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How incubation temperature affects hatchling performance in reptiles: an integrative insight based on plasticity in metabolic enzyme

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Abstract

Evaluating the effects of temperature variations on animals plays an important role in understanding the threat of climate warming. The effects of developmental temperature on offspring performance are critical in evaluating the effects of warming temperatures on the fitness of oviparous species, but the physiological and biochemical basis of this developmental plasticity is largely unknown. In this study, we incubated eggs of the turtle *Pelodiscus sinensis* at low (24 °C), medium (28 °C), and high (32 °C) temperatures, and evaluated the effects of developmental temperature on offspring fitness, and metabolic enzymes in the neck and limb muscles of hatchlings. The hatchlings from eggs incubated at the medium temperature showed better fitness-related performance (righting response and swimming capacity) and higher activities of metabolic enzymes (hexokinase, HK; lactate dehydrogenase, LDH) than hatchlings from the eggs incubated at high or low temperatures. In addition, the swimming speed and righting response were significantly correlated with the HK activities in limb (swimming speed) and neck (righting response) muscles, suggesting that the developmental plasticity of energy metabolic pathway might play a role in determining the way incubation temperature affects offspring phenotypes. Integrating the fitness-related performance and the activities of metabolic enzymes, we predict that the *P. sinensis* from high latitude would not face the detrimental effects of climate warming until the average nest temperatures reach 32 °C.

Key words: developmental plasticity, energetic metabolism, enzyme activity, hatchling, turtle.

Climate warming has imposed significant threats to animals across the world (e.g., Walther et al. 2002; Thomas et al. 2004; Parmesan 2006; Bestion and Cote 2018). Evaluating the effects of warming temperatures on animals thus plays an important role in understanding the threat imposed by climate warming (Sinervo et al. 2018). Phenotypic plasticity is one way that animals can respond to climate warming (Williams et al. 2008; Logan and Cox 2020; Li et al. 2020a, 2020b). Therefore, determining the phenotypic plasticity to temperature variation is not only important to understand the effects of warming temperatures on animals, but also critical to predicting the vulnerabilities of animals to climate warming (Huey et al. 2012; Miller et al. 2018; Sun et al. 2022).

Offspring phenotypes are integrated results of interactions among genetic determination, maternal effects, and environmental influences on embryos during development (Deeming 2004; Du et al. 2013; Noble et al. 2018b). Unlike viviparous species, oviparous mothers lay their eggs in field nests, causing embryonic development to occur in complex and frequently fluctuating thermal environments (Du et al. 2019; Du and Shine 2022). In oviparous reptiles, the thermal environment during embryonic development can influence a variety of phenotypic characteristics of hatchlings, including morphology, behavior (e.g., locomotion and thermoregulation), growth, and even the sex of hatchlings in species showing temperature-dependent sex determination (TSD) (see reviews in Deeming 2004; Valenzuela and Lance 2004; Booth 2017). Therefore, investigating the phenotypic plasticity of offspring to various developmental temperatures facilitates understanding the effects of warming temperatures on reptiles (Obregon et al. 2021). Despite the large number of studies investigating the effects of incubation temperature on post-hatching phenotypes in reptiles, little is known about physiological and biochemical mechanisms that underlie the phenotypic effects (Booth 2018; Noble et al. 2018a, 2018b).

The temperature may regulate multiple physiological and biochemical processes that determine offspring phenotypes. Energetic metabolism is one of the most important processes related to offspring phenotypes. For example, the thermal sensitivity of energy source utilization (e.g., yolk sac and albumen components) and metabolic consumption during embryonic development plays a major role in determining the body size

of reptile hatchlings when eggs are incubated under different thermal regimes (Storm and Angilletta 2007). Locomotion determines predation, mating, and other fitness-related traits (Angilletta 2009), and depends directly on the individual capacity of energetic metabolism (Le Galliard et al. 2004). In living animals, energetic metabolism is the main source of energy for physiological processes, and therefore, it is the focus of studies on the energetic basis of behavioral and physiological performance (e.g., locomotion, growth, and thermal tolerance) (Johnston and Temple 2002; Seebacher 2009; Sun et al. 2022). The metabolic enzymes play critical roles in determining organismal metabolism. For example, locomotor plasticity involves enzyme concentrations (Weinstein and Somero 1998) and enzyme activities (Seebacher and Franklin 2005). Among various metabolic enzymes, hexokinase (HK), lactate dehydrogenase (LDH), Alanine Aminotransferase (ALT), Na–K ATPase, and Ca–Mg ATPase are widely employed in evaluating the metabolic contribution to behavioral and physiological functions such as locomotion (e.g., Seebacher et al. 2010, 2015). Therefore, identifying the effect of developmental temperature on the activities of metabolic enzymes in offspring would provide new insights into the physiological and biochemical mechanisms underlying the phenotypic effects of incubation temperature.

Generally, reptile hatchlings from medium temperatures are larger and have better functional performance than those from high or low temperatures, as the "best balance hypothesis" predicts (see details in Deeming 2002, 2004; Booth 2017). Given that the efficiencies of organismal functions are determined by the thermal sensitivities of energetic metabolism and related biochemical components (e.g., enzyme activities) (Angilletta et al. 2002; Somero et al. 2017), we hypothesized that hatchlings from medium temperatures would have higher activities of metabolic enzymes than siblings from high or low temperatures, corresponding with the temperature effect on hatchling phenotypes. In the present study, we measured the functional performances (locomotion and righting response), as well as the activities of important metabolic enzymes of HK, LDH, ALT, Na–K ATPase, and Ca–Mg ATPase hatchlings of Chinese soft-shelled turtles *Pelodiscus sinensis*, emerged from eggs incubated at low (24 °C), medium (28 °C), and high (32 °C) temperatures. Based on the above hypothesis, we expect that hatchlings that emerged from eggs incubated at the medium temperature would have higher activities of metabolic enzymes underpinning better locomotion performance as compared to their siblings that emerged from eggs incubated at low or high temperatures.

Materials and Methods

Study species
Chinese soft-shelled turtles *Pelodiscus sinensis* are widespread in China and South-East Asia (Zhao and Adler 1993). The effect of temperature on embryonic development and hatchling phenotypes in this species has been extensively studied in the past decade. Constant and fluctuating incubation temperatures can both significantly affect the developmental rate (incubation duration) and energy utilization of *P. sinensis* embryos, as well as hatchling phenotypes, including morphology (e.g., body size and shape), behavior (locomotion), and post-hatching growth (Du et al. 2001, 2009, 2013; Du and Ji 2003; Ji et al. 2003; Sun et al. 2015). The well-known effects of incubation temperature

on hatchling phenotypes in *P. sinensis* make this species an ideal model for further investigation of the physiological and biochemical mechanisms underlying these phenotypic effects.

Egg collection and incubation
We collected 32 clutches of freshly laid *P. sinensis* eggs from a private hatchery in Hebei province (38.87°N, 115.46°E), North China. After collection, the fertilized eggs were weighed (±0.001 g) and incubated in a temperature-controlled incubator (KB240, Binder, Germany). We used a clutch-split design to incubate the eggs (e.g., Sun et al. 2018a; Li et al. 2020a, 2020b). In brief, three eggs from each clutch were evenly and randomly assigned to three different constant incubation temperatures in a temperature-controlled incubator (KB240, Binder, Germany) of 24 °C, 28 °C, and 32°C; these 3 temperatures represent the low, medium, and high temperatures experienced by the eggs in natural nests, respectively (Figure 1). Eggs were half-buried in plastic containers with moist vermiculite (–220 kPa). We checked the moisture in the containers every week and added water if required (Chen et al. 2011). According to the published protocol, we checked the incubator twice every day for fresh hatchlings toward the end of incubation (Sun et al. 2021). The incubation duration of eggs was calculated as the number of days between egg collection and the emergence of new hatchlings (Sun et al. 2018b). We incubated 96 eggs (32 for each incubation temperature at 24 °C, 28 °C, and 32 °C initially. During the incubation, we collected 10 and 12 eggs incubated at 28 °C and 32 °C to conduct the preliminary experiment for enzyme-activity determinations. Accordingly, the sample sizes in the incubation experiment were 32, 22, and 20 for 24 °C, 28 °C, and 32 °C, respectively.

Righting response and swimming speed
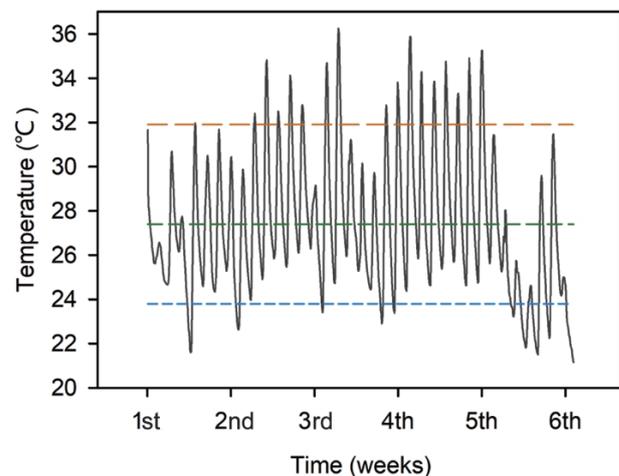Once hatched, the hatchlings were collected and the body mass was determined by weighing (±0.001 g) immediately

Figure 1 Thermal profiles of the *P. sinensis* nests in natural habitat. The graph shows nest temperatures from late June to early August, spanning the majority of incubation duration in nature for *P. sinensis*. The reference lines in the graphs indicate the average temperature (27.4 °C, green dash) and 90% confidence intervals (lower, 23.8 °C, blue dash; upper: 31.9 °C, orange dash).

after emergence, and then they were kept individually within cylindrical terraria (diameter = 120 mm; height = 90 mm) with ~10 mm water. The terraria were set in a temperature-controlled room at 24 ± 0.5 °C, with a 12L:12D photoperiod. Within photoperiods, supplementary heating underneath the terraria increased the water temperature to 32 °C. Sufficient commercial turtle food was provided for hatchlings.

The righting response of hatchlings were tested within 72 h after hatching in the temperature-controlled room at 24 °C, 28 °C, and 32 °C, respectively. Before tests, the hatchlings were acclimated at the test temperature for 2 h. Then we placed each hatchling upside down in a terrarium (320 × 250 × 350 mm) and used a digital camera (SONY DCR-SR220E) to record the test processes. We then analyzed the recording and calculated the time used by the hatchlings to right themselves after they began to move (Zhao et al. 2014). Each hatchling was tested twice with an interval of 1 h, and the average time of each hatchling used in the 2 tests was calculated as the righting response.

After the day of the righting response test, the swimming capacity of the hatchlings was tested at 24 °C, 28 °C, and 32°C, respectively, using a custom-made swim-way of transparent glass, with 20 mm temperature-controlled water. Each hatchling was tested twice with a 1-h resting period between each test. Before the test, hatchlings were acclimated for 2 h at the test temperature. During tests, the hatchlings were stimulated by a paintbrush to swim across the swim-way and the process was captured by the digital camera (SONY, DCR-SR220E). Videos were then examined to score swimming capacity. The highest speed per 200 mm was recorded for each test, and the average of 2 tests was calculated as the swimming speed, expressed as m/s.

Metabolic enzyme activities

To determine the physiological mechanisms underlying the variation of righting responses of hatchlings, we measured the activities of metabolic enzymes in the muscles of the neck photometrically (see details in the following paragraph). Synchronously, we also photometrically measured the activities of metabolic enzymes in the muscles of the limb, to determine the mechanisms underlying swimming speed. Hatchlings were euthanized with an overdose of isoflurane (Underwood and Anthony 2020). We collected and weighed (±0.001 g) muscles from the neck and limbs of hatchlings, respectively. Then we pooled the muscles from the 4 limbs of each turtle into 1 biological replicate. Only some hatchlings were sacrificed for the measurement of enzyme activities. Accordingly, the sample size for all biochemical assays of the hatchlings incubated at 24 °C, 28 °C, and 32 °C was 7, 7, and 5, respectively.

Following our established methods (e.g., Sun et al. 2015, 2022), the metabolic enzyme activities were assessed in duplicates at 24 °C, 28 °C, and 32°C, respectively. In brief, the muscle tissues were homogenized with the buffer on ice, then the tissue homogenates were centrifuged for 15 min at 4 °C, 2,000 r/min. After, the supernatants were collected for determining the protein concentration and activities of the metabolic enzymes. The protein concentrations of the samples were assayed by the BCA Protein Assay (Pierce, Jiancheng; Nanjing, China), according to the manufacturer's protocol. For each test, 10 μL supernatant was placed into each hole of a 96-multiwell plate, followed by adding 250 μL of BCA

Protein Assay reagent. Before the enzyme-activity assay, the supernatant was diluted to an appropriate concentration for the determination of enzyme activity. Then the enzyme activities of hexokinase (HK), lactate dehydrogenase (LDH), Alanine Aminotransferase (ALT), Na–K ATPase, and Ca–Mg ATPase were determined by a microplate reader (BioTek Synergy H1; California, US) according to the protocols of assay kit (Jiancheng, Nanjing, China). The activities of HK were expressed as nmol/min/mg protein, while the activities of LDH, ALT, Na–K ATPase, and Ca–Mg ATPase were expressed as U/g protein.

Statistical analysis

Prior to analysis, *Kolmogorov–Smirnov* test and *Levene*'s test were used to test the normality of distributions and homogeneity of variances for all residuals, respectively. One-way Anovas were used to detect among-treatment differences in incubation durations and One-way Ancovas were conducted to detect the difference in hatchling body mass among incubation temperatures, with initial egg mass as covariate. A chi-square analysis was conducted to detect the differences in hatching success. The effects of temperature on righting response, swimming capacity, and metabolic enzyme activities of HK, LDH, ALT, Na–K ATPase, and Ca–Mg ATPase were analyzed using repeated-measures Anova, with incubation temperature as the main factor and test temperature as the repeated measures. Further *post-hoc* Tukey's HSD tests were carried out to determine the among-treatment differences. Linear regression analysis was performed on locomotion (righting response and swimming capacity) and enzyme activity to determine the relationship between the enzyme and locomotor performance if significant differences were detected in enzyme activities. In correlation analysis, we correlated righting responses with the activities of enzymes in neck muscles, as well as swimming capacity with the activities of enzymes in limb muscles.

Results

Incubation duration, hatching success, and hatchling body mass

Incubation duration was significantly shortened as the incubation temperature increased, but hatching success was not affected by incubation temperature (Table 1). Incubation temperature significantly affected the hatchling body mass. Hatchlings were heaviest if eggs were incubated at the medium temperature, lightest at the high temperature, with those from the low temperature in between (Table 1).

Righting response and swimming speed

Both incubation temperature ($F_{2, 62}$ = 31.183, $P < 0.001$) and test temperature ($F_{2, 124}$ = 3.538, $P = 0.032$) considerably affected the righting response of hatchlings. However, the interaction between incubation temperatures and test temperatures did not affect the righting response of hatchlings ($F_{4, 124}$ = 0.301, $P = 0.877$). Hatchlings from the medium incubation temperatures righted themselves the most rapidly and siblings from low temperature righted the most slowly, with the hatchlings from the high temperature being in between (Figure 2A). Generally, the hatchlings from all incubation temperatures righted more rapidly as test temperature increased (Figure 2A).

Table 1 Embryonic development and hatchling mass of *P. sinensis* incubated at 24 °C, 28 °C, and 32 °C

	Incubation temperatures (°C)			Statistical analysis
	24	**28**	**32**	
Incubation duration (days)	84.3 ± 0.3[a]	51.0 ± 0.1[b]	38.0 ± 0.1[c]	$F_{2, 62} = 15073$
	82 ~ 86	50~ 52	37 ~ 39	$P < 0.001$
Hatching success (%)	87.5	90.9	85	$\chi^2 = 0.955$, $df = 2$
	28/32	20/22	17/20	$P = 0.620$
Hatchling body mass (g)	3.168 ± 0.097[a]	3.301 ± 0.120[a]	3.047 ± 0.104[b]	$F_{2, 61} = 10.295$
	2.080 ~ 4.120	2.538 ~ 4.485	2.228 ~ 3.991	$P < 0.001$

Values are reported as mean ± *SE*, and min ~ max for incubation duration and hatchling body mass, while are reported as percentage and number of hatchlings/eggs for hatchling success. The different superscripts indicate statistically different values. Statistically significant results are shown in bold. The sample sizes for hatchlings from 24 °C, 28 °C, and 32 °C were 28, 20, and 17, respectively.

Similarly, both incubation temperature ($F_{2, 62} = 18.037$, $P < 0.001$) and test temperature ($F_{2, 124} = 51.420$, $P < 0.001$) significantly affected the swimming speed of hatchlings. Hatchlings incubated at medium and high temperatures swam faster than those from low incubation temperature. In addition, hatchlings generally swam faster as the test temperature increased. However, the interaction between incubation temperature and test temperature did not affect the swimming speed of hatchlings ($F_{4, 124} = 1.306$, $P = 0.271$) (Figure 2B).

Activities of metabolic enzymes

For the limb muscle of hatchlings, the enzyme activities of hexokinase (HK) and dehydrogenase (LDH) were significantly affected by incubation temperatures (Figure 3, Table 2). The HK was significantly higher in hatchlings incubated at 28 °C than siblings at 24 °C and 32 °C (Figure 3A), while the LDH was highest in hatchlings incubated at 28 °C, and lowest at 32 °C, with at 24 °C in between (Figure 3B). The enzyme activities of HK and LDH were enhanced by increasing test temperatures. As the test temperature increased, the difference between incubation temperatures was greater in HK and LDH (Figure 3A,B). In contrast, the incubation temperature did not affect the enzyme activities of Alanine Aminotransferase (ALT), Na–K ATPase, or Ca–Mg ATPase, although the activities of ALT, Na–K ATPase, and Ca–Mg ATPase were enhanced as test temperature increased. However, the interaction between incubation temperature and test temperature did not affect the enzyme activities (Figure 3C–E, Table 2).

For the neck muscle of the hatchlings, the activities of HK, LDH, ALT, and Ca–Mg ATPase were significantly affected by incubation temperature, whereas Na–K ATPase was similar among incubation temperatures (Figure 4, Table 2). In detail, the activities of HK were significantly higher in the hatchlings incubated at 28 °C, than siblings at 24 °C and 32 °C (Figure 4A). The activities of LDH in the hatchlings incubated at both 24 °C and 28 °C were similar, and were both significantly higher than siblings incubated at 32 °C (Figure 4B). The activities of ALT were significantly higher in the hatchlings incubated at 24 °C than siblings at both 28 °C and 32 °C (Figure 4C). The activities of Ca–Mg ATPase were significantly higher in the hatchlings incubated at 32 °C than 24 °C and 28 °C (Figure 4E). The activities of all these 5 enzymes were significantly enhanced by increasing test temperatures (Table 2). However, the interaction between
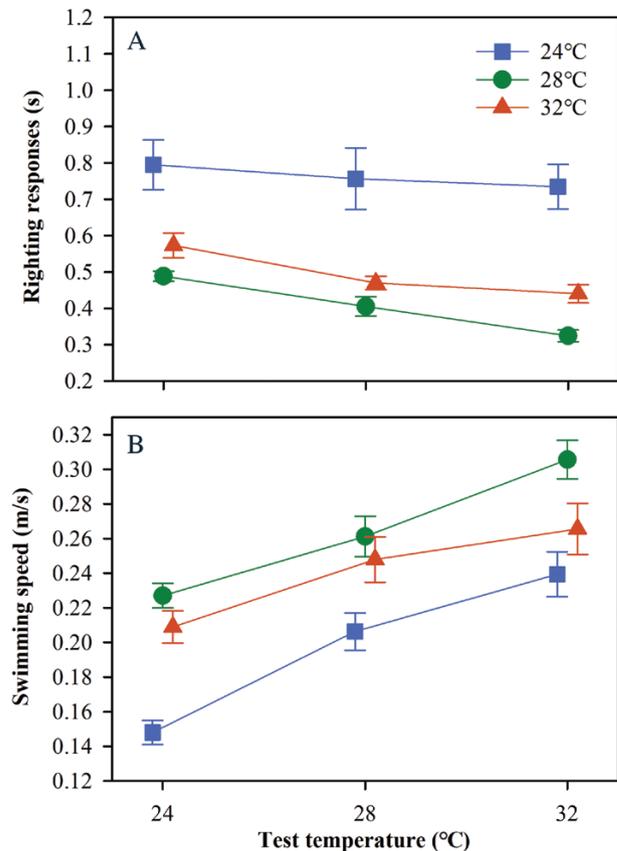

Figure 2 Righting response (A) and swimming speed (B) of hatchlings incubated at 24 °C, 28 °C, and 32 °C. The sample size for 24 °C (cube), 28 °C (circle), and 32 °C (triangle) were 28, 20 and 17, respectively. The significance level was defined as $P < 0.05$.

incubation temperature and test temperature did not affect the activities of all 5 enzymes measured in this study (Table 2).

Correlation between locomotion and activities of metabolic enzymes

The swimming speed was positively correlated with HK activities in limb muscles ($R^2 = 0.338$, Swimming speed = 0.0028 × HK activities + 0.153, $P < 0.0001$) (Figure 5A). However, the swimming speed was not correlated to the
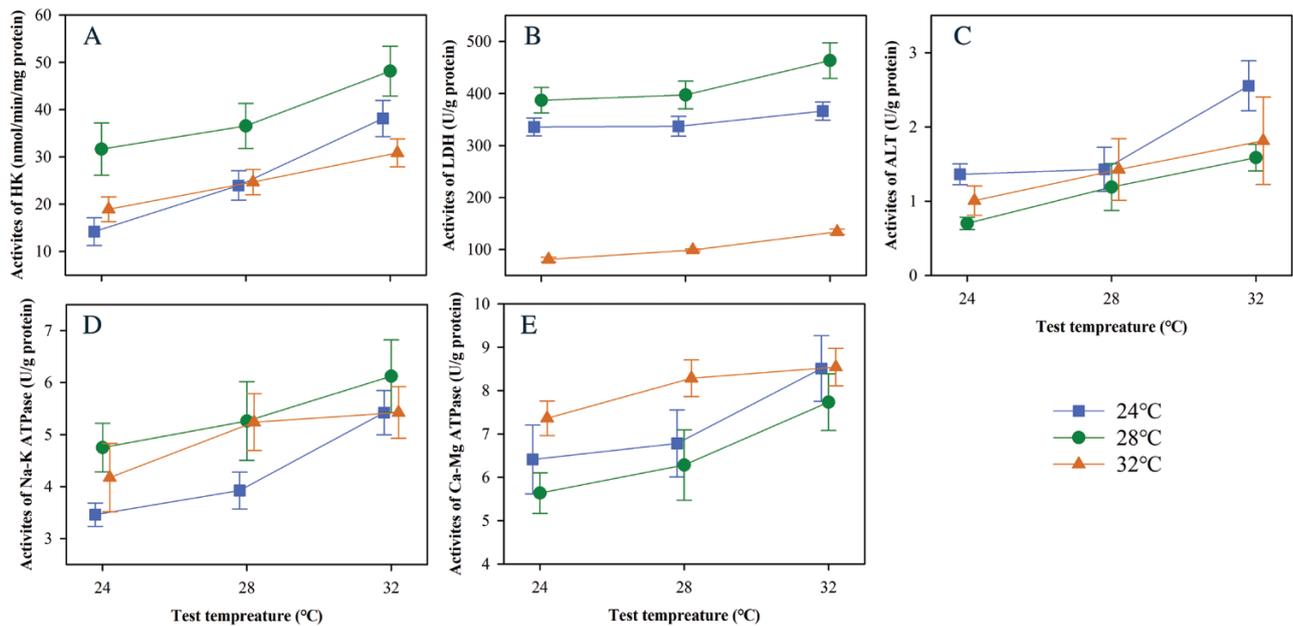
Figure 3 Activities of metabolic enzymes of (A) HK, (B) LDH, (C) ALT, (D) Na–K ATPase, and (E) Ca–Mg ATPase of the muscle of limbs. The blue cubes, green circles and orange triangles indicate the hatchlings incubated at 24 °C, 28 °C, and 32 °C, respectively. The data are shown as mean ± SE.

LDH activities in limb muscles ($P = 0.956$), even though the LDH was significantly different among incubation temperatures (Figure 5B). Similarly, the righting responses of hatchlings were positively correlated with HK activities in neck muscle ($R^2 = 0.111$, righting responses = 0.007 × HK activities – 0.757, $P = 0.014$) (Figure 5C). However, the righting responses of hatchlings were not correlated with any other enzymes (LDH: $P = 0.987$; ALT: $P = 0.598$; Ca–Mg ATPase: $P = 0.085$) (Figure 5D–F).

Discussion

As expected, the functional performance of swimming speed and righting response was enhanced if the eggs were incubated at medium temperature (i.e., 28 °C), relative to siblings at 24 °C and 32 °C. In addition, the functional performance generally matched with enzyme activities of HK in turtle hatchlings, with better performance and higher metabolic enzymes of HK observed in hatchlings from the medium incubation temperature (i.e., 28 °C) than those from the high or low incubation temperatures. Therefore, incubation temperature probably modifies the enzyme activities of HK and thereby influences the plasticity in the functional performance of reptile hatchlings.

Generally, the hatchlings of oviparous reptiles have better functional performance if the embryos are developing at moderate temperatures (see reviews in Deeming 2002, 2004; Booth 2017). Even though low temperatures sometimes produce hatchlings with better growth or survival in some reptile species, moderate incubation temperatures are considered to provide the best balance between developmental rate, hatching success, and post-hatch performances (e.g., Tang et al. 2012; Booth 2017; Noble et al. 2018a, 2018b). For example, the hatchlings of leatherback sea turtle *Dermochelys coriacea* exhibit higher body size and righting response if they were incubated at a medium depth of nest (i.e., medium temperature) (Rivas et al. 2019). In this

study, better functional performance in medium-temperature hatchlings supported the widely accepted prediction of the "best balance at moderate temperature" hypothesis. Integrating the locomotion of hatchlings incubated at different temperatures and thermal environments in the nest, we predicted that the hatchlings from the high-latitude population of *P. sinensis* would not be severely threatened by climate warming in near future before the average nest temperatures reached 32 °C.

Interestingly, we provided a novel piece of evidence at the biochemical level (i.e., HK and LDH) for the "best balance" hypothesis. This evidence also provided new insights into the biochemical mechanisms underlying the phenotypic effects of incubation temperature on the locomotion of hatchlings in this study. Energetic metabolism provides the basis for behavioral and physiological functions, including the righting response and swimming performance, which may determine the capability of predation, escape, and territory defense, and hence, the fitness of the animals (e.g., Hertz et al. 1988; Gibb and Dickson 2002; Seebacher and James 2008; Sun et al. 2011, 2020). Our study provides a piece of robust evidence that the effects of incubation temperature on offspring performance are associated with the developmental plasticity of the activities of metabolic enzymes in HK, which could regulate the cellular metabolism significantly (Sun et al. 2015, 2022). The significantly different activities of HK in the limb and neck muscles (Figures 3A and 4A) and their correlation with the swimming speed and righting response respectively (Figure 5A,C), indicated that HK activities have a significant impact on the plasticity in locomotion of *P. sinensis* hatchlings. Similarly, the important contribution of HK activities to metabolism in determining locomotion is universally demonstrated across various other animal groups (e.g., Ford and Candy 1972; Newsholme et al. 1972; Suarez 2000; Dawson et al. 2020).

Although it is a consensus that functional performances (e.g., locomotion) are dependent on the energy states of

Table 2 Statistical analysis in metabolic enzyme activities of muscles in limbs and neck, respectively

	Statistical analysis		
	$T_{incubation}$	T_{test}	$T_{incubation} \times T_{test}$
Enzyme activities of muscles in limbs			
HK	$F_{2,15} = 4.315$	$F_{2,30} = 107.395$	$F_{4,30} = 4.708$
(nmol/min/mg protein)	$P = 0.033$	$P < 0.001$	$P = 0.005$
LDH	$F_{2,15} = 59.340$	$F_{2,30} = 59.172$	$F_{4,30} = 4.346$
(U/g protein)	$P < 0.001$	$P < 0.001$	$P = 0.007$
ALT	$F_{2,16} = 2.391$	$F_{2,32} = 11.333$	$F_{4,32} = 0.767$
(U/g protein)	$P = 0.123$	$P < 0.001$	$P = 0.555$
Na–K ATPase	$F_{2,15} = 1.723$	$F_{2,30} = 23.666$	$F_{4,30} = 1.670$
(U/g protein)	$P = 0.212$	$P < 0.001$	$P = 0.183$
Ca–Mg ATPase	$F_{2,15} = 1.039$	$F_{2,30} = 31.675$	$F_{4,30} = 2.049$
(U/g protein)	$P = 0.378$	$P < 0.001$	$P = 0.113$
Enzyme activities of muscle in neck			
HK	$F_{2,15} = 10.303$	$F_{2,30} = 19.230$	$F_{4,30} = 0.748$
(nmol/min/mg protein)	$P = 0.002$	$P < 0.001$	$P = 0.567$
LDH	$F_{2,15} = 32.998$	$F_{2,30} = 22.626$	$F_{4,30} = 0.647$
(U/g protein)	$P < 0.001$	$P < 0.001$	$P = 0.632$
ALT	$F_{2,15} = 3.857$	$F_{2,30} = 3.942$	$F_{4,30} = 0.613$
(U/g protein)	$P = 0.045$	$P = 0.030$	$P = 0.656$
Na–K ATPase	$F_{2,15} = 2.935$	$F_{2,30} = 3.535$	$F_{4,30} = 0.305$
(U/g protein)	$P = 0.086$	$P < 0.001$	$P = 0.872$
Ca–Mg ATPase	$F_{2,15} = 11.034$	$F_{2,30} = 24.633$	$F_{4,30} = 2.035$
(U/g protein)	$P = 0.001$	$P < 0.001$	$P = 0.117$

$T_{incubation}$, T_{test}, HK, LDH, and ALT indicate incubation temperature, test temperature, Hexokinase, Lactate dehydrogenase, and Alanine aminotransferase, respectively. The significant level is defined as $\alpha = 0.05$. Statistically significant results are shown in bold.
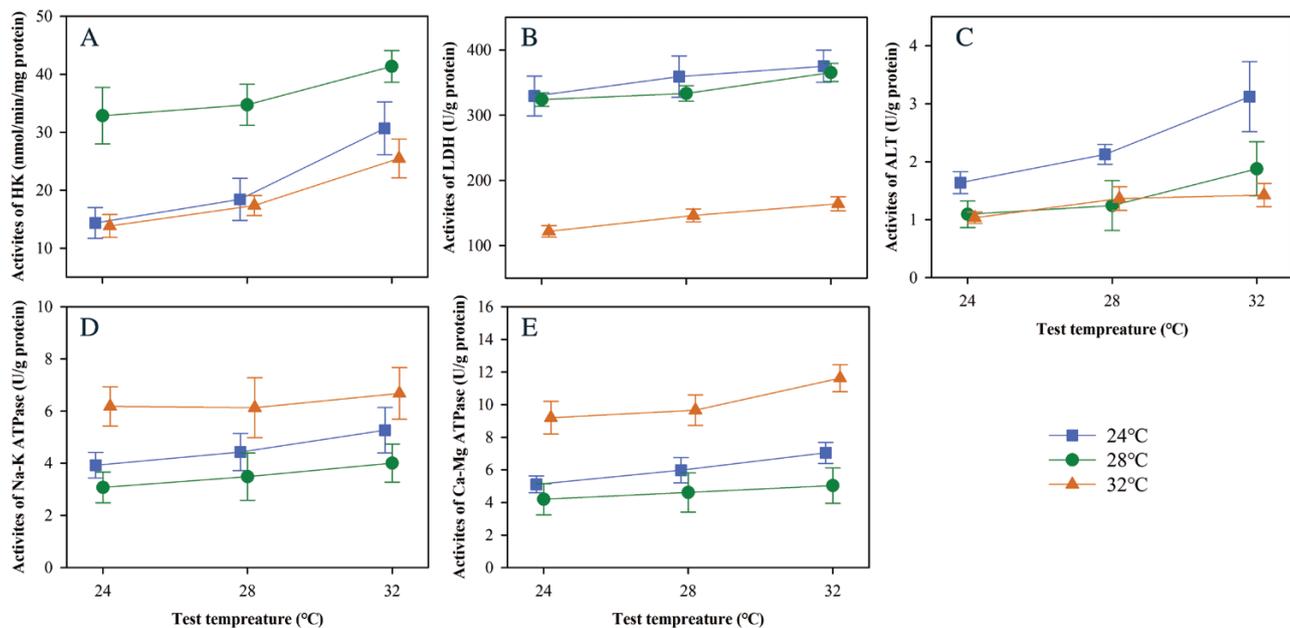

Figure 4 Activities of metabolic enzymes of (A) HK, (B) LDH, (C) ALT, (D) Na–K ATPase, and (E) Ca–Mg ATPase of the muscle of necks. The blue cubes, green circles and orange triangles indicate the hatchlings incubated at 24 °C, 28 °C, and 32 °C, respectively. The data are shown as mean ± SE.

animals (Gibb and Dickson 2002; Seebacher and James 2008; Sun et al. 2020), the functional performance may also be affected by other physiological and biochemical processes. For example, locomotor performance may depend on various properties of muscle such as muscle fiber types, maximal shortening velocity, activation and relaxation rates, and maximal force production. (Herrel et al. 2007; James et al. 2007; Seebacher and James 2008; Higham and Biewener 2011). In
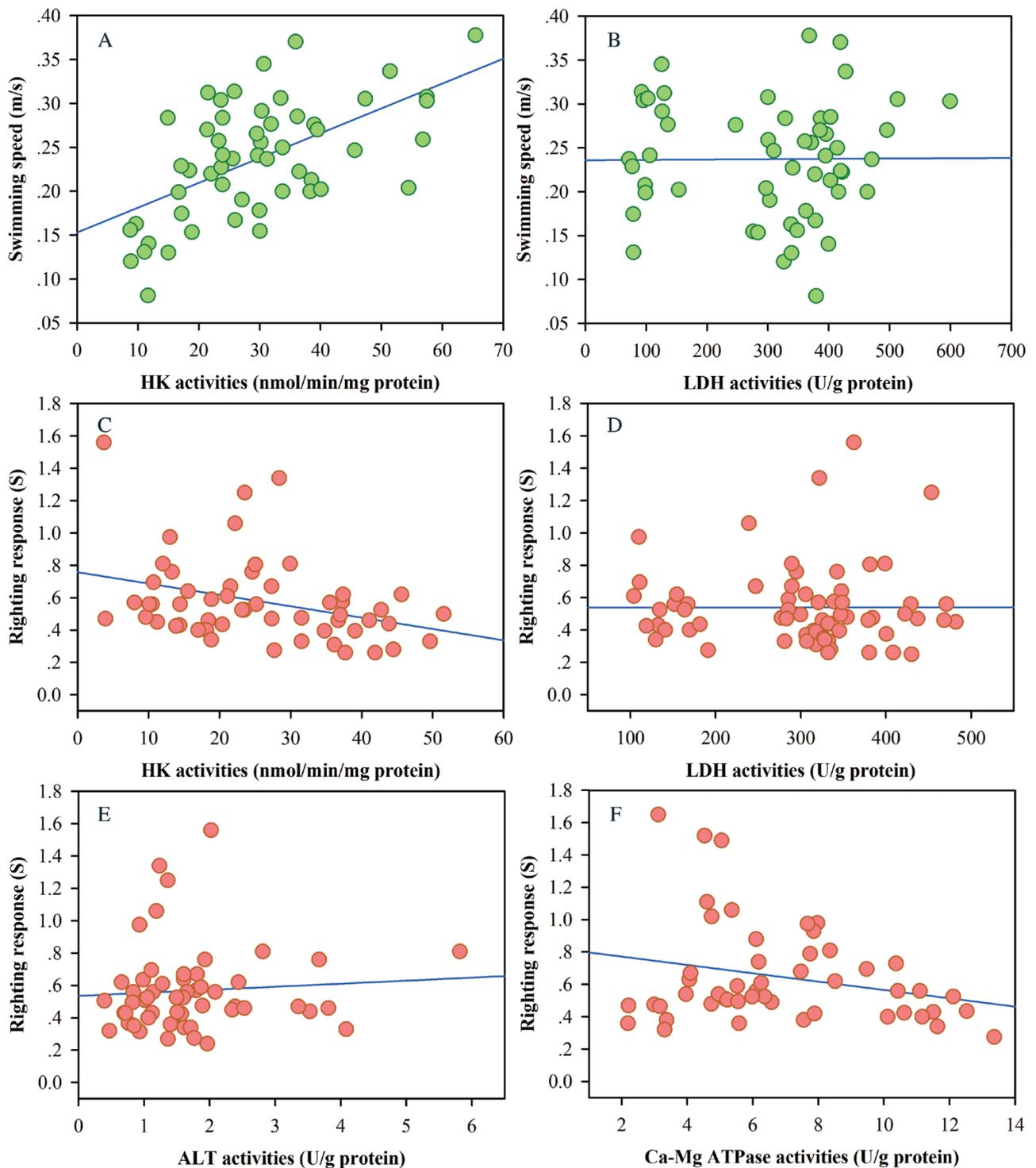
Figure 5 Correlation between swimming speed (A, B) and righting responses (C–F) and activities of metabolic enzymes. (A, B) indicate the correlation between the activities of HK and LDH in limb muscles and the swimming speed. Each green spot indicates an individual. (C–F) indicate the correlation between the activities of HK, LDH, ALT, and Ca–Mg ATPase in neck muscles and the righting responses, respectively. Each orange spot indicates an individual.

addition, the differential effects of incubation temperature on enzyme activities (Figures 3 and 4) may also indicate the complexity of the relationship between functional performances and the physiological and biochemical processes in organisms. The network by which incubation temperature regulates energy metabolism, which in turn determines functional performance, involves multiple levels of physiological and biochemical modification (Higham and Biewener 2011). For

example, in addition to the metabolic enzymes investigated in this study, other traits such as mitochondrial density, structure, and respiration that play critical roles in cellular metabolism and function could also be involved (Hochachka and Somero 2002; Li et al. 2010). Although we do not know how incubation temperature would affect mitochondrial traits, these mitochondrial features do adjust to change the energy supply in response to variations in environmental temperature

in natural populations (O'Brien and Mueller 2010; Coppe et al. 2013; Kake-Guena et al. 2017; Michaelsen et al. 2021).

Given the complexity of the relationship between the plasticity of performance and underlying physiological and biochemical bases, future studies exploring these mechanisms at multiple levels would be of great significance to increase our understanding of the effects of incubation temperature on post-hatching phenotypes (e.g., Sun et al. 2022). First, we should consider the effect of incubation temperature on critical physiological systems (e.g., muscles and cardiovascular system) that play important roles in physiological and behavioral functions such as metabolism and locomotor performance (de Assis et al. 2004; Tattersall et al. 2012; Booth 2018; Sun et al. 2022). Second, we may focus on the regulation of some primary metabolic pathways based on the activities of key metabolic enzymes. The tricarboxylic acid cycle (TCA, Kreb's cycle), oxidative phosphorylation, fatty acid degradation, and glycolysis are critical to energetic metabolism in organisms (Voet and Voet 1995; Brown et al. 2004). For example, oxidative phosphorylation and fatty acid degradation are related significantly to metabolic responses to thermal variations in reptiles (Sun et al. 2015, 2022). Third, in the post-genomics era, in light of the advanced technology and the rapidly decreasing cost, the application of proteomics, metabolome, and transcriptome would allow us to investigate the mechanisms underlying developmental plasticity in depth (e.g. Coppe et al. 2013).

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Author Contributions

B-J. Sun and X-Y. Qin designed the study, analyzed the data, and wrote the manuscript. D-Y. Wu, X-Z. Han and T. Li performed the experiments.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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