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The influence of rearing temperature on early embryonic development of *Pelophylax nigromaculata*

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Abstract. Temperature influenced the developmental rate and survival of embryos of *Pelophylax nigromaculata*. The total incubation period decreased from 254.07hrs at 18.20 ± 0.49°C and 162.80hrs at 20.87 ± 0.57°C in two experiments. The proportion of normal embryos and survival of eggs until hatching were higher (85.2%) when the eggs were incubated at 20.87 ± 0.57°C. However, death of eggs until hatching has reached 27.2% when the eggs were incubated at 18.20 ± 0.49°C. Therefore, it is appropriate to raise incubation temperature, and fit for enhancing developmental rate and survival of eggs. Also, this information can be valuable for optimizing laboratory culture and facilitating future use of this species as a test organism in toxicity tests.

Key words: *Pelophylax nigromaculata*; temperature; embryonic development.

Optimal environmental conditions are important for successful cultivation of species in aquaculture and temperature is a major factor influencing the developmental rate of anuran (Pollister & Moore 1937, Moore 1939, Stewart 1956, Herreid & Kinney 1967, Zhang 1989, Bradford 1990, Zhang 1990, Han & Lu 2001, Feng et al. 2004). Low temperatures, causing slower development (Moore 1939), may limit where a species can breed if temporary aquatic habitats dry before metamorphosis can occur or if prolonged exposure to predators reduces the number of eggs hatching or larvae metamorphosing. High temperatures may result in developmental abnormalities which likewise reduces hatching success (Howard 1978). Generally, the developmental response to temperature variation has been studied in a number of amphibians (Pollister & Moore 1937, Moore 1939, Stewart1956, Herreid & Kinney 1967, Bradford 1990).

Pelophylax nigromaculata is abundant and widespread throughout Chinese mainland (Fei & Ye 2001). These frogs prefer to lay eggs in paddy field, because paddy field is shallow, high clarity and lacks predators (Unpublished data). Similarly, Wang et al. (2007) observed *P. nigromaculata* selected spawning sites with higher percentages of water and vegetation cover and avoided deeper water. Also, the clutch size of this frog was 4643.04 ± 253.9 eggs and egg size was 1.60 ± 0.005mm. Zheng et al. (2002) found correlation between SVL of female size or weight and clutch size, which revealed that larger females were more fecund. However, it is surprising that few analyzed early embryonic development of these frogs. Owing to large body size of frogs, these frogs often were hunted by humans. Moreover, pesticides and herbicides were heavily used in agriculture, and urbanization destroyed their habitats. Also, endoparasites of anuran may influence on the population of these species (Fabricante & Nuñeza 2012). Consequently, the population of *P. nigromaculata* has drastically declined in recent years. As bases for further analysis, this work provides a normal table of development and the methods for the handling of *P. nigromaculata* embryos.

Field work at Nanchong was conducted in March 2006. Eggs were obtained naturally from *P. nigromaculata*, which usually bred in the dawn in nature. The used eggs were all from one batch. Temperature was controlled by the use of lagged boxes, with or without an electric bulb for heating, kept in rooms with regulated temperatures. Twenty-five eggs were placed in a single bowl containing 100-120 cc of water, respectively. Dissolved oxygen concentration and pH were monitored and adjusted when necessary. The water was changed every three days. Observation of the total developmental time period was performed based on a group of intact eggs kept at two kind of temperatures: 20.87 ± 0.57°C, and 18.20 ± 0.49°C. We staged each excavated egg clutch according to the modified Gosner (1960) stages of Anstis (2002). Every 15 min, the eggs were observed through a stereoscopic microscope in order to verify the embryonic developmental stage in stage1-11. Then, we observed the embryonic development every 30 min, because it spent a long time to the next stage (> 1h) after stage11.

The results were analyzed statistically using Chi-square test to determine differences in duration in every stage and total incubation duration; significance accepted was $p < 0.05$.

The proportion of normal embryos and survival of eggs until hatching were higher (85.2%) when the eggs were incubated at 20.87 ± 0.57°C. However, death of eggs until hatching has reached 27.2% when the eggs were incubated at 18.20 ± 0.49°C. The embryos of *P. nigromaculata* spent 254.07hrs at 18.20 ± 0.49°C completing their embryonic development. On the contrary, it spent less time (162.80hrs) at 20.87 ± 0.57°C (Chi-square test: $\chi^2 = 19.86$, df = 1, $P < 0.001$). Similarly, there was significant reduction in duration of stage 17, 21, 22 and 24 at 20.87 ± 0.57°C compared to 18.20 ± 0.49°C (all $P < 0.05$, Table 1).

The age in hours represents the period of time required for 50 per cent or more of the individuals to reach a particular stage. The dimension given for each stage is an average value of the total lengths of the individuals that exhibited the characteristics of the stage at the hour indicated. Stages 1-26 (Fig. 1) comprise the embryonic period.

Stage 1 (Fertilization): The egg at fertilization rotates so that the animal hemisphere is uppermost. The animal hemisphere is brown-black; the vegetal hemisphere is cream-white.

Stage 2 (Two cell stage): The first cleavage forms two blastomeres.

Stage 3 (Four cell stage): The second cleavage forms four blastomeres.

Stage 4 (Eight cell stage): The third cleavage forms eight blastomeres.

Table 1. The stages of early embryonic development in *Pelophylax nigromaculata* at two different temperatures.

Stage	20.87 ± 0.57℃		18.20 ± 0.49℃	
	Hrs in each of stage	Hrs from fertilization	Hrs in each of stage	Hrs from fertilization
1	1.93	0.00	2.50	0.00
2	1.05	1.93	1.38	2.50
3	0.55	2.98	0.67	3.88
4	0.58	3.53	0.73	4.55
5	0.75	4.12	1.00	5.28
6	0.72	4.87	0.98	5.28
7	0.75	5.58	1.07	7.27
8	2.30	6.33	2.63	8.33
9	11.42	8.63	14.12	10.97
10	2.95	20.05	5.73	25.08
11	1.17	23.00	1.67	30.82
12	6.33	24.17	6.83	32.48
13	8.25	30.50	9.83	39.32
14	5.60	38.75	5.98	49.15
15	3.63	44.35	10.60	55.13
16	3.57	47.98	7.67	65.73
17	7.82	51.55	17.60	73.40
18	9.62	59.37	19.93	91.00
19	13.17	68.98	15.78	110.93
20	8.97	82.15	13.08	126.72
21	11.40	91.12	24.58	139.80
22	7.65	102.52	19.72	164.38
23	17.82	110.17	18.62	184.10
24	19.53	127.98	34.28	202.72
25	15.28	147.52	17.07	237.00
26		162.80		254.07

Stage 5 (Sixteen cell stage): The fourth cleavage forms 16 blastomeres.

Stage 6 (Thirty-two cell stage): The fifth cleavage forms 32 blastomeres.

Stage 7 (Large-cell blastula stage): The sixth cleavage forms 64 blastomeres.

Stage 8 (Medium-cell blastula stage): After many times cell division, the surface of the embryo is not flat, but the blastomeres border can be distinguished. Owing to increasing blastomeres number, embryos present mulberry.

Stage 9 (Advanced blastula stage): Continue to cells division, the blastomeres border is vague because of increasing blastomeres number, but the embryo surface becomes smooth.

Stage 10 (Early gastrula stage): Involution occurs at the dorsal lip of the blastopore.

Stage 11 (Mid-gastrula stage): The dorsal lip of the blastopore expands into a semicircle, and involution occurs along the semicircular surface.

Stage 12 (Late gastrula stage): A complete blastopore forms, resulting in a circular yolk plug.

Stage 13 (Neural plate stage): The embryo flattens dorsally and thickened. The yolk bolt sank into the blastopore, and the neural plate forms.

Stage 14 (Neural folds stage): The neural folds form as lateral ridges of the neural groove, embryo elongation is not obvious.

Stage 15 (Embryo rotation stage): Both sides of the neural folds gradually move to the center, the neural groove to form a linear crack. Under the microscope, the embryo clockwise rotates slowly inside the egg jelly.

Stage 16 (Neural tube stage): The neural folds close to form a neural tube. The sensory panels, gill plate and mouth sucker primordial were formed.

Stage 17 (Tail bud stage): The tail bud develops. There is vertical uplift in embryo back; tail bud is obviously in the back-end. The sensory panels and gill plate become apparent.

Stage 18 (Muscular movement stage): The corresponding to stimulation, embryo showed a simple flexure. Simultaneously, pronephric ridges, visceral arches, and olfactory pits are recognizable.

Stage 19 (Incubative stage): The egg membrane partially dissolved, then, the embryo attached membrane. Later, when egg membrane dissolved, the embryo was detached from the yolk membrane, and the embryo adsorbed on membrane. The embryo gives a flexure in response to stimulation, but not to maintain body balance.

Stage 20 (Heart beats stage): The heart begins to beat in the junction of yolk sac and the mouth fossa. Heart rate is 20/ min in the beginning, 50/ min is at the end of the heart beats' stage. External gill buds are conspicuous.

Stage 21 (Gill circulation stage): The external gills gradually stretched and branched. Corpuscles moved in gill filaments. Embryos dispersed the bottom, the mouth remains attached to the membrane. The embryo gives a short swimming, in response to stimulation, but not to maintain body balance.

Stage 22 (Mouth open stage): The mouth opens, and suckers begin to disappear. The cornea is transparent; see the black eye. The external gills enlarge. The embryo did not adsorb on membrane, swim for a long time, but not to maintain body balance.

Stage 23 (Tail circulation stage): Blood corpuscles circulate in the tail fin. The tail epidermis becomes transparent. The external gills are in full development. Black chromatophores appear in the head region and on the dorsal tail musculature. The embryo can swim freely and maintain body balance.

Stage 24 (Opercular fold stage): The opercular fold forms at the base of the external gills, and gradually extended to the end of the gill. The horny teeth become hard and black, and see bent intestine and white excrement. The number of melanophores increases.

Stage 25 (Operculum closed on right side stage): The operculum closes on the right side. The external gills are visible only on the left side.

Stage 26 (Operculum completely closed stage): The operculum closes on the left side. The embryos hatch and have the appearance of a tadpole.

Incubation time constitutes a significant ecological and physiological parameter to embryonic amphibians. In this study, the results showed that it spent less time in the total incubation period at 20.87 ± 0.57°C compared to 18.20 ± 0.49°C, because increased temperature may reduce the duration of each stage. Similarly, Zhu and Shi (1957) studied early development of *P. nigromaculata,* which spent 202.3 ± 5.9hrs at 20°C.

The temperature action is different for each stage development of anurans. For some frog species, temperature mainly reduced time on the early stages of embryo development (Zou et al. 2001); others acted on the late stages

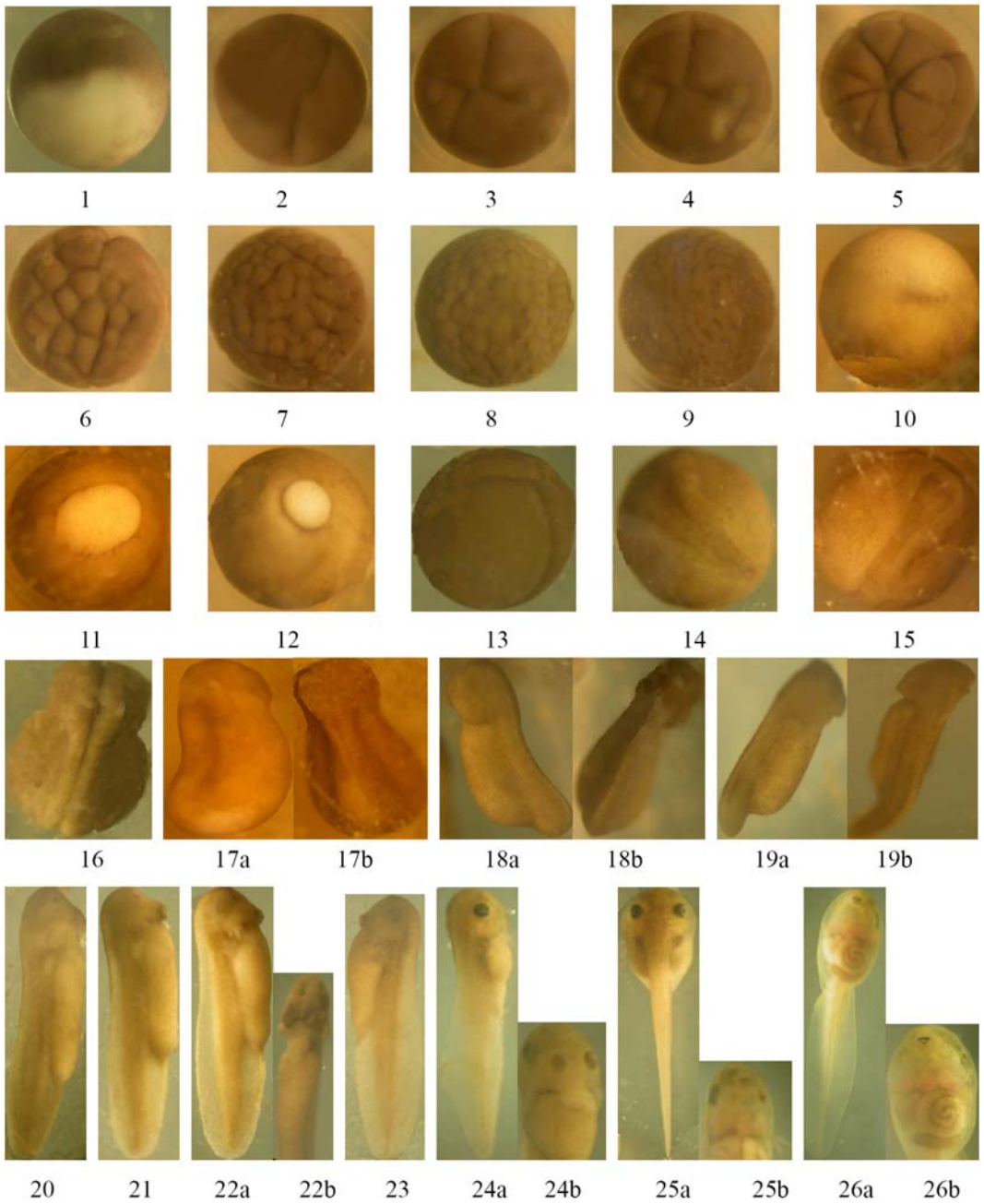

Figure 1. The early embryonic development of *Pelophylax nigromaculata* (stages 1-26)
(1) The fertilized egg. (2) Two cell stage. (3) Four cell stage. (4) Eight cell stage. (5) Sixteen cell stage. (6) Thirty-two cell stage. (7) Large-cell blastula stage. (8) Medium-cell blastula stage. (9) Advanced blastula stage. (10) Early gastrula stage. (11) Mid-gastrula stage. (12) Late gastrula stage. (13) Neural plate stage. (14) Neural folds stage. (15) Embryo rotation stage. (16) Neural tube stage. (17) Tail bud stage, a: profile; b: rearface. (18) Muscular movement stage, a: profile; b: rearface. (19) Incubative stage a: profile; b: rearface. (20) Heart beats stage. (21) Gill circulation stage. (22) Mouth open stage, a: profile; b: abdomen. (23) Tail circulation stage. (24a) Opercular fold stage, a: profile; b: abdomen. (25) Operculum closed on right side stage, a: rearface; b: magnifying head and abdomen. (26) Operculum completely closed stage, a: a whole tadpole; b: magnifying head and abdomen.

(Zhang 1990). Compared to 20.87 ± 0.57°C, tail bud stage, gill circulation stage, mouth open stage and opercular fold stage spent 2-3 times at 18.20 ± 0.49°C. Therefore, *P. nigromaculata* belonged to reduction time on late stage.

Compared to *Gastrotheca riobambae* (reviewed in del Pino & Elinson 2003) and *Bufo valliceps* (Limbaugh & Volpe 1957), the embryo development of *P. nigromaculata* lacked gray crescent, and increased incubative stage. *Colostethus machalilla* (del Pino et al. 2004) lack incubative stage and medium-cell blastula stage. However, some studies on *Bufo* (Ge et al. 1982, Wang 1984, Ye et al. 1986)did not show heart beats stage and tail circulation in embryo development because embryo color was dark. Our study was similar with embryo development of *Hylarana guentheri* (Zou et al. 2001), *Hoplobatrachus tigerinus* (Geng et al. 1999), *Fejervarya limnocharis* (Wu & Sun 1981, Zhang 1987), *Microhyla ornata* (Geng et al. 1996, Liu et al. 1996). Studies on *Kaloula borealis* (Li et al. 1998) and *Rana chensinensis* (Liu et al. 1989), found incubat-

ive stage posterior to heart beats stage during embryo development. However, incubative stage of embryo development of *P. nigromaculata* was prior to heart beats' stage. Thus, it belonged to the early-hatch (Zou et al. 2001).

The early development of *P. nigromaculata* was not harsh for water quality, because tap water may be used for incubating frog eggs. Moreover, when incubation temperature were appropriated to increase, and fit for enhancing developmental rate and survival of eggs, because it reduced abnormality and mould development. In this case, this information provides the reference for protecting this frog species.

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Reference

Anstis, M. (2002): Tadpoles of south-eastern Australia. Reed New Holland, New South Wales, Australia.

Bradford, D.F. (1990): Incubation time and rate of embryonic development in amphibians: the influence of ovum size, temperature, and reproductive mode. Physiological Zoology 63: 1157-1180.

del Pino, E.M., Avila, M.E., Pérez, O.D., Benitez, M.S., Alarcón, I., Noboa, V., Moya. I.M. (2004): Development of the dendrobatid frog *Colostethus machalilla*. International Journal of Developmental Biology 48: 663–670.

del Pino, E.M., Elinson, R.P. (2003): The organizer in amphibians with large eggs: Problems and perspectives. pp. 359-374. In: Grunz, H (ed.), The Vertebrate Organizer. Springer Verlag.

Fabricante, K.M.B., Nuñeza, O.M. (2012): Diet and endoparasites of *Rana grandocula* (Amphibia, Ranidae) and *Limnonectes magnus* (Amphibia, Dicroglossidae) in Mt. Sambilikan, Diwata Range, Agusan del Sur, Philippines. AES Bioflux 4: 113-121.

Fei, L., Ye, C.Y. (2001): The colour handbook of amphibians of Sichuan. China Forestry Publishing House, Beijing.

Feng, Z.J., Li, W., Ge, G.K., Xu, J.J., Xu, F., Bai, D.Y. (2004): The Effect of Temperature on the Late Embryonic Development of *Bufo raddei*. Journal of Xuzhou Normal University (Natural Science Edition) 22: 54-56

Ge, R.C., Feng, B.S., Tong, Y.X. (1982): The stages and early embryonic development of *Bufo raddei*. Journal of Lanzhou University (Natural Science) 18: 125-136.

Geng, B.R., Chen, Y.L., Zhnag, Q.J., Cai, M.Z. (1999): The early embryonic development of *Hoplobatrachus tigerinus*. Journal of Fujian Normal University (Natural Science) 15: 85-90.

Geng, B.R., Wen, Q., Zhang, Q.J. (1996): The early embryonic development of *Microhyla ornata*. Chinese Journal of Zoology 31: 17-21.

Gosner, K.L. (1960): A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16: 183–190.

Han, Y.P., Lu, X.Y. (2001): The early embryonic development of *Rana plancyi*. Chinese Journal of Zoology 1: 6-11.

Herreid, C.F., Kinney, S. (1967): Temperature and Development of the Wood Frog, *Rana Sylvatica*, in Alaska. Ecology 48: 579–590.

Howard, R.D. (1978): The influence of male defended oviposition sites on early mortality in bull-frogs. Ecology 59:789-798.

Li, Z.Y., Sun, J.M., Hu, B., Wang, J.M. (1998): The early embryonic development of *Kaloula borealis*. Chinese Journal of Zoology 33: 4-8.

Limbaugh, B.A., Volp. E.P. (1957): Early development of the Gulf Coast Toad, *Bufo valliceps* Wiegmann. American Museum Novitates 1842: 1-32.

Liu, S.L., Song, Z.M., Zhang, J.Y., He, M.Y., Zhu, K., Li, Y. (1996): The early embryonic development of *Microhyla ornata*. Journal of Sichuan University (Natural Science) 33(3): 323-329.

Liu, Y.W., Liu, Z.G., An, J.Y., Hu, H.W. (1989): Morphology of early embryonic development of *Rana chensinensis*. Journal of Shenyang Agricultural University 20: 49-54.

Moore, J.A. (1939): Temperature tolerance and rates of development in the eggs of Amphibia. Ecology 20: 459-478.

Pollister, W., John, A.M. (1937): Tables for the normal development of *Rana sylvatica*. The Anatomical Record 68: 489–496.

Stewart, M.M. (1956): The separate effects of food and temperature differences on the development of marbled salamander larvae. Journal of the Elisha Mitchell Scientific Society 72: 47-56.

Wang, C. (1984): The primary research for the early embryonic development of *Bufo gargarizans*. Acta Herpetologica Sinica 3: 39-48.

Wu, Y.L., Sun, Y.C. (1981): The early embryonic development of *Rana limnocharis*. Chinese Journal of Zoology 3: 28-31.

Ye, D.Q., Hong, X.F., Chen, X.Z. (1986): The early embryonic development stages of *Bufo melanostictus*. Acta Herpetologica Sinica 5: 185-188.

Zhang, H.G. (1989): The influence of rearing temperature on early embryonic development of *Rana japonica*. Journal of Southwest University (Natural Science) 14: 74-79.

Zhang, H.G. (1990): Different temperatures influence on early embryonic development of *Bufo gargarizans*. Chinese Journal of Zoology 25: 22-26.

Zhang, J. (1987): The stages and early embryonic development of *Fejervarya limnocharis*. Acta Herpetologica Sinica 6: 26-33.

Zhu, Z.P., Shi, L.J. (1957): A table of normal embryonic development of *Pelophylax nigromaculata*. Acta Anatomica Sinica 2: 59-64.

Zou, P.Z., Wen, C.Y., Xu, J., Chen, J.R. (2001): The primary research for the early embryonic development of *Hylarana guentheri*. Chinese Journal of Zoology 36: 15-19.