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Research Paper

Digital malformation and chromosomal abnormality in *Rana tigirina* – the warning signal of declining amphibian population

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Abstract

Amphibians are in most cases, diverse and sensitive to environmental variability. They are considered reliable bioindicators of environmental pollution. In recent years, much attention has been given towards the detection of genotoxic agents in the aquatic environment. The presence of such agents is suggested to have adverse effects on animal as well as human health via the food chain. Previous studies on reproductive toxicology in amphibians showed that different types of pesticides adversely affect larval development and induce complete feminization. The present study demonstrates the phenomenon of hermaphroditism and digital malformation in adult amphibians. The morphology of reproductive system of abnormal male Rana tigirina was studied which comprised of testis with well developed long oviduct. Histological structures of kidney, testis and ovary were investigated by examining the sections of the organs in both sexes. The studies indicated that particularly the outer part of the section of kidney in male abnormal individual revealed the ovarian structure which is an indication of hermaphroditism. Somatic chromosomes of both male and female specimens were prepared from intestinal epithelium by mitotic division inhibition technique. Conventional Giemsa stained metaphase chromosomes of both male and female Rana tigirina revealed the existence of 2n = 26 biarmed chromosomes. An examination of well spread metaphase stages in both sexes revealed a variety of chromosomal abnormalities. Comparative analysis of chromosomal abnormalities in both normal and morphologically abnormal (with digital malformation) individuals revealed that the frequencies of chromosomal abnormalities were maximum in morphologically abnormal individuals. Germ cell cytotoxicity was also assessed by studying the different types of sperm shape abnormalities in the morphologically abnormal individuals. So this study emphasizes that excessive genome damage caused by genotoxic agents in aquatic environment may be an important factor for such reproductive consequences as well as digital malformation.

Keywords: Bioindicators, Karyotype, Hermaphroditism, Metaphases, Mitotic division inhibition technique, Reproductive organs.

Introduction

Amphibians have been the subject of various cytogenetic investigations [1-5] because of their transitional phylogenetic position in the animal kingdom, co-existence of the primitive and bimodal karyotype along with a symmetrical and supposed to be advanced karyotype, low diploid numbers with enormous in length and ambiguous nature of sex chromosome. The introduction of amphibian as a novel model in

mutagenicity or carcinogenicity bioassay has made it further important for detailed knowledge of cytogenetical aspects of amphibia [6,7].

Over two decades ago, the widespread decline of amphibian populations raised an alarm amongst herpetologists [8 - 10]. The declines are widespread and have been particularly serious since late 1990s [11]. The Indian subcontinent is known to be very rich in Amphibian fauna yet contribution toward amphibian cytogenetics is rather poor. Chakrabarti and Banerjee have monitored some Amphibian populations at different ponds, fields, moist bushes and canals in some districts of West Bengal, India for more than thirty years (since 1982) for cytogenetic study [5,12-18]. In the early 1980s, numerous different types of toad and frog were found but now a day, they were not so abundant.

Daniels et al have studied different types of amphibians in Western Ghats [11,19] and Eastern Ghats [20,21] of India. It is seemed that different factors such as loss of habitat, UV-B radiation and chemical contaminants are primarily responsible for population decline [9,22,23]. Fungal infections are common in freshwater fish in both the wild and in Aquarians. Lips [24] reported a chytridiomycete fungal infection is found in the skin of amphibians may be one of the important factors for population decline. Studies have also shown that insecticides such as DDT, dieldrin and malathion affect the immune systems [25] and the herbicide atrazine causes sex reversal in amphibians [26, 27].

Recently, in the course of the investigations on the Nucleolar organizer region (NOR) polymorphism in *Bufo melanostictus* from different parts of West Bengal we have found few common Indian frogs (*Rana tigirina*) with digital abnormalities. Only three individuals (two males with digital abnormality and one normal female) were collected and kept in laboratory for chromosome preparation. An interesting feature has been noticed in the male abnormal ranid, *Rana tigirina* (No – 1 and 2) after dissection. After a critical examination of the internal organs of the abnormal male, it has been noticed that both testis and oviduct were present indicating sexual dimorphism in ranid anuran populations. It is the purpose of the present study to know whether the karyological and histological abnormalities along with digital malformation play any impact with sexual dimorphism of ranid anurans. So the present paper has been oriented to analyse the chromosomal constitution and histological structures of few organs of the abnormal male and female ranids *Rana tigirina*.

Materials and Methods

Specimens: Five individuals (Rana *tigirina*) were collected from Kestopur water bodies, North 24 Parganas, West Bengal, India in the month of August during rainy season. Out of five, three individuals (two females and one male) were morphologically normal and two individuals (males) were morphologically abnormal with digital malformation in the forelimb (Figure 1, Table 1). These specimens were almost dead. Three individuals (two abnormal males [No 1 and 2] with digital malformation and the other normal female [No - 3] which were almost dead) were selected and kept in laboratory for further investigations. Other two living normal specimens [No 4 and 5] were freed in the water bodies.

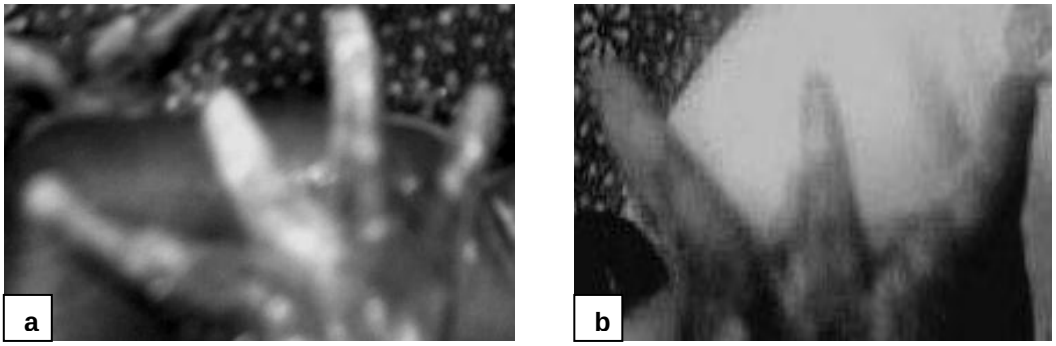

Figure 1: Adult common Indian frog (*Rana tigirina*) a) Normal (four digits in forelimb) b) Morphologically abnormal with digital malformation in the forelimb (Three digits instead of four)

Table 1: Number of specimens (Male and Female Rana *tigirina*) used during investigation

S. No.	Weight (gms)	Sex	Alive (A) or almost dead (AD)	Experimental Parameters				Remark
				Observation of digit externally	Histology	Chromosomal abnormality	Sperm structural abnormality	
1.	300	M	AD	Digital abnormality (3 instead of 4)	Kidney, Testis	Simple & Complex chromosomal aberrations	small, ring shaped, distorted etc.	Used for experiment
2.	280	M	AD	Digital abnormality (3 instead of 4)	Kidney, Testis	Simple & Complex chromosomal aberrations	small, ring shaped, distorted	Used for experiment
3.	350	F	AD	Normal	Kidney, Ovary	Simple & Complex chromosomal aberrations	-	Used for experiment
4.	280	M	A	Normal	-	-	-	Freed in water body
5.	300	F	A	Normal	-	-	-	Freed in water body

Gross anatomy analysis: For the anatomical analysis, the specimens were dissected out. Then the internal organs of both male and female were observed and photographed with Canon digital camera. The gross morphology of the reproductive systems of the males is comprised of paired elongated, cream coloured testis with well developed long oviduct which is the indication of hermaphroditism. The reproductive system of female *Rana tigirina* (No – 3) revealed normal features.

Histology: For the histological analysis, the testis, kidney of specimens no - 1 & 2 and ovary, kidney of specimen no – 3 were fixed in Bouin's fluid for 24 h. After that, the tissues were washed, dehydrated in a graded series of ethanol solutions (70%, 80%, 90%, and 100%) and finally embedded in paraffin. Paraffin sections were cut into 6 µm slides and stained with Haematoxylin and Eosin for light microscopic observation. The sections were observed and photographed by using MIPS (micro-imaging projection system) with Olympus microscope.

Chromosome preparation: Somatic chromosomes from three specimens (No. 1, 2 and 3) were prepared from intestinal epithelium by mitotic division inhibition technique as standardized by Chakrabarti et al [12]. Technique in brief: Intestinal tissue was treated with 0.3 % Colchicine (Sigma, St Louis, U.S.A) for 2 hours in normal saline. Then the tissue was kept in distilled water for 30 min. at room temperature and fixed in fresh methanol: acetic acid fixative (3:1 v/v) for five minutes. The fixed tissue was homogenized in fresh Aceto-alcohol fixative and followed by centrifugation at 1500 rpm for 10 min. The pellet was fixed in fresh methanol: acetic acid fixative (3:1 v/v). The whole process was repeated thrice. Three drops of cell suspension were dropped on clean grease-free slide (soaked previously in chilled 50% ethanol) and allowed to dry in air. For conventional staining, slides were stained in 5% phosphate buffered Giemsa stain (pH 6.8) for 30 minutes and washed in running tap. Then dried slides were observed under the 100X oil immersion lens of an Olympus Binocular Research Microscope using 10X wide field eye pieces.

Criteria for selecting metaphases for scoring of data: Only those metaphases displaying well differentiated darkly stained sister chromatids and in which chromosomes showed good spread with little or no overlapping were chosen for the purpose. Statistical analyses of data (particularly in case of chromosomal aberrations) were made by student's t-test [28].

Sperm preparation from testis: Cream coloured testis was kept in 45% Glacial Acetic acid solution. Then the testis was homogenized and aspirated gently in a homogenous cell suspension. The cell suspension was kept for 5 min. and then centrifuged at 1000 rpm for 10 min. The supernatant was discarded and the pellet was fixed in chilled methanol: glacial acetic acid fixative (3:1 v/v). The whole process was repeated twice. Two drops of cell suspension were dropped on clean grease-free slide (soaked previously in chilled 50% ethanol) and allowed to dry in air. Then slides were stained in 5% phosphate buffered Giemsa stain (pH 6.8) for 20 minutes and washed in running tap. The dried slides were observed under the 100X oil immersion lens of an Olympus Binocular Research Microscope using 10X wide field eye pieces.

Results and Discussion

Gross anatomy analysis: The gross morphology of the reproductive system of the male (specimen No- 1 and 2) is comprised of paired elongated, cream coloured testis with well developed long oviduct which is the indication of hermaphroditism. The other specimen (No- 3) was normal female.

Histology: The gonad and kidney of specimen No – 1, 2 and 3) were observed (Figure 2). The testis (Specimen No – 1 and 2) is almost normal in inner structure. Seminiferous tubules are filled with close bundles of normal spermatozoa (Figure 2a). Interestingly, the inner parts of the section of the kidney were normal while the outer parts revealed or remained as an ovarian structure (i.e. ovarian follicles) – an indication of hermaphroditism (Figure 2b). The gonad of female individual (Specimen No – 3) was normal ovary filled with growing follicles (Figure 2c).

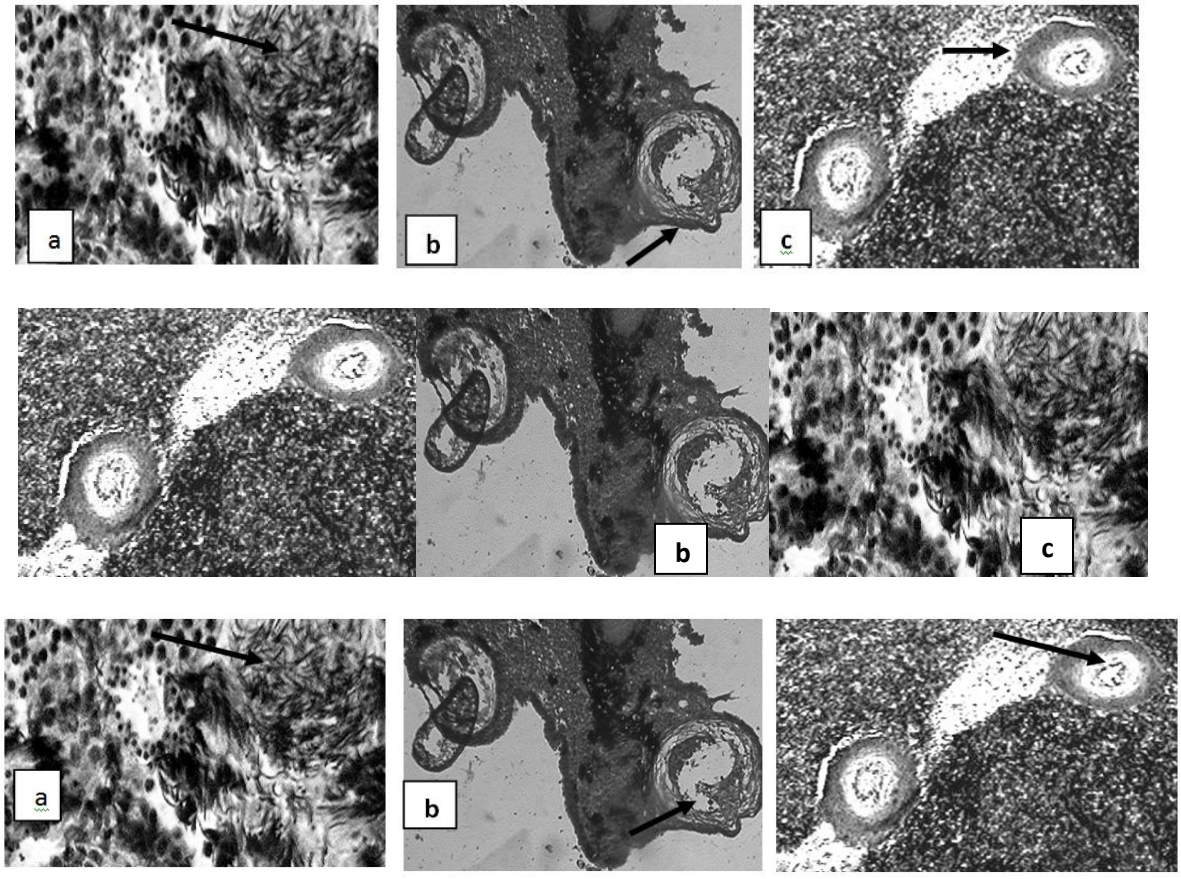

Figure 2: Histological sections of Testis and Kidney (Specimen No - 1) and Ovary (Specimen No – 3) of *Rana tigirina* a) Section of testis showing seminiferous tubules with spermatozoa (arrowed) b) Section of kidney with the ovarian structure (arrowed) in the male specimen c) Section of ovary with follicles (arrowed) in female individual

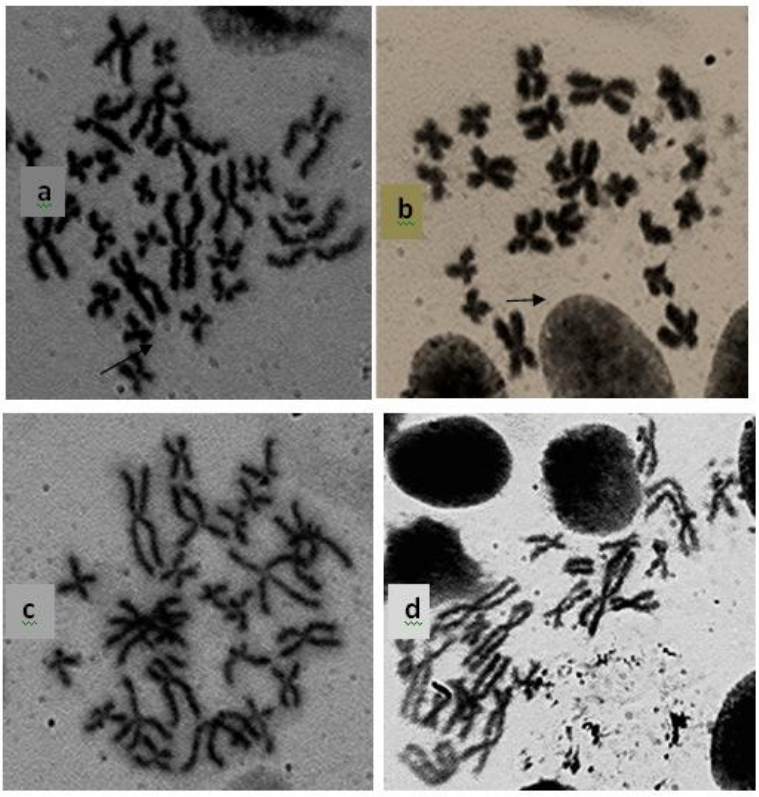

Figure 3: Somatic metaphase chromosomes of *Rana tigirina* in normal and abnormal individuals prepared from intestinal epithelial cells a) Normal metaphase plate with 26 chromosomes, b) Metaphase stage showing aneuploid condition with 22 chromosomes, c) Metaphase stage showing chromatid break (arrowed), d) Metaphase stage showing centric fission (arrowed) { magnification 10 x 100}

Chromosomal aberration analysis: Conventional stained metaphase chromosomes of *Rana tigirina* (Specimen No – 1, 2 and 3) revealed the existence of 26 biarmed chromosomes (Figure 3a). An examination of well spread metaphase stages from specimen No – 1 and 2 revealed a variety of chromosomal abnormalities (Figure 3b,c,d) The abnormalities were in the form of simple chromosomal abnormalities or SCA (i.e. chromatid and isochromatid breaks, gaps, deletions etc.) and complex chromosomal abnormalities or CCA (i.e. unequal condensations, centric fissions, exchanges, multiple chromatid breaks etc.). The frequencies of different aberrations recorded from specimen No – 1 and 2 were significantly higher in comparison to specimen No- 3 (Figure 4).

Sperm head or sperm shape abnormalities: Germ cell cytotoxicity was also evaluated by assessing different types of sperm head and sperm shape abnormalities in the specimens No- 1 & 2 (Figure 5). These are amorphous, small, ring shaped, distorted in shape etc. On the basis of high incidence of sperm head and shape abnormalities and chromosomal abnormalities, it is suggested that these changes possess a constant threat to the amphibian population.

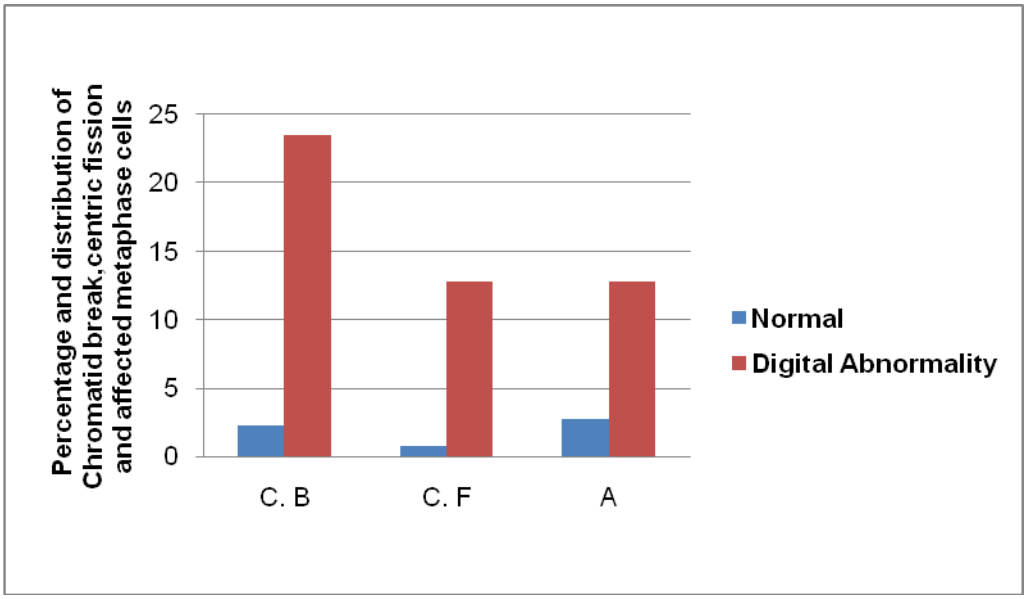

Figure 4: Percentage and distribution of chromatid break (C.B.), centric fission (C.F.) and affected metaphase (A) cells in normal and abnormal (Digital abnormality) individuals. 100 metaphases studied from four slides (four replications) in each case

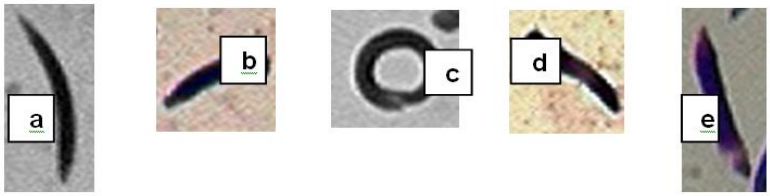

Figure 5: Different types of sperm abnormalities (arranged from cut out photomicrographs) recorded in abnormal individual a) normal sperm, b) – e) abnormal sperms (magnification 10 x 100)

Conclusion

Amphibians are known as bioindicators of environmental quality. Because early stages of life cycle of many species are restricted to the aquatic environment and many adults respire through moist skin [29, 30]. So amphibians are susceptible or vulnerable to dermal absorption of toxic chemicals in water. Chakrabarti et al.[6], Banerjee[5] , Chakrabarti and Banerjee[7] have established the amphibian system as an ideal test model for biomonitoring of environmental mutagens and carcinogens due to unique responsiveness to different genotoxicity test including sister chromatid exchanges (SCEs), chromosomal aberration and micronuclei test.

The present study thoroughly examines the gross morphology of reproductive organs (i.e. testis and oviduct in male and ovary in female) in both male and female ranid individuals with their histological structures, chromosomal aberrations analysis and also sperm abnormalities in the morphologically abnormal male individual. It is interesting to note that ranid with digital abnormality (three digits instead of four in the forelimb) shows the phenomenon of hermaphroditism with the presence of both testis and oviduct in same individual. Quantitative analysis of chromosomal abnormalities in both normal and morphologically abnormal (with digital malformation) ranid revealed that the frequencies of abnormality were significantly higher in the morphologically abnormal individuals. The occurrence of a remarkably high number of cells with chromosomal abnormalities as well as sperm abnormalities in morphologically abnormal individuals can be attributed to the concentration of mutagens or clastogens in the tissues of

the animals used in the present study. Moreover, different types of pesticides or mutagens in the water body have led to the formation of mixed gonads or reproductive organs in Rana tigirina. Long term effect of pesticides in the tissue of the animals causes excessive genome damage which may be one of the causal factors for digital malformation with reproductive abnormality and the warning signal of declining amphibian population.

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References

1. Schmid M., Chromosome banding in amphibia. I.Constitutive heterochromatin and nucleolar organizer regions in *Bufo* and Hyla, Chromosoma, 66, 361-388 (**1978**).

2. Morescalchi A., New developments in vertebrate cytotaxonomy I. Cytotaxonomy of the amphibians, Genetica, 50 (3), 179-193 (**1979**).
3. Chakrabarti S., Somatic chromosomes of Indian burrowing frog *Uperodon globulosum* (Gunther Anura , Amphibia), Experientia, 35,743-744 (**1979**)

4. Manna G.K., Cytogenetic studies on fish and amphibian., Conference Proceedings, Genetical research in India,XV International Genetics Congress, New Delhi, 243-273 (**1983**)

5. Banerjee S.N., Chromosomal endophenotype of some Indian Anura with reference to C-band distribution and sensitivity of heterochromatin to induced aberration, Ph.D. Thesis, University of Burdwan, Burdwan, West Bengal, India , 1-149 (**1986**)

6. Chakrabarti S., Banerjee S.N. and Chakrabarti A., *In vivo* sister chromatid exchanges in amphibia – a convenient model for mutagenicity bioassay, Indian Journal of Experimental Biology 22, 347 – 349 (**1984**)

7. Chakrabarti S. and Banerjee S.N., *Bufo melanostictus*, an anuran amphibian, as a novel model for biomonitoring of environmental mutagens and carcinogens, Hamadryad 31 (2), 242 – 249 (**2007**)

8. Philips K., Where have all the frogs and toads gone, Bioscience 40, 422 – 424 (**1990**)

9. Wake D.B., Declining amphibian populations, Science 253, 860 (**1991**)

10. Pechmann J.K.H., Scott D.E., Semlitsch R.D., Caldwell J.P., Vitt L.J. and Gibbons J.W., Declining amphibian populations: the problem of separating human impacts from natural fluctuations, Science 253, 892 – 895 (**1991**)

11. Daniels R.J.R., Impact of tea cultivation on anurans in the Western Ghats, Curr. Sci. 85, 1415 – 1422 (**2003**)

12. Chakrabarti S., Banerjee S.N., Neogi L.N. and Roychowdhury S., C-band positive W-chromosome in female Indian frog *Rana tigrina*, Experientia 39, 321 - 322 (**1983**)

13. Banerjee S.N. and Chakrabarti S., A simple technique to detect satellite and satellited stalk in *Bufo melanostictus* , Current Science 56, 41-42 (**1987**)

14. Banerjee S.N. and Chakrabarti S., Differential clastogenic sensitivity of heterochromatin and euchromatin: Evidence from an in vivo study made on a submammalian vertebrate, In vivo 3, 55-60 (**1989**)

15. Banerjee S.N. and Chakrabarti S., In vivo sister chromatid differentiation in an amphibian model – application of reverse banding, Current science 58 (7), 401- 403 (**1989**)

16. Banerjee, S.N., Bhattacharya A.K. and Chakrabarti S., Two Indian anuran populations on the decline, Proc. Zool.Soc. 45 (Suppl. A), 141 – 144 (**1992**)

17. Banerjee S.N. and Chakrabarti S., C-band polymorphism in three bufonid species, Persp. Cytol. Genet. 8, 177-183 (**1995**)

18. Banerjee S.N. and Chakrabarti S., Chromosomal sex determination in Anura, Hamadryad 27 (2), 248-253 (**2003**)

19. Daniels R.J.R., Geographical distribution patterns of amphibians in the Western Ghats, India. J. Biogeogr. 19, 521-529 (**1992**)

20. Daniels R.J.R. and Ishwar N.M. Rarity and the herpetofauna of the Eastern Ghats, India, Cobra 16, 2-14 (**1994**)

21. Ravi Kumar M. and Daniels R.J.R., Checklist of reptiles and amphibians of Kolli Hills, Cobra 38, 21-22 (**1999**)

22. Blaustein A.R. and Wake D.B., Declining amphibian populations: a global phenomenon? Trends in Ecology and Evolution 5, 203 (**1990**)

23. Daniels R.J.R., The problem of conserving amphibians in the Western Ghats, Current Science 60, 630-632 (**1991**)

24. Lips K.R., Mass mortality and population declines of anurans at an upland site in Western Panama, Conservation Biology 12, 117 – 125 (**1998**)

25. Kleiner K., No defence: weak immunity is frog's undoing, New Scientist 174, 12 (**2002**)

26. Dalton R., Frogs put in the gender blender by America's favourite herbicide, Nature 416, 665 - 666 (**2002**)

27. Hayes T.B. et al., Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis),*Proceedings of National Academy 107(10) , 4612 – 4617(**2010**)

28. Panse V.G. and Sukhatme P.V., Statistical methods for agricultural workers., Indian Council of Agricultural Research, New Delhi, 46-58 (**1985**)

29. Dunson W.A., Wyman R.L. and Corbett E.S., A symposium on amphibian declines and habitat acidification, Journal of Herpetology, 26, 349 - 352 (**1992**)

30. Duellman W.E. and Trueb L., Biology of amphibians, McGraw-Hill Book Company , New York, 445 - 458, (**1986**)