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Exploring Altitude's Influence: Contrasting Embryonic Development of *Hyla annectans* Jerdon in High and Low Altitude Environments

P. Wankitlang Shangpliang [a*], Rupa Nylla K. Hooroo [b], Graham Bakynson Ranee [a], Sudhanya Ray Hajong [b], Annu Kumari [c] and Eugene Lyngkhoi [b]

[a] Department of Zoology, St. Edmund's College, Shillong, 793 003, Meghalaya, India.
[b] Department of Zoology, North Eastern Hill University, Shillong, 793 022, Meghalaya, India.
[c] Department of Zoology, The Assam Royal Global University, Guwahati, 781 035, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The study investigated the embryonic development of *Hyla annectans* Jerdon, a frog species, at high and low altitude breeding sites of Meghalaya, North East India. Results revealed significant differences in the duration of embryonic development and hatching between the two altitudes. Embryos at low altitude sites exhibited a faster development, hatching approximately four days earlier than those at high altitude sites. Early embryonic stages displayed similar characteristics in size between the two sites, but distinct differences emerged during later stages, notably in stages associated with heart development, muscular response, and gill circulation. Furthermore, metamorphosis completion varied between altitudes, with a shorter duration observed at low

**Corresponding author: Email: shangpliang1992@gmail.com;*

altitude compared to high altitude. Environmental conditions, particularly water properties, also differed significantly between the two sites. Water temperature and free carbon dioxide levels were higher at low altitude, while pH and dissolved oxygen levels were higher at high altitude. These findings suggest a potential influence of altitudinal gradients as environmental factors on embryonic development in *H. annectans*.

Keywords: Altitude; anurans; embryonic development; Hyla annectans Jerdon.

1. INTRODUCTION

Hyla annectans Jerdon, a species of frog known for its preference for undisturbed, clear, and pristine waters, has been the subject of considerable research attention. Earlier studies have highlighted its annual breeding cycle and spawning habits in the elevated regions of Nagaland, ranging from 1400 to 2440 meters above sea level, as well as in Meghalaya, where oviposition sites have been observed at elevations ranging from 1400 to 1650 meters [1-3]. However, recent findings have expanded our understanding, revealing that the distribution range of *H. annectans* extends to lower elevations, ranging from approximately 35 to 700 meters above sea level [4].

While taxonomic descriptions of adult *H. annectans* specimens and staging of their normal development have received considerable attention [5], comparatively fewer studies have delved into the embryonic and tadpole development in natural settings, particularly regarding the influence of ecological factors. Furthermore, scant attention has been paid to investigating the effects of altitude on embryonic development rates among conspecific anurans [6].

Against this backdrop, the present study aims to assess the impact of ecological variables—specifically water temperature, pH, dissolved oxygen, and free carbon dioxide—on the developmental parameters of *H. annectans* in its natural breeding habitats at both high and low altitude water bodies in Meghalaya, Northeast India. By examining factors influencing the size of eggs or tadpoles and the duration of development, this research seeks to shed light on adaptive mechanisms and compare embryological and other developmental processes among different anuran species, potentially contributing to our understanding of their phylogenetic relationships. These findings promise to enrich the database on the quality of breeding habitats for anurans, offering valuable insights for conservation efforts and future research endeavors in amphibian biology.

2. MATERIALS AND METHODS

Over the course of 2018-2020, two distinct study sites were chosen to observe the development of *Hyla annectans*. One site was situated at a high altitude (25.29745ºN, 91.58512ºE; elevation 1424 m; Fig. 1) in the northern ranges of Mawsynram, while the other was at lower elevations (25.17203ºN, 91.561336ºE; elevation

(A) (B)

Fig. 1. The two breeding sites of *Hyla annectans* located at: (A). High altitude (25.29745ºN, 91.58512ºE; elevation 1424 m) on the northern ranges of Mawsynram and (B) Low lying elevations (25.17203ºN, 91.561336ºE; elevation 38 m) on the southern slopes of Meghalaya bordered by Bangladesh on its south

38 m; Fig. 2) on the southern slopes of Meghalaya, near the Bangladesh border.

To assess the environmental conditions influencing development, various physico-chemical parameters of water were recorded at each site using specialized instruments, including temperature, pH, dissolved oxygen, and free carbon dioxide, during each observation period. The staging of embryos followed the criteria outlined by Gosner in 1960 [7]. Specifically, ten early embryonic Gosner stages (Gosner stage 1, 5, 10, 14, 15, 16, 17, 18, 19, and 20) were considered for analysis across four clutches observed annually at each site. For each clutch in each year, the mean size (measured in millimeters), age (measured in hours), and developmental stage were recorded.

To discern differences between the high and low altitude study sites, parametric tests, specifically paired-sample Student's t-tests, were employed to evaluate disparities in the physico-chemical variables of the water at both locations.

3. RESULTS

The eggs of *H. annectans* were discovered attached to aquatic vegetation at the center of both breeding sites (Fig. 2). Upon comparing the two study sites, it became evident that the duration of embryonic development from fertilization to hatching occurred more rapidly at the low altitude site (106±1.40 hours or approximately 4 days; hatched at stage 18) than at the high altitude site (243±2.06 hours or approximately 10 days; hatched at stage 20).

Early embryonic development of *H. annectans* revealed similarity in mean size (measured in mm diameter) at both study sites for early embryonic stages (Gosner stage 1, 5, 10, 14, 15) as depicted in Table 1 and Fig. 4. Notably, during the early embryonic stages, embryo sizes at fertilization stage (stage 1, Fig. 3A), the eight-celled stage (stage 5, Fig. 3B), formation of the blastoporal dorsal lip (stage 10, Fig. 3C), formation of lateral ridges of neural folds (stage 14, Fig. 3D), and closure of the neural folds (stage 15, Fig. 3E) were relatively similar at both sites. However, a gradual increase in embryo size was observed during rotation (stage 16, Fig. 3F) and formation of the neural tube (stage 17, Fig. 3G) in embryos observed at the high altitude site.

The dissimilarity in embryo size between the two study sites was particularly distinct at three stages of development: when the heartbeat is visible (stage 18, Fig. 3H), during muscular response (stage 19, Fig. 3I), and at the gill circulation stage (stage 20, Fig. 3J). Interestingly, embryos laid at the low altitude site hatched earlier at stage 18 compared to those at the high altitude site, which hatched at a later stage 20 (Fig. 4). Additionally, complete metamorphosis occurred in approximately 48 days at low altitude and 54 days at high altitude, with tail-less metamorphosed frogs observed at the pool's edges.

Fig. 2. Egg clutches of *Hyla annectans* attached to vegetation in the centre of the breeding pool

Table 1. The recorded data of the 10 selected common embryonic stages of *Hyla annectans* at the selected study site of high altitude and low altitude with the Mean age of development in hours (N=12) and Mean size (mm) diameter/TOL±SD (N=80) of the ten selected developmental stages with the range and mean (N=45) of the water physico-chemical variables (water temperature, water pH, dissolved oxygen and free carbon-dioxide) obtained during field observations from the two study sites (2018-2020). All the data are represented as Mean±SD

	Study site of high altitude						Study site of low altitude					
Ten (10) Common Gosner stage of development	**Mean age of development in hours (h) ±SD; N=12**	**Mean size (mm) diameter/TOL±SD; N=80**	**Water temperature (ºC)**	**Water pH**	**Dissolved oxygen (Mg/L)**	**Free carbon-dioxide (Mg/L)**	**Mean age of Development (h) ±SD; N=12**	**Mean size (mm) diameter/TOL±SD; N=80**	**Mean water temperature (ºC)**	**Mean water pH**	**Mean dissolved oxygen (Mg/L)**	**Mean free carbon-dioxide (Mg/L)**
1	0±0	2.4±0	Range: 18.00-23.00 Mean ±SD : 19.6±1.16	Range; 6.02-7.06 Mean ±SD: 6.59 ±0.12	Range: 4.56.-7.22 Mean ±SD: 6.05 ±0.56	Range: 3.33- 4.66 Mean ±SD: 4.02 ±0.24	0±0	2.4±0.01	Range: 19.00-25.00 Mean ±SD : 23.60±1.08	Range: 4.56-5.88 Mean ±SD : 4.98±0.88	Range: 3.22-4.44 Mean ±SD : 4.02±0.12	Range: 5.12-7.68 Mean ±SD : 6.62±0.74
5	8.4±0.40	2.56±0					8±0	2.56±0.04				
10	20.8±0.40	3.2±0					18±0	3.2±0.06				
14	86±1.20	3.3±0					76±0.20	3.3±0.08				
15	98±1.46	3.4±0					82±0.80	3.36±0.04				
16	102±2.30	3.5±0.04					90±0.90	3.8±0.06				
17	126±2.26	4.3±0.12					98±1.21	4±0.075				
18	156±2.45	4.4±0.66					106±1.40	4.02±0.094				
19	180±1.60	5±0.88					116±1.78	4.66±0.06				
20	243±2.06	5.4±0.04					158±2.18	4.78±0.04				

Table 2. Paired sample t-test results of the water physico-chemical variables (water temperature, water pH, dissolved oxygen and free carbon-dioxide) obtained during field observations from the two study sites of *Hyla annectans* (high altitude and low altitude) (2018-2020)

Parameters	Year	t-value	df	*P*-value
Water temperature (ºC)	2018	-6.98	14	0.000
	2019	6.56	14	0.000
	2020	-1.19	14	0.264
Water pH	2018	6.08	14	0.000
	2019	6.12	14	0.000
	2020	-6.22	14	0.000
Dissolved Oxygen (mg/L)	2018	8.11	14	0.000
	2019	8.22	14	0.000
	2020	8.03	14	0.000
Free carbon-dioxide (mg/L)	2018	-10.50	14	0.000
	2019	9.67	14	0.000
	2020	-7.34	14	0.000

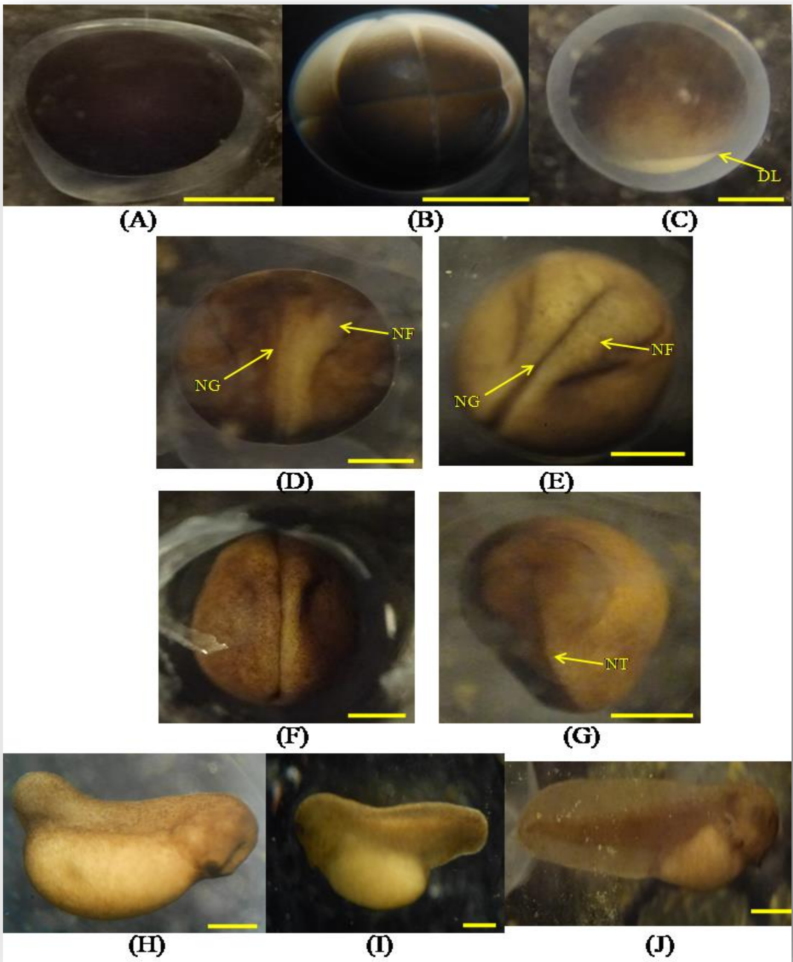

Fig. 3. (A to J): The 10 selected embryonic developmental stages (Gosner stages 1,5,10, 14,15,16,17,18,19,20) of *Hyla annectans* documented during field observations.
DL- Dorsal Blastoporal Lip, NF-Neural Fold, NG-Neural Groove, NT-Neural Tube. Scale bars are 1 mm

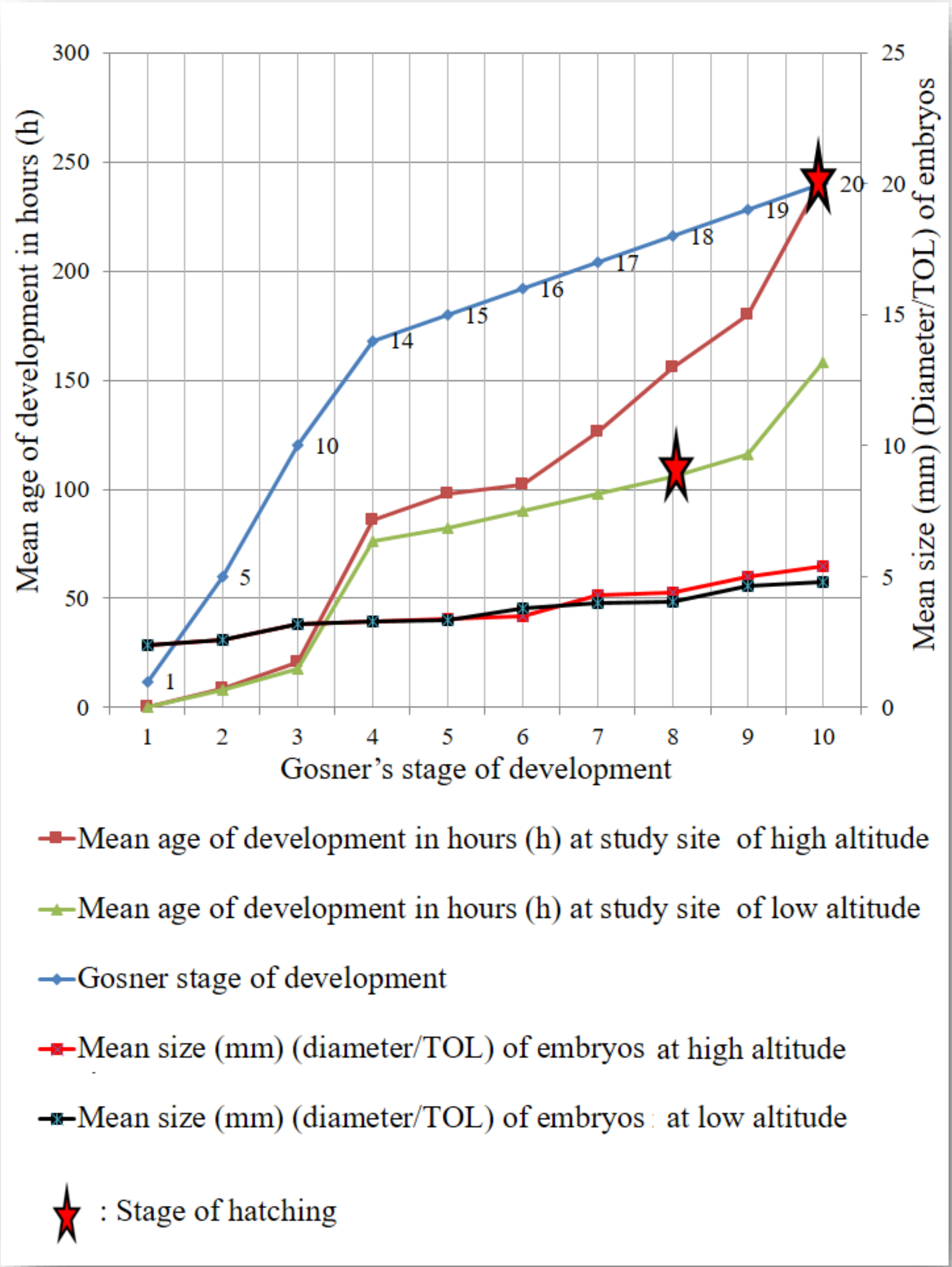

Fig. 4. Graphical representation illustrating the variations in the age of development (measured in hours) and the size of embryos (measured in millimeters) between high and low altitude study sites

Recorded physico-chemical properties of water are detailed in Table 1. A paired samples t-test (2-tailed) revealed significant differences in water temperature ($P<0.001$), water pH ($P<0.001$), dissolved oxygen ($P<0.001$), and mean free carbon dioxide ($P<0.001$) across all three years when comparing the two study sites of high and low altitude (Table 2). Water temperature (Fig. 5A) and free carbon dioxide levels (Fig. 5D) were relatively higher at the low altitude site compared to the high altitude site, while water pH (Fig 5B) and dissolved oxygen levels (Fig. 5C) were higher at the high altitude site compared to the low altitude site.

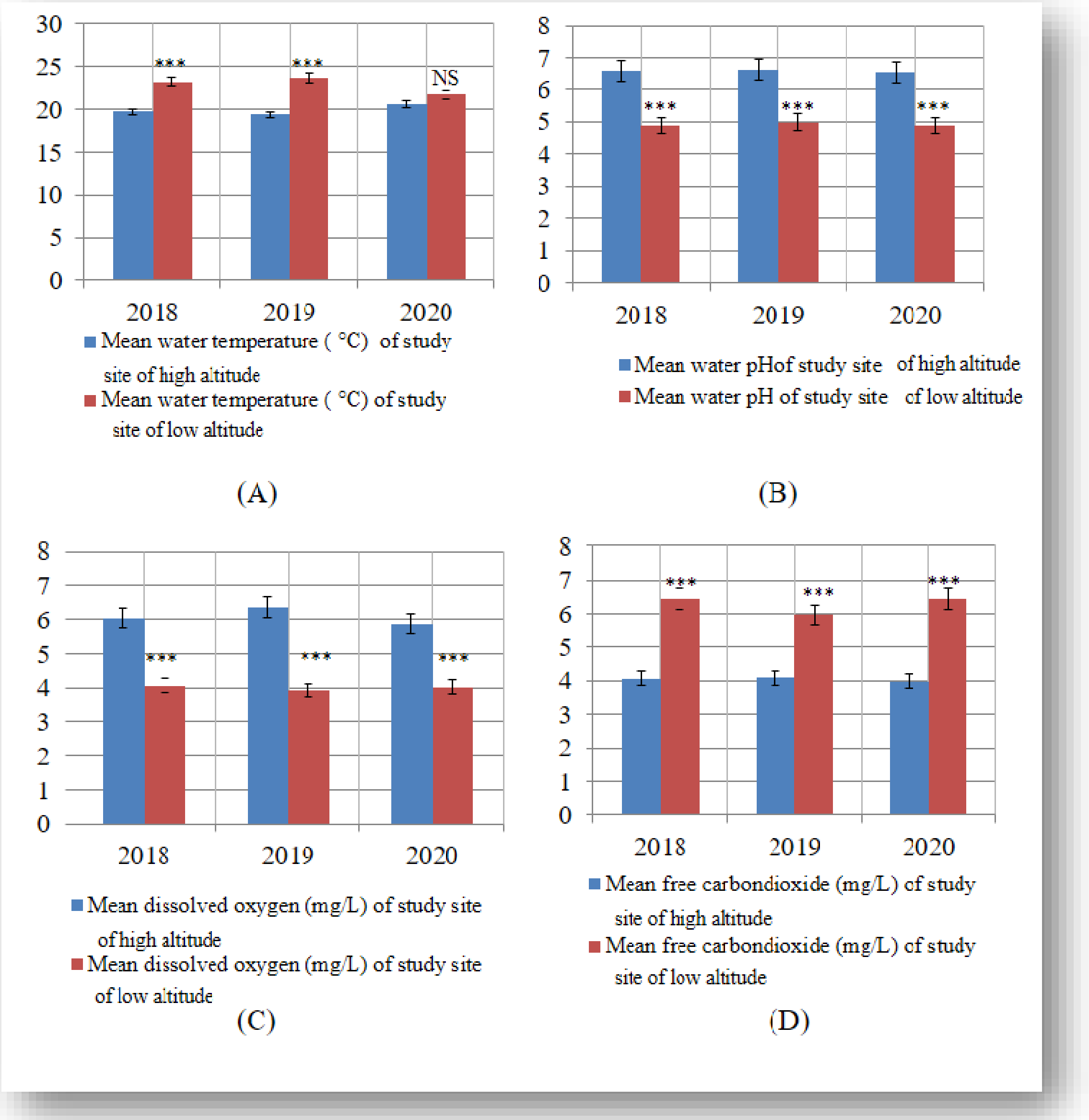

Fig. 5. (A) Mean water temperature (ºC), (B) Mean water pH, (C) Mean dissolved oxygen (mg/L), (D) Mean free carbon-dioxide (mg/L) of study site of high altitude and study site of low altitude recorded during development of *Hyla annectans*

** indicate significant differences from study site of high altitude (***p<0.001),*
NS= not significant at p>0.05
All values are given Mean ± SD (N=15)

4. DISCUSSION

The current study unveils notable distinctions between sites at varying altitudes regarding their environmental conditions and developmental processes of *Hyla annectans*. At lower altitudes, temperatures are higher, accompanied by elevated levels of free carbon dioxide and lower pH and dissolved oxygen levels, which aligns with previous findings suggesting increased CO2 concentrations in water bodies due to organic matter decomposition [8]. This observation underscores the potential role of Sphagnum moss, prevalent in the lower altitude site, in contributing to water acidification.

Geographical variance in temperature emerges as a key factor influencing intraspecific differences in developmental rates [6]. The inverse relationship between water temperature and dissolved oxygen, particularly evident at the low altitude site, follows general water chemistry patterns. The shorter metamorphic duration observed at lower altitudes may result from the combined influence of ecological variables, potentially acting in concert. Smith-Gill & Berven noted temperature's differential effects on larval growth and development, with slower differentiation at lower temperatures but increased growth at higher temperatures [9]. Additionally, the presence of acidic waters with elevated carbon dioxide levels at lower altitudes may impose stressors, prompting accelerated development as an adaptation [10].

The reduced morphometrics and accelerated development of *H. annectans* embryos at lower altitudes are conspicuous. Dissolved oxygen plays a pivotal role in further developmental processes, physiology, and behavior of anuran aquatic life stages. Despite experiencing lower oxygen levels, embryos at lower altitudes may possess adaptive mechanisms to meet oxygen demands, such as surface film deposition or aerial respiration.

The novel finding of early hatching at stage 18 in embryos at lower altitudes may be attributed to various factors. Environmental cues, such as hypoxia, can induce early hatching [11, 12], suggesting an adaptive strategy evolved by *H. annectans* to cope with environmental stressors, including water molds and reduced hydro-periods at breeding sites.

This study enriches our understanding of anuran species' developmental variations in high and low altitude habitats in Meghalaya, offering valuable insights for predicting climate change impacts. It underscores the need for further research into the effects of ecological factors on anuran development to elucidate species-specific ecological requirements.

5. CONCLUSION

In conclusion, this study provides valuable insights into the developmental parameters of *Hyla annectans* in both high and low altitude breeding habitats in Meghalaya, Northeast India. By examining the influence of ecological variables such as water temperature, pH, dissolved oxygen, and free carbon dioxide on embryonic development, the research sheds light on adaptive mechanisms and differences in developmental processes among conspecifics anuran species. Moreover, the findings emphasize the importance of considering ecological factors in understanding species-specific developmental patterns and highlight the potential impacts of climate change on amphibian populations. Hence, this research contributes to our understanding of amphibian biology and underscores the importance of preserving diverse habitats to ensure the continued survival of species like *Hyla annectans*. By providing insights into the developmental dynamics of amphibians in different ecological settings, this study informs conservation strategies and the importance of protecting pristine breeding habitats for amphibian populations.

ETHICAL APPROVAL

This study received financial support from a project sanctioned to R.N.K. Hooroo by the Science and Engineering Research Board (SERB), New Delhi, under grant number EEQ/2017/000081. The ethical guidelines provided by the North Eastern Hill University Institutional Ethics Committee for Animal Models were strictly adhered to throughout the course of this work.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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