). Sahoo and Chand (2006), evaluating the effect of nitrite on the immune response of adult *Macrobrachium malcolmsonii*, measured a LC_{50} of 3.14 mg L^{-1} after 96 h. Thus, it seems that *M. amazonicum* adults are slightly less tolerant to nitrite than *M. malcolmsonii* adults as it shows lower LC_{50} values: 2.34 mg L^{-1}. Mallasen and Valenti (2006) recommend nitrite values during the production of freshwater prawns below 2.0 mg L^{-1}. Hayd et al. (2014) claim that safe levels below 0.8 mg nitrite/L may be used as a general reference for production system. However, in the present study results pointed out that the safe levels for the production of *M. amazonicum* are much lower (0.14–0.23 mg L^{-1}) (Table 2).

Regarding the classification of intoxication risk by nitrite to *M. amazonicum*, the values were of 1.34 to postlarvae, 0.85 to juveniles and 0.85 to adults, suggesting moderated risk to postlarvae and low risk to juvenile and adult (Urban and Cook 1986).

Comparing sensitivity to nitrite in the different life stages of *M. amazonicum*, it was found that the sensitivity to this compound is related to the animal's stage of development, where the later stages showed higher resistance. The sensitivity of an organism to a toxic agent can vary depending on its size, age and stage of development (Wajsbrot et al. 1990), because several enzymes may have differential activities along development or aging (Barbieri et al. 2002). In the early stages of development the organisms are more sensitive generally due to increased mitotic activity (Barbieri 2008), and also possibly their higher surface to volume ratios, as they are smaller organisms. This variation in sensitivity of different ontogenetic stages has been observed previously in *Macrobrachium*, in studies with other compounds such as pesticides (Dai et al. 2014), heavy metals (Asih et al. 2013) and nitrogen compounds (ammonia and nitrite) (Lin et al. 1993; Mallasen and Valenti 2005, 2006).

In production systems, the nitrite values generally are below the safe limit determined in this study. Marques et al. (2012) observed in production systems in net pens nitrite values ranging from 0.004 to 0.011 mg L^{-1}; Kimpara et al. (2011) found in ponds values ranging from 0.026 to 0.028 mg nitrite/L, while the recommended nitrite limits here are of 0.1 mg L^{-1} for post larvae and 0.2 mg L^{-1} for juveniles and adults. Thus, the results obtained have important implications for the production of this prawn and allow the indication of precise limit levels for the exposure of the different life stages of this species to nitrite, enabling the handling and management of these animals in production systems. Thus, monitoring this variable is important to avoid losses, mainly in larviculture and recirculation systems.

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