Morphological Alterations in the External Gills of Some Tadpoles in Response to pH

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Abstract

Introduction Water pH affects the breeding, hatching, development, locomotion, mortality and habitat distributions of species in nature. The external gills of anuran tadpoles were studied by several authors in relation to abiotic factors. Exposure to low and high pH has been found to adversely affect the different tissues of various organisms. On that consideration, the present investigation was performed with tadpoles of the species *Hyla annectans* and *Euphlyctis cyanophlyctis*.

Material and Methods The maximum and the minimum pH thresholds were determined prior to the detailed experiments on the effects of pH. The pH that demonstrated 50% mortality was taken as the minimum and maximum pH thresholds. The hatchlings of both the species were then subjected to different pH (based on the minimum and maximum pH thresholds). After 48 hours of exposure, the external gills of the hatchlings were anesthetized and observed under a scanning electron microscope.

Results After 48 hours, clumping, overlapping and curling of the secondary filaments of the external gills and epithelial lesions in response to both acidic and alkaline pH were observed. The lengths of the secondary filaments were also affected by pH in both the species studied when compared with the control groups.

Conclusion Scanning electron microscopic approaches are relevant in assessing the adverse effects of pH on the morphology of the external gills of *H. annectans* and *E. cyanophlyctis* tadpoles, which included problems with osmoregulation, acid-base balance and respiratory function.

Keywords
- ► Hyla annectans
- ► Euphlyctis cyanophlyctis
- ► low pH
- ► high pH
- ► scanning electron microscopy

Introduction

Over the past few years, the understanding of the effects of acidification on amphibians has increased, as this is the main group of organisms potentially affected by the acidification of fresh water systems.[1] Amphibians mostly breed in small shallow ponds or in the stagnant littoral zone of large water bodies, in which they may encounter increasing levels of environmental stresses, like acidification.[2] Most anuran tadpoles use aquatic habitats for their growth, development and metamorphosis. During the different developmental periods, an increase in hydrogen ion concentration may bring forth several effects: decreased sperm motility,[3] increased embryonic mortality, and reduced hatchability.[4,5] Further, tadpoles exhibit an array of sublethal responses to increased hydrogen ion concentration, such as alterations in growth and development or morphological defects, especially after hatching.[6–9] Amphibian larvae are affected by acidic pH through different mechanisms, and one of the consequences of this is a change in the ionic equilibrium.[10,11] In anuran tadpoles, the gills not only perform the respiratory function but they also are the sites for

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ion exchange.[12] The respiratory gill apparatus of anuran tadpoles is distinguished by two modes of development that are determined by their position as internal and external gills.[13] All gills and their associated structures are external before the opercular fold is formed. There is a variation among the anurans in relation to the stage of the development at hatching, which indicated that the external gills are fully developed only for a short period of time after hatching.[14] Thus, it would be inappropriate to consider the external gills as having a negligible function in anurans. The anuran gill apparatus is considered a multifunctional organ,[13] and alterations in the water can bring about a change in its morphology and functions resulting from altered physiological and metabolic processes such as respiration, osmoregulation and acid-base balance.[15]

Hyla annectans has a tendency to breed in pristine habitats away from stressful environments, such as human settlements, construction sites etc. *Euphlyctis cyanophlyctis*, on the other hand, breeds in quiet or stressful environments, and thrives well in these habitats. During the breeding season, the larvae of both species were found to inhabit waters with pH that ranges from low to slightly high. In general, the pH of water has been reported to be high in the winter months and low in the monsoon season and summer in India. During summer, low pH is caused by high turbidity resulting from heavy rain, algal blooms, human activity etc.[16,17] The increased temperature promotes microbial activities that cause a rise in the production of CO_2.[16,17] During winter, high pH could be attributed to increased primary productivity. The high growth of algal population due to increased CO_2 leads to an increase in the pH levels.[18] Anuran external gills were chosen for this investigation because they were in direct contact with the aquatic environment. Further, after hatching, the external gills only last for a short time (72 hours) before they start to regress. Hence, the external gills play a major role as an indicator of water quality. Anuran gills that were exposed to varying pH conditions are likely to exhibit morphological changes that, in turn, could affect the physiology of the animal. In the present study, the effects of low and high pH on the morphological features of the external gills of two anuran species belonging to two different families, a tree frog, *H. annectans*, and a skipper frog, *E. cyanophlyctis*, which breed during the early part of the monsoon season, were analyzed.

Materials and Methods

Study Materials

Hyla annectans

Hyla annectans, a species of the genus *Hyla* described by Laurenti in 1768, is the only species of this genus found in India.[19] It has been recorded in Meghalaya, Nagaland and upper Burma.[20] It breeds from March to June, and is categorized as a lower-risk near threatened species.[21] This species inhabits temporary ponds, rain pools, puddles and terraced paddy fields at the edge of the forest, where water logging is observed. The tadpoles of this species (►**Fig. 1**) are found till the month of July.

Fig. 1 **(A)** Lateral view of *Hyla annectans* tadpole at stage 39. **(B)** Lateral view of *Euphlyctis cyanophlyctis* tadpole at stage 39.

Euphlyctis cyanophlyctis

Euphlyctis cyanophlyctis belongs to the family Dicroglossidae (Anura, Amphibia). It is distributed all over India, Pakistan, Bangladesh, Sri Lanka, Nepal and Thailand.[20] It breeds from March to August. The tadpole has an olive to gray brown body, with loosely scattered black pigmentation. It can tolerate a wide range of oxygen, temperature and pH variations, from fresh water to considerably brackish and polluted refuse water.

Collection of Eggs

The eggs of *H. annectans* used in the study were collected from a temporary pool in Sohra (Cherrapunjee) Meghalaya, India (altitude of 4,869 ft above sea level [ASL]). The eggs of *E. cyanophlyctis* were collected from a paddy field in the Ri Bhoi district, Meghalaya, (altitude of 2,916 ft ASL). The eggs were collected with the help of a close mesh and were transported to the laboratory, where they were kept in plastic trays filled with pond water under room temperature.

Determination of Maximum and Minimum pH Thresholds

The eggs of both species were exposed to different levels of low pH (3.0; 3.5; 4.0; 4.5; 5.0 5.5; and 6.0) and high pH (7.5; 8.0; 8.5; 9.0; 9.5; 10.0; and 10.5) to test the maximum and minimum threshold pH they could withstand. The control groups of tadpoles were maintained in water collected from their natural habitat with a pH of 6.5 for both species. The pH at which 50% mortality occurred was taken as the minimum and maximum pH thresholds for the tadpoles. The experiments were performed at a pH level higher than the minimum threshold and lower than the maximum threshold. The pH level was measured with an APX 175 digital pH meter (CD

Hightech Pvt. Ltd., Malleswaram, Bangalore, India) with automatic temperature compensation. The developmental stage was determined through appropriate developmental chronological tables.[22]

Experiment on Hatchlings

The embryos of both species were reared up to stage 19, a stage in which the gill buds are formed. The experimental groups for low pH were maintained in water in which the pH was adjusted by adding dilute HCl. For the high pH group, water was made alkaline with the addition of dilute NaOH.[23] The pH was adjusted to 4.5 and 8.5 for *H. annectans*, and to 3.5 and 9.5 for *E. cyanophlyctis* respectively (based on the minimum and maximum pH thresholds). The tadpoles were maintained for ~ 48 hours, till they attained stage 22 to 23[22] in the laboratory. After 48 hours of exposure to low and high pH, the hatchlings were anaesthetized with tricaine methanesulfonate (MS-222; Sandoz, Sigma-Aldrich, St. Louis, MO),[24] and were processed for optical and scanning electron microscopy (SEM).

Optical Microscopy

For light microscopy, the external gills were fixed in a 1:1 solution of formaldehyde and ethanol. The observations were made with a Leica ES2 microscope (Leica Microsystems, Wetzlar, Germany), and were photographed using a Nikon Coolpix S3200 16.0 Mp 6X wide optical zoom camera (Nikon, Minato, Tokyo, Japan).

Scanning Electron Microscopy

For the SEM, the specimens were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 4 hours at 4°C. Following the primary fixation, the tissues were washed for 15 to 30 minutes in sodium cacodylate buffer. The postfixation was performed with 1% osmium tetroxide prepared in 0.1 M sodium cacodylate buffer (pH 7.2), and the tissues were dehydrated in graded acetone with 2 changes of 15 to 30 minutes in each grade. The dehydrated samples were dried using the tetramethylsilane (TMS) drying technique of Dey et al.[25] The samples were secured horizontally to brass stubs (10 mm in diameter by 30 mm in height) with double adhesive tape. A conductive coating of gold was applied to the samples using the JFC 1100 (JEOL Ltd., Akishima, Tokyo, Japan) ion sputter. A relatively low vacuum was maintained in the sputtering chamber for the ionization of air particles, which was followed by the application of high voltage. The ionized air particles, while moving randomly, hit the target metal (gold), resulting in the release of fine gold particles that coat the samples. Gold coating prevents sample damage due to radiation, and also increases the conductivity. The observations were made with a JSM 6360 (JEOL Ltd., Akishima, Tokyo, Japan) scanning electron microscope in the secondary electron emission mode at an accelerating voltage of 20 KV, and at a working distance of 12 mm.

Statistical Analysis

All data are presented as means ± standard error (SE). The relationships within each treatment were assessed by one-way analysis of variance (ANOVA), followed by Tukey multiple comparison tests. A significance level of $p \leq 0.05$ was used.

Results

The Minimum and Maximum pH Thresholds

The minimum pH threshold for the *H. annectans* tadpoles was 4.0, with a mortality rate of 80%, and the maximum pH threshold was 9.0, with a mortality rate of 60%. The minimum pH threshold level for the *E. cyanophlyctis* tadpoles was 3.0, with a mortality rate of 70%, and the maximum pH threshold was 10.0, with a mortality rate of 70%. No mortality occurred in the control groups of each species, which were maintained at a pH of 6.5 during the experimental period.

Morphology of the Gills

Light Microscopy

Three days after fertilization, the embryos of both the species attained stage 20,[22] which is defined by gill circulation and tail elongation. The development of the external gills in both species reached its peak at stage 23 (Gosner, 1960),[22] and the gills at this stage were chosen for the morphological study, as they begin to regress 3 days after that. The observations made with light microscopy on the external gills of both species revealed a pair of well-developed branched gill filaments. They are present laterally on each side of the tadpole, posterior to the head (►**Fig. 2A**, **B**).

Scanning Electron Microscopy

Control group (pH 6.5)

***Hyla annectans* tadpoles**

Scanning electron microscopy of the external gills in the control group (6.5) of *H. annectans* tadpoles revealed

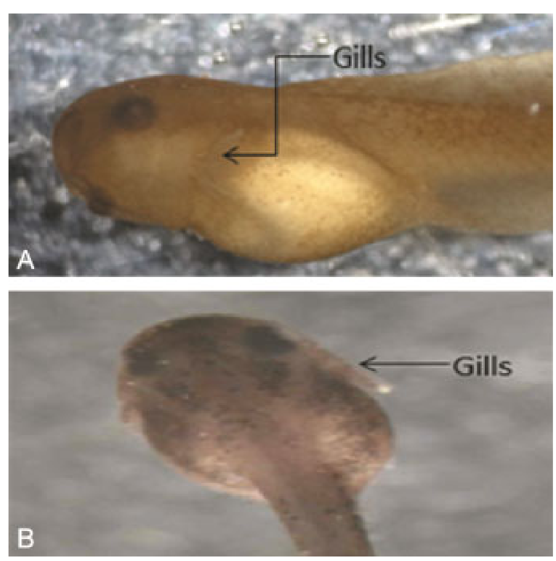


Fig. 2 **(A)** Lateral view of the external gills of *Hyla annectans* larvae at stage 23 **(B)** Dorsal view of the external gills of *Euphlyctis cyanophlyctis* larvae at stage 23.

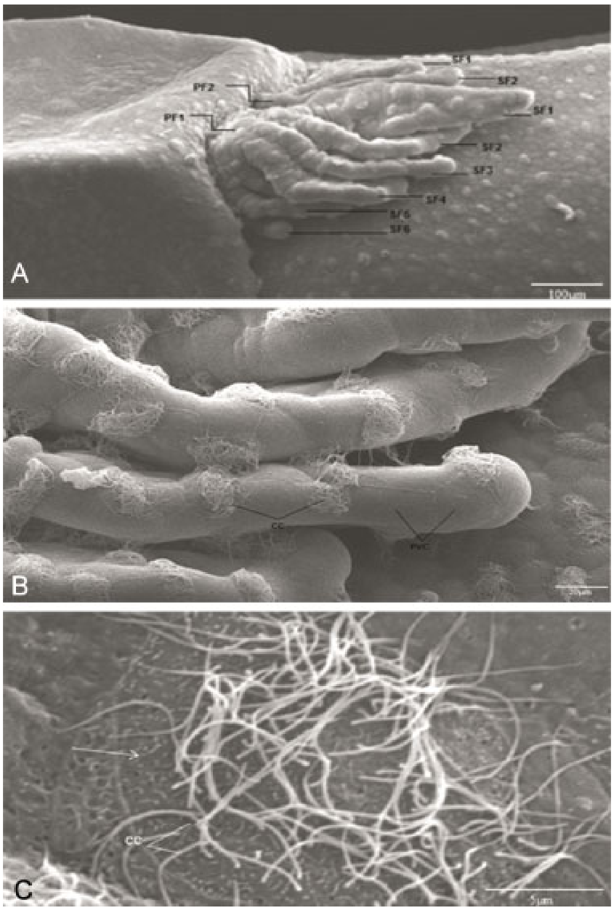


Fig. 3 (A) Scanning electron micrographs showing the morphology of the external gills of *Hyla annectans* tadpoles, under control conditions, comprising the primary filaments (PF1 and PF2) and the secondary filaments (SF1 to SF6). **(B)** Scanning electron micrographs showing the pavement cells (PVCs) and the ciliated cells (CCs) in *H. annectans* tadpoles. **(C)** Scanning electron micrographs showing the CCs and the microridges (arrow) in *H. annectans* tadpoles.

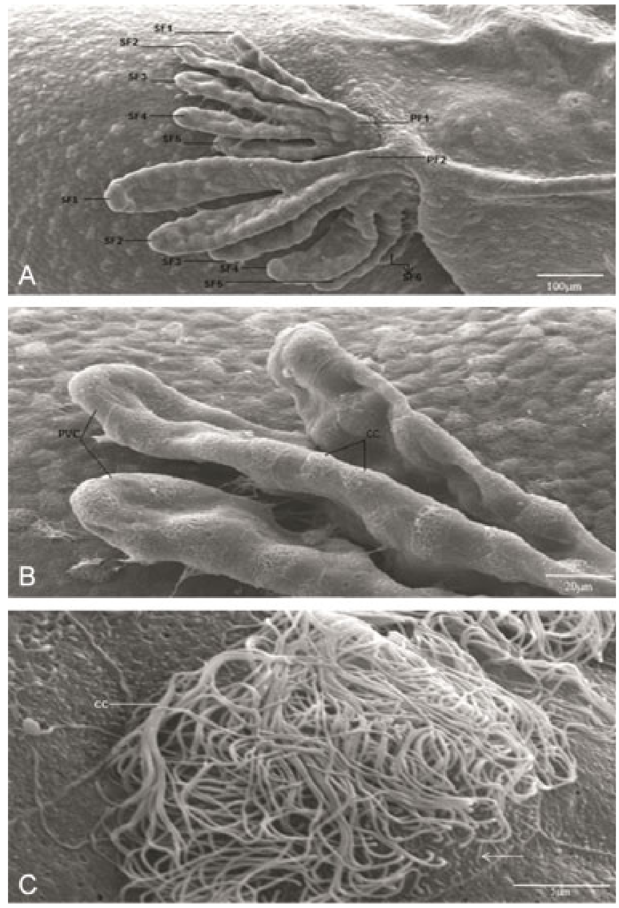


Fig. 4 (A) Scanning electron micrographs showing the morphology of the external gills of *Euphlyctis cyanophlyctis* tadpoles, under controlled conditions, comprising the primary filaments (PF1 and PF2) and the secondary filaments (SF1 to SF6). **(B)** Scanning electron micrographs showing the ciliated cells (CCs) and the pavement cells (PVCs) in *E. cyanophlyctis* tadpoles. **(C)** Scanning electron micrographs showing the CCs and the microridges (arrow head).

that it consists of 2 main primary filaments, and each primary filament is made of 2 to 6 secondary filaments (►**Fig. 3A**). The epithelium of the gill filaments observed with SEM is composed of two types of cells; the polygonal cells, known as pavement cells, which represent the most frequent cellular types with well-developed boundaries, and the ciliated cells, which are less common when compared with the pavement cells (►**Fig. 3B**). They bear long tufts of cilia, which often extend above the pavement cells. Pavement cells and ciliated cells are characterized by the presence of short superficial microridges (►**Fig. 3C**).

***Euphlyctis cyanophlyctis* tadpoles**

The external gills of *E. cyanophlyctis*, on the other hand, are composed of two primary filaments with 5 to 6 well-developed secondary filaments arising from each primary filament (►**Fig. 4A**). The morphological features of the external gills of these tadpoles were observed to be similar to those of the *H. annectans* tadpoles. The epithelium is composed of cells known as the pavement cells, and ciliated cells. Microridges were also observed (►**Fig. 4B**, **C**).

Experimental groups

The gill apparatus of the tadpoles of both species that were reared in water with low and high pH did not reveal any morphological changes when compared with the control groups after 24 hours of exposure. However, microstructural modifications occurred in the external gills only after 48 hours of exposure.

Low pH group (4.5)

***Hyla annectans* tadpoles**

Scanning electron microscopy revealed a reduction in the length of the secondary filaments and clumping of the adjacent filaments at certain places (►**Fig. 5A**, **9**, ►**Table 1**). The pavement cells exhibited a distinctive swelling appearance (►**Fig. 5B**). The breakage of cilia at certain places and the occurrence of epithelial lesions were also observed (►**Fig. 5C**).

Low pH group (3.5)

***Euphlyctis cyanophlyctis* tadpoles**

After exposure to low pH, it was revealed that the morphology and orientation of the gill filaments was disturbed. An overlapping of the secondary filaments was evident when

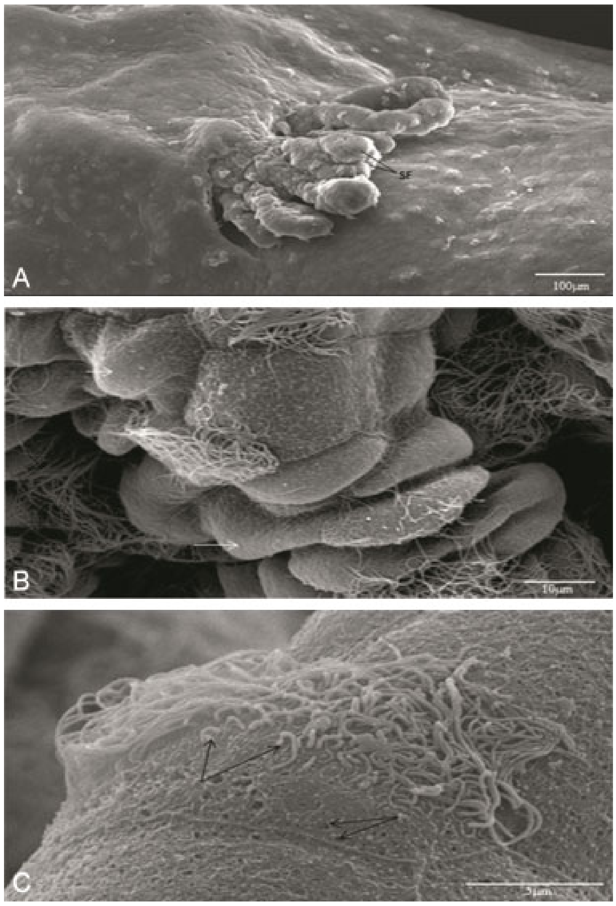


Fig. 5 (A) Scanning electron micrographs of *Hyla annectans* larvae after 48 hours of exposure to low pH showing the reduction in the length and clumping of adjacent secondary filaments (SFs). **(B)** Scanning electron micrographs showing the swelling of the pavement cells (arrow) under low pH. **(C)** Scanning electron micrographs showing breakage of the cilia and epithelial lesions under low pH (arrow).

compared with the control group (►**Fig. 6A**). The gills revealed swellings in the pavement cells and breakage of the cilia at certain places (►**Fig. 6B**). Epithelial lesions were also evident (►**Fig. 6C**). There was no significant increase or decrease in the length of the secondary filaments in the tadpoles maintained in water with a low pH ($p > 0.05$) when compared with the control group. (►**Table 1**; ►**Fig. 9B**).

High pH group (8.5)

***Hyla annectans* tadpoles**

At a higher pH (8.5), morphological changes were also observed in the gill tufts of *H. annectans* tadpoles, and some of the secondary filaments exhibited a curling and enfolding effects (►**Fig. 7A**). The pavement cells were swollen in certain portions along the secondary filament. On the other hand, the ciliated cells maintained their original structural features. However, swellings of the pavement cells were observed (►**Fig. 7B**). Epithelial lesions were also observed at certain places (►**Fig. 7C**). There was no significant increase or decrease in the length of

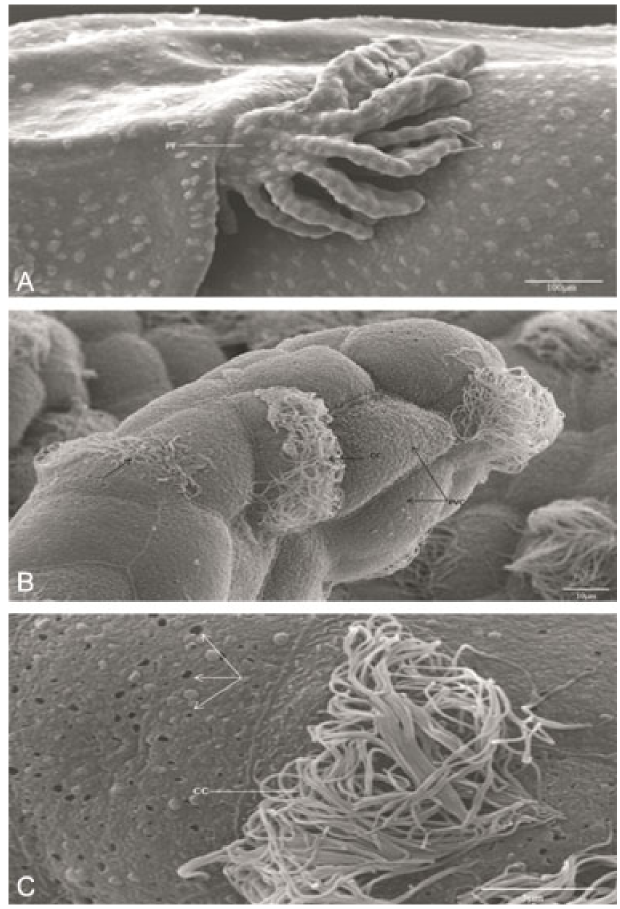


Fig. 6 (A) Scanning electron micrographs of the external gills of *Euphlyctis cyanophlyctis* larvae after 48 hours of exposure to low pH showing the primary filament (PF) and the secondary filaments (SFs) along with the overlapping filaments (arrow). **(B)** Scanning electron micrograph showing the morphology of the swellings of the ciliated cells (CCs) on the pavement cells (PVCs). Breakage of the CCs was also observed at certain places (arrow). **(C)** Scanning electron micrographs showing the morphology of the CCs and the epithelial lesions (arrow).

Table 1 Mean length (in μm) of the secondary filaments of *Hyla annectans* and *Euphlyctis cyanophlyctis* tadpoles under the effects of low and high pH ± standard error (SE)

Observations	N	*Hyla annectans*	Observations	N	*Euphlyctis cyanophlyctis*
Control (6.5)	5	237.68 ± 20.38	**Control (6.5)**	5	199.22 ± 17.21
Low pH (4.5)	5	140.76 ± 14.76	**Low pH (3.5)**	5	179.99 ± 14.39
High pH (8.5)	5	240.75 ± 25.74	**High pH (9.5)**	5	263.83 ± 17.01

Abbreviation: N, number of secondary filaments.

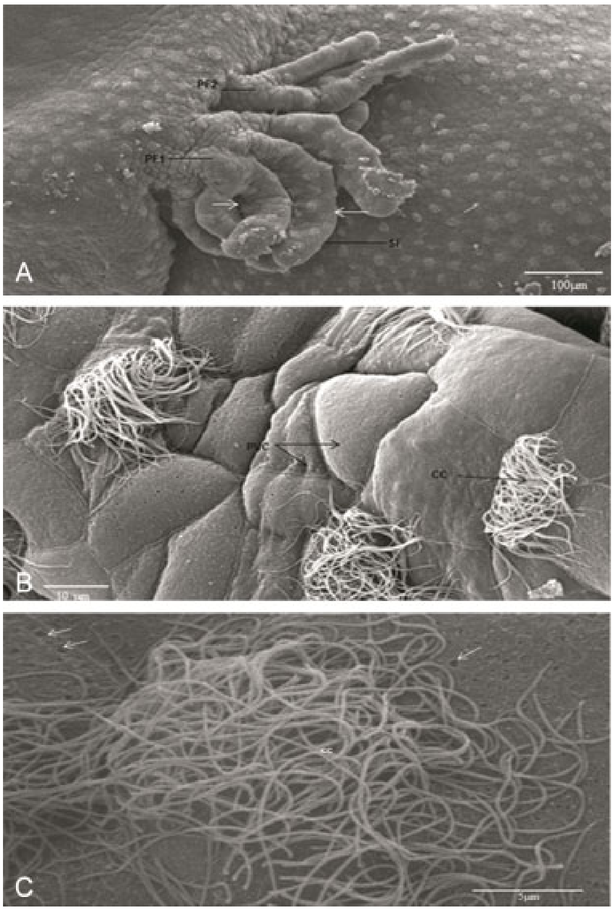


Fig. 7 (A) Scanning electron micrographs of the external gills of *Hyla annectans* tadpoles showing the curling and enfoldings (arrow) of the secondary filaments (SFs) after 48 hours of exposure to high pH. **(B)** Scanning electron micrographs showing swellings of the pavement cells (PVCs) on portions of the SFs along with the morphology of the ciliated cells (CCs). **(C)** Scanning electron micrographs showing the CCs and the epithelial lesions (arrow).

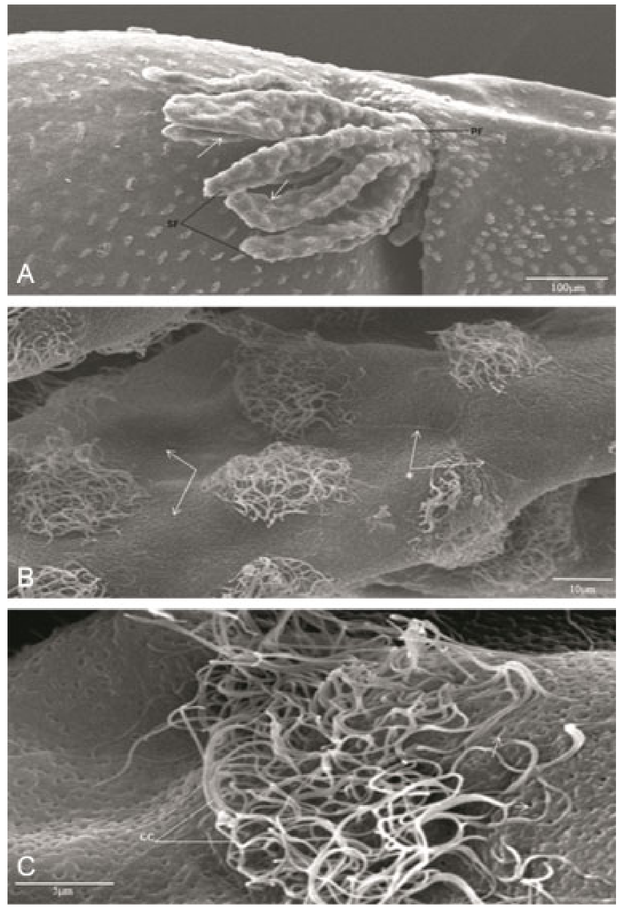


Fig. 8 (A) Scanning electron micrographs of the external gills of *Euphlyctis cyanophlyctis* tadpoles showing the overlapping and bending of the secondary filaments (SFs) observed after 48 hours of exposure to high pH (arrow). **(B)** Scanning electron micrographs showing the boundaries of adjacent pavement cells (PVCs) to be affected under high pH (arrow head) along with the boundaries of adjacent PVCs that are not affected (*). **(C)** Scanning electron micrographs showing the morphology of the ciliated cells (CCs) and the epithelial lesions (arrow).

the secondary filaments of the tadpoles maintained in water with a high pH ($p > 0.05$) compared with the control group (►**Table 1**; ►**Fig. 9A**).

High pH group (9.5)

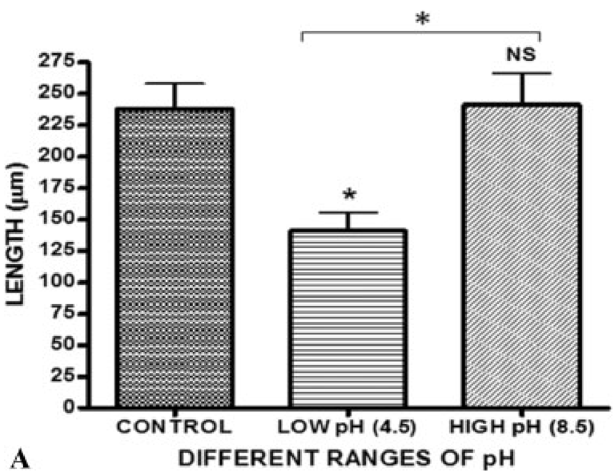
***Euphlyctis cyanophlyctis* tadpoles**

At a higher pH (9.5), the secondary filaments of *E. cyanophlyctis* tadpoles were observed to be overlapped, and tips of the secondary filaments were bent toward one another at certain portions (►**Fig. 8A**). The boundaries of the adjacent pavement cells were also affected to the point that their demarcation could not be distinguished (►**Fig. 8B**). The morphology of the ciliated cells remained unaffected. However, epithelial lesions throughout the surface of the secondary filament were evident (►**Fig. 8C**). There was a significant increase ($p < 0.05$) in the length of the secondary filaments when compared with the control group (►**Table 1**, ►**Fig. 9B**).

The similarities and differences in gross morphology and micro structural features of various components of the external gill apparatus of the two species of tadpoles are shown in ►**Table 2**.

Discussion

One of the most important ecological variables guiding the biology of amphibians is water pH.[2] It affects the breeding, hatching, development, locomotion, mortality and habitat distributions of species.[2,26–30] Moreover, the variations in acid tolerance of different amphibian species are associated with environmental pH, which indicates that adaptation to acidity does occur among them.[26,31] Several studies reported that amphibian embryos are less tolerant than larvae to acidity in an aquatic medium. Greater tolerance to acidic pH has been reported in amphibians as development progresses.[4,9] Fertilization, embryonic development, hatching, larval development and metamorphosis may be affected by an acidic pH. Egg development ceases within hours after exposure to a very low pH.[3,4,32] Although larvae do hatch successfully under low pH, the number of developmental abnormalities was found to be high.[4,5,28,32] Similar types of abnormalities in response to alkaline and acidic water pH



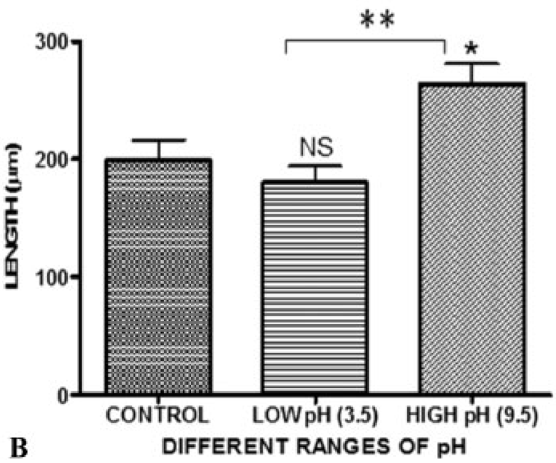


Fig. 9 **(A)** Length of the secondary filaments (in μm) in tadpoles of *Hyla annectans* under the effect of different pH. The error bar represents the standard error (SE). All of the values are expressed as mean $\pm$ standard error of the mean (SEM); $N = 5$. The asterisks represent different levels of significance as compared with the values of control: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; NS, not significant at $p > 0.05$ respectively. **(B)** Length of the secondary filaments (in μm) in tadpoles of *Euphlyctis cyanophlyctis* under the effect of different pH. The error bar represents the SE. All of the values are expressed as mean $\pm$ SEM; $N = 5$. The asterisks represent different levels of significance as compared with the values of control: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; NS, not significant at $p > 0.05$ respectively.

have been reported in other aquatic animals such as fish. Certain studies have demonstrated that trout that are actively exposed to alkaline pH develop metabolic alkaloses.[33,34] In rainbow trout (*Salmo gairdneri*), waters having high pH promote CO_2 excretion, while low pH inhibits it.[35] In addition, it has been suggested that high pH inhibits the Na^+/NH_4^+ exchange mechanism.[36]

The importance of the gill apparatus in terms of respiration has been discussed by some scholars.[37] However, there are a few studies on the external gills of tadpoles with regard to the effects of low pH.[24,38] In fact, no recent reports are available in the existing literature on the effects of low and high pH on the external gills of anuran tadpoles, except our recent findings.[39] With regards to pH, studies about the effects of low and high pH on the oral morphology of *H. annectans*,[40] *Polypedates teraiensis* and *Hylarana leptoglossa* tadpoles[41] have been conducted in our laboratory. It is worthy of mention in this context that the adverse effects of pH on various tissues of some aquatic organisms such as fish are amply highlighted in the existing literature. Fish exposed to waters with low pH (< 5.2) showed certain morphological alterations that include lifting, sloughing and necrosis of the branchial epithelium; increased mucous secretion; reduced length of the tight junctions between the cells of the branchial epithelium; and distortion of primary and secondary lamellae.[42–48] Studies on salamander embryos revealed that gastrulation and gill development were truncated in water with low pH.[32] The latest finding suggested that amphibian gills play a crucial role in oxygen uptake and osmoregulation.[37] However, it has been suggested that gill damage in amphibian larvae may not be the sole cause of death among the larvae, as oxygen uptake is not merely restricted to the gills; it is also absorbed through the skin.[49]

It should be noted that the comparative morphology of the external gills of some species of anurans has received less attention possibly due to the fact that they do not last long.[50] Furthermore, it has been suggested that the external gill morphology in anurans exhibits significant diversity at the anatomical and ultra-structural levels.[50] The present findings on the external gill surfaces in *H. annectans* and *E. cyanophlyctis* support the earlier reports.[50] The morphological observations made in the current study on the pavement cells, microridges and ciliated cells were similar to those made by other authors on the external gills of *Rana dalmatina* tadpoles.[13] There have been many reports on the functional role of the microridged epithelial cells of the primary epithelium.[51–54] In addition to their role in attaching the mucus produced by the epithelial cells to the gill surface, the microridges of the primary epithelium may stimulate the microturbulence of water.[55] Various functions have been assigned to the ciliated cells on the surface of amphibian embryos, such as: movement of mucus films; inhibition of micro- organisms and debris attachment; increase in respiratory gas exchange on the surface fluid films by decreasing “dead-space”; enhancement of mobility during prehatching and posthatching gliding; and determination of the quality of water flowing over the external gills.[56–59] The breakage of cilia on the surface of the gills observed in the current study in response to both low and high pH suggests that pH alterations may affect the functions of the gills of the two anuran species studied.

The swellings in the secondary filaments observed in the present study could be due to the entry of solute and water into the matrix. In brook trout (*Salvelinus fontinalis*) exposed to pH 4.5 and 5.0 for 456 hours, similar results were found: swelling of primary and secondary lamellae; fusion of secondary lamellae; increased mucus production; and thickening and shortening of the secondary lamellae.[60] Reports of the deleterious consequences of pH have been meticulously documented in the literature, and gills exposed to toxicants produced similar effects, such as separation and lifting up of the epithelium, which may be considered a defense response.[61] The swellings in the secondary filament

Table 2 A comparison of the external gills of tadpoles exposed to low and high pH of *Hyla annectans* and *Euphlyctis cyanophlyctis* with the controls

Observations		**Controls**		**Low pH**		**High pH**	
		Tadpoles of *Hyla annectans*	**Tadpoles of *Euphlyctis cyanophlyctis***	**Tadpoles of *H. annectans***	**Tadpoles of *E. cyanophlyctis***	**Tadpoles of *H. annectans***	**Tadpoles of *E. cyanophlyctis***
Secondary gill filaments	**Shape**	Cylindrical, club-shaped	Cylindrical, club-shaped	Distortion in shape	Cylindrical, club-shaped	Cylindrical, club-shaped	Cylindrical, club-shaped
	Length	–	–	Significant decrease	No significant increase	No significant increase	Significant increase
	Clumping	No clumping	No clumping	Clumping observed	No clumping	No clumping	No clumping
	Overlapping	No overlapping	No overlapping	No overlapping	Overlapping observed	No overlapping	Overlapping and bent
	Curling	No curling effect	No curling effect	No curling effect	No curling effect	Curling effect	No curling effect
Pavement Cells	**Shape**	Polygonal in shape	Polygonal in shape	Polygonal in shape	Polygonal in shape	Polygonal in shape	Polygonal in shape
	Swelling	No swelling	No swelling	Swelling observed	Swelling observed	Swelling observed	No swelling
	Boundaries of pavement cells	Clear boundaries	Clear boundaries	Not affected	Not affected	Not affected	Affected
Cilia	**Morphology**	Interwoven thread-like structures	Interwoven thread-like structures	Interwoven thread-like structures	Interwoven thread-like structures	Interwoven thread-like structures	Interwoven thread-like structures
	Breakage	No breakage	No breakage	Breakage observed	Breakage observed	No breakage observed	No breakage observed
Epithelium	**Gill epithelium**	More or less smooth	More or less smooth	Epithelial lesions present	Epithelial lesions present	Epithelial lesions present	Epithelial lesions present

of the gills observed in the present study could be attributed to a decrease in the Na^+- and K^+- activated ATPase and/or a decline in blood Na^+ and Cl^- concentrations.[62] The depletion of electrolytes, in particular Na^+ and Cl^-, could bring about lethal iono-/osmoregulatory disruption.[63] Regarding the occurrence of epithelial lesions in response to low and high pH, we may conclude that it reduced the functional surface of the gill for gaseous exchange, impairing respiratory function.[64]

It should be noted that acidic environments clearly had an effect on natural amphibian populations.[9] The aforementioned studies revealed that hydrogen ions interfered with sodium flux in amphibian larvae. Sodium uptake was depressed under low pH, and this leads to larval mortality when sodium levels are reduced to 50%. Interspecific variations in larval acid tolerance are related to differences in sodium flux and body sodium content. Alterations in the water pH also had adverse effects on the morphology of the gill apparatus of the anuran larvae of *Litoria fallax*.[38] These included the lifting of the branchial epithelium, necrosis of the integument, and tight junction damage at both the gills and body surface. Similarly, in the present study, changes in water pH had an effect on the gills and surface epithelium of *H. annectans* and *E. cyanophlyctis* larvae. These alterations in the morphology of the gills result in altered respiration and iono-/osmoregulation.[46,65] A high sensitivity to acidic pH resulted in lower survival time and loss of body ions, as observed in fish and amphibians.[66] The morphological alterations observed in the external gills of *H. annectans* and *E. cyanophlyctis* in the present study can thus interfere with the respiratory and osmoregulation processes.

The present study provided an understanding of the detrimental effects of low and high pH among amphibians. Changes in the morphology of the gills could result in respiratory complications, which, in turn, may affect the development and survival of individuals.

Conclusion

The present study involving SEM revealed several changes in the external gills of *H. annectans* and *E. cyanophlyctis* larvae exposed to low and high pH. Abnormalities in the arrangement and shape of the gills filaments, and changes in the external gill surface, which included swelling of the gills, epithelial lesions, clumping, breakage of the cilia, and effects on the pavement cell boundaries, were determined. Scanning electron microscopic approaches are relevant in assessing the effects of pH on the external gills of *H. annectans* and *E. cyanophlyctis* tadpoles.

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