Chapter 2

Evaluation of the Frog, *Rana tigerina* (Daudin) as an Indicator Species to Assess the Genotoxicity of Aquatic Pollution

Contamination of watersources by pesticides and industrial effluents is a serious environmental problem in both developed and developing countries. In order to assess the impact of pollution on the genome of aquatic species relevant toxicological system must be employed. These should either be the target species themselves or some representation well-suited for genetic analysis.

Fishes are often used as a mutagenic model to assess the aquatic pollution (Klingerman, 1975; Krishnaja and Rege, 1982; Baker and Rachman, 1979). However, the smaller size of the chromosome complements and their higher 2n number cause inconvenience in chromosomal studies. Amphibians, on the other hand, possess larger metaphase complements and less 2n number which enhance the suitability of this model for mutagenic bioassays of aquatic and semiaquatic environments (Chakrabarti *et al.*, 1984). Amphibian hemopoietic cells can be used as a reliable test system for the detection of cyto- and genotoxic compounds as well as for the genetic monitoring of aquatic or semiaquatic environment (Kraskowski *et al.*, 1986; Zakhidov *et al.*, 1993.)

The selection of a species for the mutagenic bioassay needs careful evaluation. Usually, the bone marrow has been considered as a target tissue for the analysis of metaphase chromosomes, primarily due to its high mitotic index. However, most of the amphibians generally lack an actively hemopoietic bone marrow unlike mammals and birds. Among amphibians, only the newt *Plethodon* and the frog *Rana pipiens* were

known to possess active bone marrow (Barret, 1947; Curtis *et al.*, 1979). Ultrastructure characteristics of granulopoetic tissues in various types of amphibians have been studied (Campell, 1969, 1970., Hightower and Haar, 1975; Mitsui, 1960, 1965; Tooze and Davies, 1968). In anurans, the important seat of the formation of new corpuscles is in the bone marrow and spleen while the major hemopoietic organs of newt are the liver, spleen and thymus (Hightower and Haar, 1975). Though it is believed that the animals which hibernate in winter and aestivates in summer have seasonal fluctuation in the bone marrow (Curtis *et al.*, 1979), little is known about the seasonal variation in the mitosis in the bone marrow cells of anuran amphibians.

In the present study, the seasonal variation in the activity of bone marrow, of the frog *Rana tigerina* has been analyzed by counting the mitotic index in the bone marrow for a period of two years, in order to evaluate the suitability of this species in clastogenic studies. The metaphase chromosome complements obtained from both the sexes were examined. Chromosome preparations were also done from the spleen.

Materials and Methods

Animals: Mature specimens of both sexes of *Rana tigerina* weighing 40-60 gms were collected from uncontaminated natural ponds in the villages near Dabhoi. The animals were maintained in aquaterraria at 20 - 25°C and fed cockroaches thrice a week. They were acclimatized to laboratory conditions for 15 days prior to experiments. At least 8 animals were collected every month and used for the experiment to examine the seasonal variation in mitosis in the bone marrow.

Seasonal variations in mitosis: Smear preparations of bone marrow cells were made according to the procedure described for the preparation of mitotic chromosomes after *in vivo* colchicine treatment (Chapter 1). At least six animals (3 males and 3 females) per month were used for the experiment and five slides were prepared from each animal. The analysis of seasonal variation was done from the slides prepared for a period of two years. Mitotic index (MI) was calculated as the number of dividing cells per thousand cells counted.

Chromosome preparations and karyotype: Mitotic Chromosomes were prepared from more than 30 males and 30 females. The preparations were made from the bone marrow after *in vivo* colchicine treatment (Chapter 1).

Results

Preliminary Assay and Karyotype

The diploid number in both sexes of *Rana tigerina* as determined from bone marrow is 26, comprising 5 pairs of larger chromosomes and 8 pairs of smaller chromosomes (Plate 1). Karyotypes from male and female frogs are shown in Plate 2 and 3 respectively. The chromosomes are arranged according to the size as described by Shah *et al.* (1973). Occasionally the long arm of both homologous pair No. 6 shows a conspicuous secondary constriction in both sexes, which is sometimes found only on one chromosomes (Plate 1, Figs. 1 & 2). Sex chromosomes are morphologically indistinguishable because both the sexes are chromosomally alike.

Seasonal Variations

Seasonal variation in the mitosis in the bone marrow cells of the test animal, obtained from a two-year data is shown in table 1.. The highest mitotic activity was recorded during the winter season in both the years. Mitotic activity in the bone marrow increases from August and reaches its peak between October to January months followed by a sudden decline in February. Mitosis was found to be negligible during the months from February to August. Mitotic activity in the spleen cells were found only in January and February months. Analyzable number of metaphase cells were not available from the spleen during other season.

Discussion

In order to conduct the experiments on the analysis of chromosomal aberrations, it is necessary to have reliable system. A good number of analyzable metaphase cells are necessary for the analysis of chromosomal aberrations.

Table 1. The mitotic index recorded in the bone marrow of the frog *Rana tigerina* during a two year period. Data presented are of mean ± SE of 5 animals in each month.

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
I year	4.08 ±0.19	0.18 ±0.12	NG	NG	NG	0.48 ±0.05	0.30 ±0.06	0.53 ±0.12	2.45 ±0.32	3.65 ±0.16	4.03 ±0.21	3.93 ±0.15
II year	3.45 ±0.55	0.18 ±0.10	NG	NG	NG	0.28 ±0.04	0.38 ±0.08	1.10 ±0.12	2.30 ±0.78	2.30 ±0.23	2.60 ±0.68	2.65 ±0.85

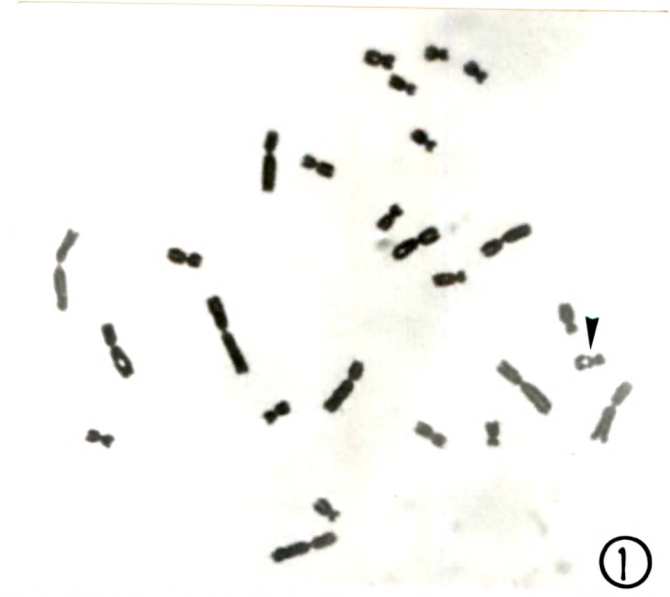
NG = Data could not be recorded as the mitosis was found negligible during this period

Plate 1. Normal metaphase complements of the frog *Rana tigerina* (all x 1650).

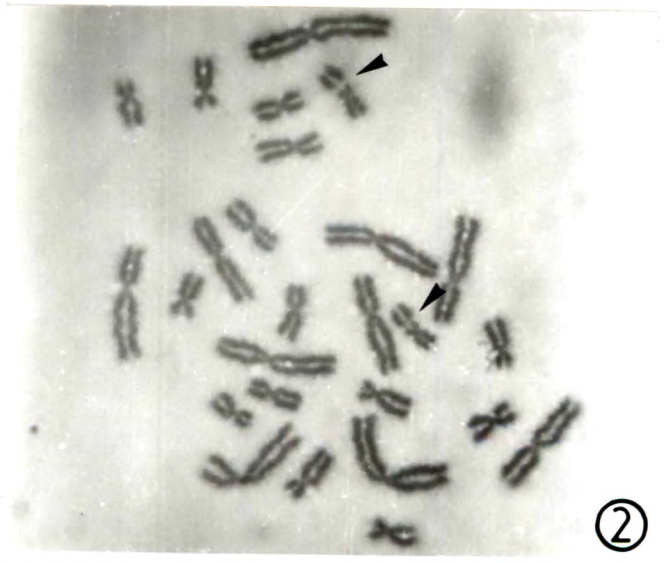
Figure 1. Metaphase chromosome preparation from the bone marrow of a male frog. Secondary constriction on a chromosome (arrow).

Figure 2. Metaphase chromosome preparation from the bone marrow of a female frog. Secondary constrictions on a pair of chromosomes (arrows).

Figure 3. Metaphase chromosome preparations from the spleen of a female frog.



①



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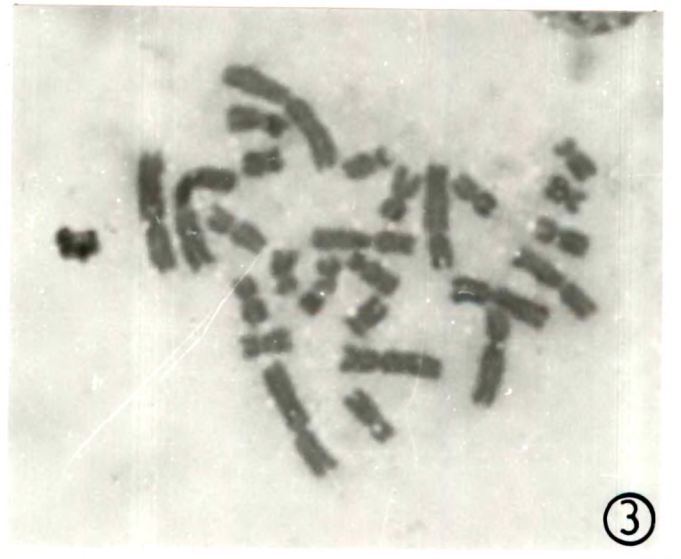
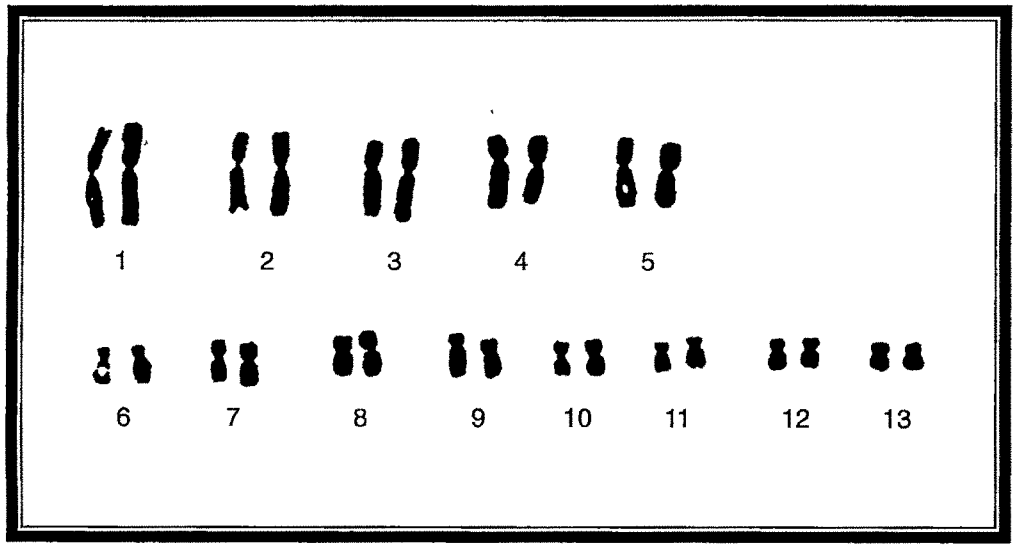


③

Plate 2. Karyotype of a male frog, *Rana tigerina* (prepared from the figure 1 of plate 1. One of the chromosomes in 6th pair shows a secondary constriction.

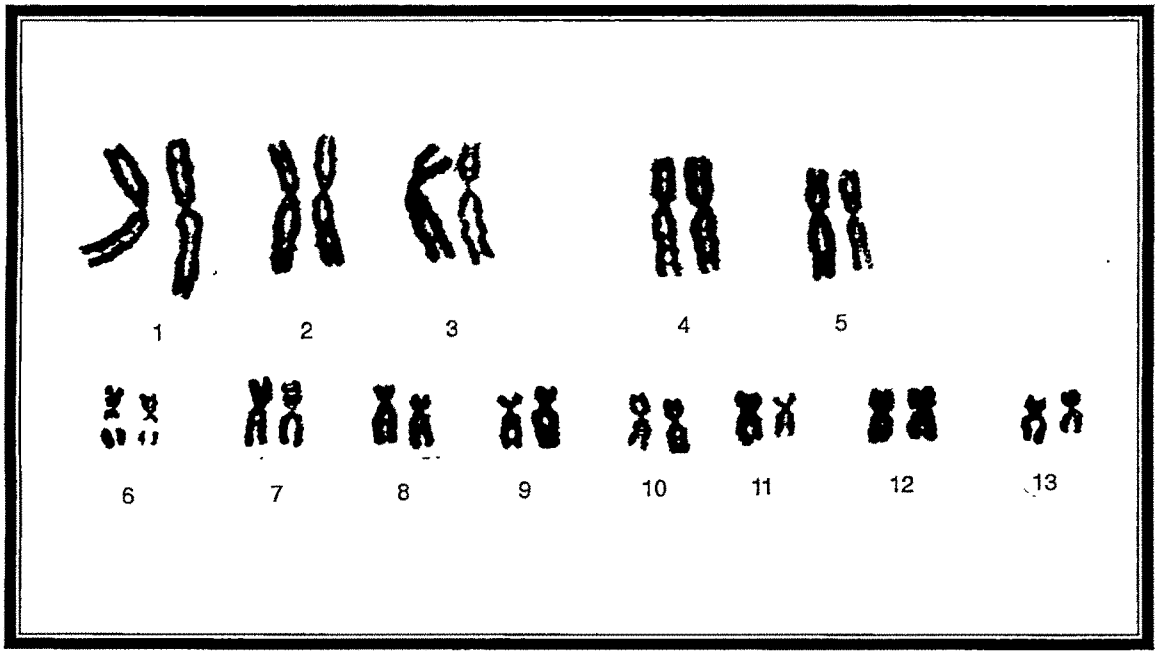
Karyotype from bone marrow of male *Rana tigerina*
(prepared from the photograph shown in figure 1 of Plate 1)



(Scanned image of the photograph)

Plate 3. Karyotype of a female frog *Rana tigerina* (prepared from the figure 2 of Plate 1. The 6th pair shows the secondary constrictions on both chromosomes.

Karyotype from bone marrow of female *Rana tigerina*
(prepared from the photograph shown in figure 2 of Plate 1)



(scanned image of the photograph)

The disparity in the activity of bone marrow cells, between the warm-blooded and cold-blooded animals is well established. It is also believed that the bone marrow undergoes periodic changes corresponding to the changes in the blood. Hutchison and Szarki (1965) found that the blood cell counts vary widely between individual frogs and that erythrocyte counts as an indicator of physiological condition should be regarded as reliable only if large samples are taken into consideration. In *Rana pipiens*, the blood counts were found to be highly variable and it was not possible to determine any seasonal influence in the total count (Rouf, 1969). There are considerable individual and seasonal differences in various species of *Rana* (Alder and Hubler, 1923; Klieneberger, 1927; Schermer, 1954). Further Hutchison and Szarki (1965) found that temperature is not related to variation in erythrocyte counts. Even in the present study no apparent correlation could be observed between the erythrocyte counts and mitotic index (data not shown). Individual difference was also greatly variable.

The result shows that the mitotic activity in the bone marrow increases after the month of August which is the breeding season of this species. However, it attains a peak in winter and gradually declines in early summer and negligible during March to July. Highest mitotic activity was recorded in December-January months when the temperature was recorded 15-20 °C, which is the minimum temperature in comparison to other months in this region. The mitosis is generally believed to be high in active animals. However, a good mitosis was observed in less active animals during winter months. During the post breeding season, the fat in the bone marrow gets completely resorbed and the tissue gets a lymphoid appearance. Lymphoid cells must be concerned with the production of new corpuscles that undergoes rapid multiplication and thus a corresponding increase in mitotic index during the post breeding season (September - October months). In fact, the highest mitotic index was expected during this period. In contrast to this, the mitosis was observed at peak during the winter season (December - January) despite the presence of a good amount of fat in the bone marrow (although no estimates of the quantity of fat or relative number of fat cells in the bone marrow was carried out, it seemed likely that the quantity of fat is highest during winter months).

Analyzable number of metaphase cells were found in the spleen only during the January-February months. The mitotic activity observed in the spleen cells was, however, less than in the bone marrow even during this period. Morphology of chromosomes was found better in bone marrow cells. Hence, bone marrow can be considered as a better target tissue than spleen for the mutagenic assays in the test animal.

When the data of mitotic indices recorded for a period of two years, were compared (Table 1) there were difference in the highest activity recorded. This could probably be due to the difference in climatic characteristics of these two years. Nevertheless, the sudden fall of mitosis in February and a high mitotic index during September to January season are clearly conclusive from the data.

The results of the present study clearly indicate that the frog *Rana tigerina* can be used as an indicator species for mutagenic bioassays only during September to January season. During other seasons the mitosis in the bone marrow is negligible.