CHAPTER IV

SPERM POLYMORPHISM

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CONTENTS

INTRODUCTION

The polymorphism of sperm i.e. existence of more than one type of morphological varieties of sperms in a species is a subject of long discussion and thought. Siebold (1836) discovered two types of spermatozoa in pond snail *Pabedina* sp.. Since then many cases of conspicuous polymorphism have been found in invertebrates and in some vertebrates. However systematic studies on this phenomenon have not been made until very recently.

In an acoelan flatworm, Hendellberg (1969) found two kinds of spermatozoa , a very peculiar "typical" one with two flagella and a smaller atypical one which during spermiogenesis cast off its nucleus with residual cytoplasm and remains in the semen as short cytoplasmic rod. Earlier Meves (1903) recorded diminution or loss of chromatin as a frequent characteristic of atypical sperm.He coined the term 'eupyrene' for cells with normal chromatin content; 'oligopyrene' for those with subnormal amounts and ' apyrene' for cell without chromatin . Chromatin may be absent or occur in abnormal amount in flatworms, mollusks, annelids, insects and vertebrates (Roosen – Runge,1977).

Sperm polymorphism has also been found in a number of amphibian forms (Ray 1978; Ray et.al.,1989; Roy and Ray 1994; Mukherjee et.al.,1999; Patra et.al., 2001).

AIMS AND OBJECTIVES

In the present study an attempt has been made :

1. To record various forms of sperms and their characteristics at light microscopic level.
2. To find out surface topography of each such form.
3. To account for various ultra-structural features, and
4. To workout the possible explanation of origin of various forms, behavior and functional aspect during the reproduction process, i.e. to accomplish normal fertilization.

MATERIALS AND METHODOLOGY

MATERIALS

Adult male Himalayan toad ,*Bufo himalayanas* males were collected during two distinct phases of reproduction, i.e. during breeding season (May to August) when spermatogenic activity is high and during non-breeding season (October to February) when spermatogenic activity ceases totally in high altitude forms and takes place in a low wave in the specimens inhabiting in low altitude (1500-2000 mt.). This is done in order to study the seasonal effect, if any, on sperm morphology.

METHODOLOGY

(1) Collection of spermatogenic cells:

Spermatogenic cells were collected following the differential centrifugation technique proposed by Roy *et.al.*,(1989). The testes were dissected medially and tissue materials were obtained, minced by a pair sharp edged scissors into small fragments in amphibian saline (pH 6.9). The tissue materials were suspended for a brief period to obtain a milky white suspension and were centrifuged at 100rpm to remove the debris. The supernatant was immediately transferred to gluteraldehyde-paraformaldehyde mixture for preservation.

A portion of the supernatant was centrifuged at 1000rpm, 2000 rpm, 3000 rpm and 4000 rpm to collect various stages of spermatogenic cells depending on their sizes. The residual supernatant centrifuged with different grades of solution ranging from 2.5% - 5.0 % to obtain pure sperms and were examined under light microscope to evaluate the degree of contamination. The best result obtained at around 3.0% - 3.5% gluteraldehyde solution.

(2) Fixation of cells for SEM and TEM study:

The details of the process of fixation was described in the chapter II of this thesis.

(3) Method of studying:

Sperm morphology

(A) Light microscopic study:

A drop of milky white supernatant suspension was spread carefully over a clean grease-free slide and after air drying the preparation was fixed with methanol for one minute. After drying Leishmann stain (E.Marck) was added and carefully spreaded with the help of a clean glass rod to cover the film entirely. This was kept for 10 minutes and then double distilled water was added in equal volume of the stain for maturation. After 15 minutes the preparation was washed thoroughly in running water for a considerable period of time.

Then the preparation was made dry and covered with DPX (E.Marck) mounting medium for permanent mounting with a glass coverslip. Observations were made under a light microscope (Leitz).

(B) Scanning electron microscopic study:

The detailed description has been given in chapter III of his thesis.

(C) Transmission electron microscopic study:

In this case the initial process of tissue preparation is somewhat dissimilar with the normal gonadal tissue preparations. As the spermatozoa were present within the fluid suspension a different procedure for the tissue block preparation was made. The maximum pure suspension with a good number of expected spermatozoa was fixed within a capsule made from araldrite mixture for block making. Then the block was cut as per procedure described in details in the chapter II of this thesis.

OBSERVATIONS

A. Light microscopic

A mature spermatozoon of *Bufo himalayanas* shows typical characteristics of an anuran sperm. It has a slender, anteriorly tappering heterochromatic head with a long slender deeply stained heteropycnotic nucleus . The nucleus gradually flattens from the tip to the broad base or posterior end. The tip of the nucleus covered with the acrosomal material, somewhat conical in shape and laterally overflows on the nuclear tip. Acrosome is achromatic in nature and lightly stained. Acrosome is also provided with a typical hook like curved structure or acrosomal barb at its anteriormost end (Plate 32, Figure a & b). Size of the head nucleus is 29X2 μm in diameter.

The middle piece is short and stout and is provided with centrosomal bodies within. From this part a flagellar tail emerge. The tail is typically biflagellate, which is also an important characteristic of this species. The length of the two long filamentous flagella is unequal (Plate 32, Figure a). In this work it has been observed that different types of morphologically non-identical mature sperms are present. Thus polymorphic nature of the spermatozoa is evident in this species.

B. Scanning electron microscopic

Under SEM all the morphological details revealed under light microscope could be visualized. The head, neck, middle piece and tail part are well documented and recognizable. **Head** is elongated with compact, elongated electron dense nuclear part of uniform diameter. Head surface is smooth

i.e. without any protrusions or accessory structures as found in related species. The anterior part of the head elongates gradually to form a slender structure called acrosome. The acrosomal barb is hook like with a cup like depression at its anterior-most end (Plate 32, Figure b).

The middle piece is situated in the somewhat flattened posterior portion and is laterally bulged out suggesting the accumulation of mitochondria and centrioles within (Plate 32, Figure c).

The **tail piece** is biflagellated and is connected by a membrane medially which is discontinuous at the posterior end. The end portion of the flagellum bears a knob like structure (Plate 32, Figure d & e).

Like light microscopy, several morphological varieties in terms of acrosomal vesicles, head structure and tail are also documentable (Plate 30A, Figure A,B, F).

C. ***Transmission electron microscopic***

Under transmission electron microscope similarly well differentiated head, neck and tail parts are visible.

Head is conical in shape, tapering towards the anterior end and with compact chromatin material. However in transverse sections, electron dense granules are found throughout the length of the nucleus and has different degree of compactness at various level (Plate 35, Figure A, B).

The anterior part of the head terminates in a electronlucent acrosomal cap. The cup shaped acrosomal barb is also visible and exhibits wooly appearance and with several discontinuous filaments in it (Plate 35, Figure D).

The middle part is elongated with median electron lucent vacuole surrounded by heavily packed multilayer of distinctly visible mitochondria that are almost circular in cross section. The two centrioles respectively called proximal and distal centrioles are also visible (Plate35,Figure C, E, F).

The proximal centriole is lodged in the nuclear pocket situated at **posteriormost part** of the head nucleus. The distal centriole is situated at the base of the middle piece and is not perpendicular to the proximal centriole. In tranverse section both the proximal and distal centrioles exhibits typical 9+2 arrangement of microtubules (Plate 35, Figure F, G).

The tail is biflagellated and each flagellum consists of an axial filament made up to 20 (18+2) sub-microscopic longitudinal microfibrils. The presence of undulating membrane has been documented by the presence of a wire like structure with electron dense materials at both ends (Plate 35, Figure G, H).

Apart from typical spermatozoon structure, megacephalic sperm with condensed head is evident. Similarly globular as well as microcephalic sperm head were also observed under transmission electron microscope (Plate 34, Figure A, B, C).

PLATE 35

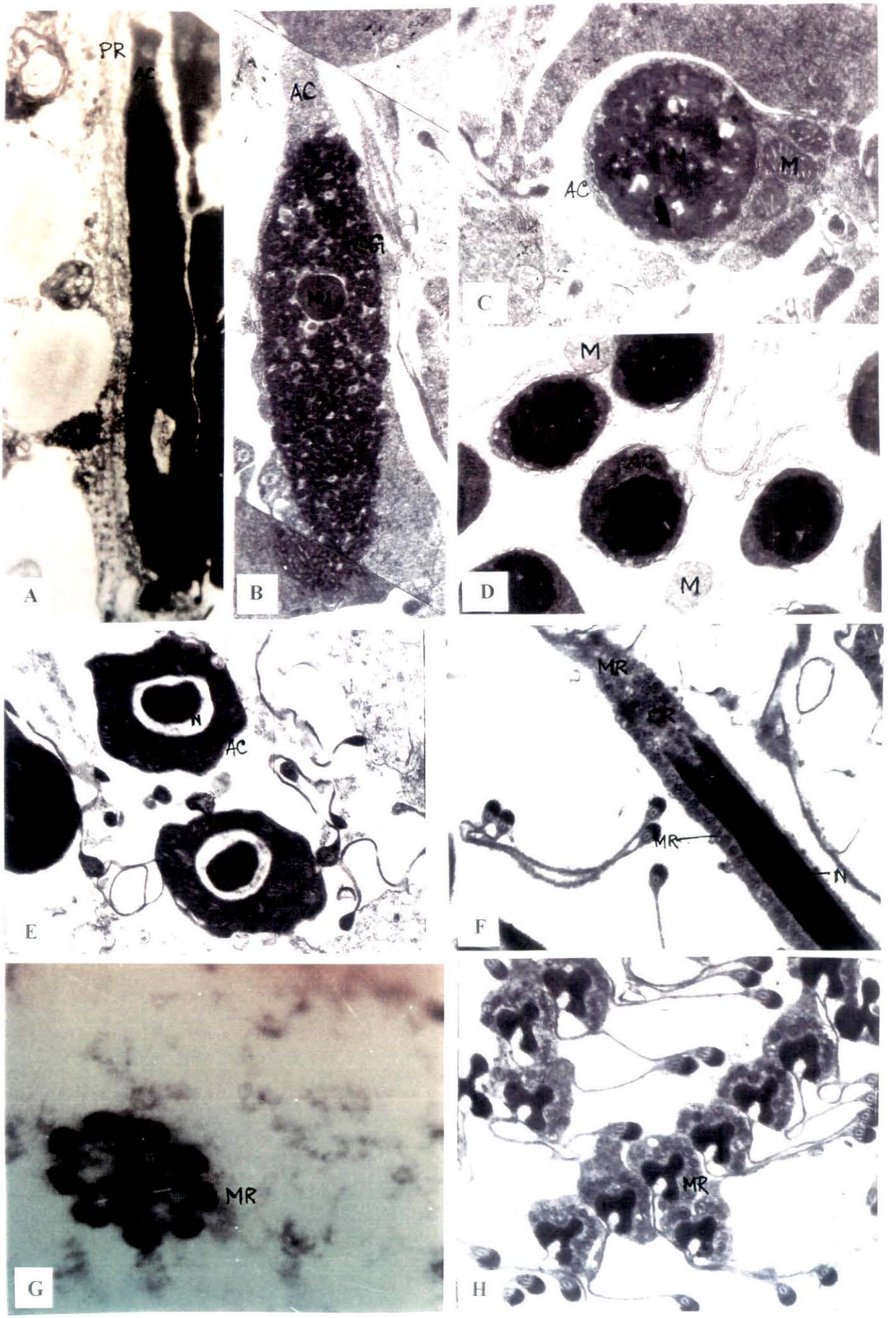
Legends

Electron micrographs of different parts of mature spermatozoa

A. A mature sperm with head and middle piece
B. Head shows compactation of nucleus and acrosomal materials
C. Head in cross section showing acrosome and middle part with a number of mitochondria
D. Heads in cross section in a sperm bundle showing nucleus and acrosomal envelop
E. Middle piece of a mature sperm in cross section showing centrosomes and microtubules
F. Tail showing filaments with typical (9+2) arrangement through its cross section
G. Tail pieces of mature sperms in a sperm bundle through cross section

N- Nucleus AC- Acrosome PR- Perforatorium NU- Nucleolus
CG- Chromatin granules M- Mitochondria MR- Microtubules

PLATE-35



DISCUSSION

Sharma and Dhinsda (1955) first provided structural details of ripe spermatozoa of toad, *Bufo stomaticus* and described that such spermatozoon consists of deeply stained head with a cylindrical nucleus and a pointed needle like acrosome situated at its anterior tip . Immediately behind the head and with its intimate contact a small deeply staining middle piece with clumped mitochondria and two centrioles lie side by side, were also described. Two axial filaments joined together by a rudimentary undulating membrane and each such filament arises from its own centriole.

Burgos and Fawcett (1956) has given a remarkable account of electron micrographs of the maturing spermatids of *Bufo arenarum* including the formation of acrosome, changes in nucleus, centriole, tail and undulating membrane. They first described the existence of a perforatorium, hitherto not described by any author in genus *Bufo* . These observations confirmed the earlier findings of Sharma and Dhindsa (1955) with light microscopy (Diagram-11).

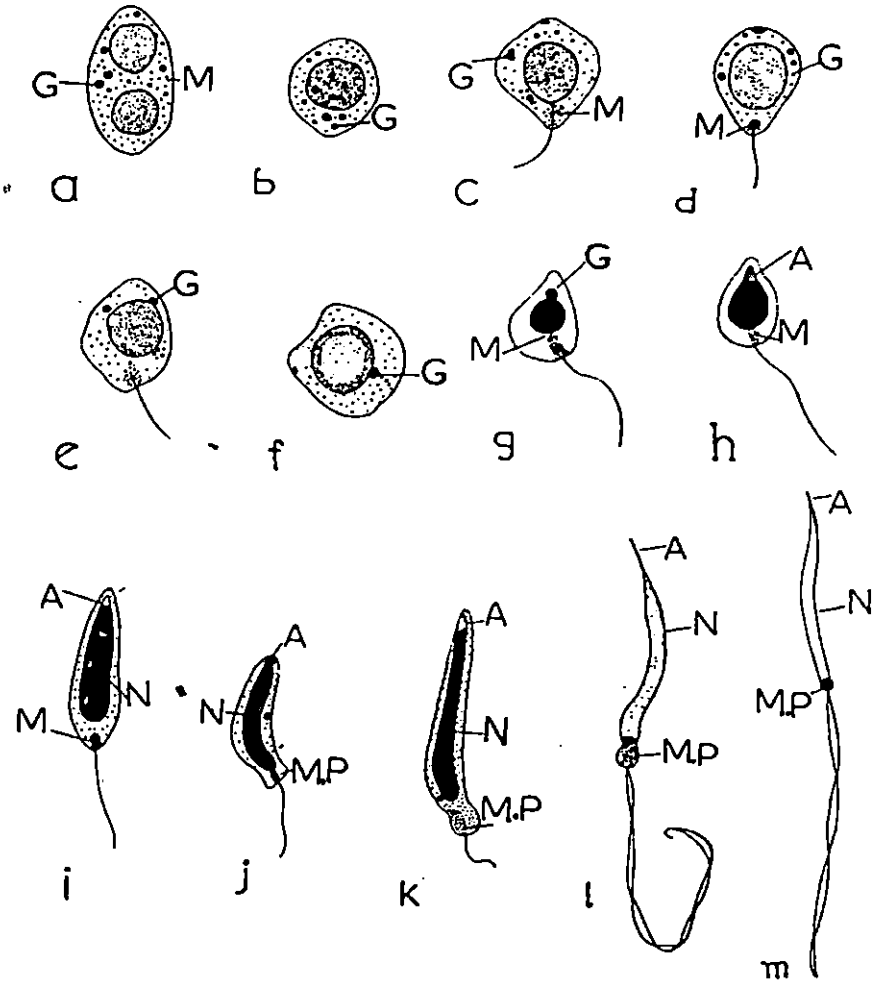
The present observations on *Bufo himalayanas* similarly observe confirmatory of the earlier descriptions of the mature spermatozoa of various species of genus *Bufo.*

In many cases of polymorphism, the aberrant types are morphologically very characteristics and sometimes show a behavior which suggest that they have become functional components of the reproductive process, although they have never been seen to accomplish normal fertilization. In the rotifer *Asplanchna* a typical spermatid produces immotile 'rod spermatozoa' which are not cells but cellular products of spermatids (Kochler and Birky,1966), the function of these rods is unknown. It may be related to the peculiar intradermal fertilization of these animals. Gupta (1964) reported the alternating development of two kinds of spermatids in *Diatomus*, one with and one without a chromatoid body. The first evelops into the typical fertilizing sperm and the second into the 'swelling spermatozoa'.

Various authors have reviewed the origin of atypical spermatozoa. Ankel (1924,1930,1933 and 1958) was able to show that in many species of viviparous *Chenopus vermetus* and some others the atypical series is recognizable already in the spermatogonial stage. In *Bythinia* and closely related species a atypical spermatozoa is morphologically very similar to typical ones, but they are oligo- or hyperpyrene and all of them arise in meiosis. Roy and Ray (1984) however recognized that in vertebrates there is a possible existence of different clones of spermatogonia mother cells and a concept of hypo-hyper diploidy was suggested. In *Bufo himalayanas* three such clones of spermatogonial cells were recorded having normal diploid chromosome sets (2n=24), hyper-diploid chromosomal set (2n=26/28) and hypo-diploid chromosomal set (2n=20-22). They recorded that there was a preponderance of particular chromosome 'duplication' or 'loss'. Roy and Ray (1989) have also established similar such clones of spermatogonial cells in the Himalayan newt, *Trilototriton verrucossus.*

The total cell loss during spermatogenesis in every case is the result of a series of specific degenerative events which occur at several identifiable stages. There are certain critical phases at which a proportion of gamets may begin to detiorate. Meiosis, specially in leptotene, zygotene and diakinesis is most frequently identified as a critical event. Spermatogonial mitosis, perhaps only certain one in the sequence, is a close second.

DIAGRAM : 11



SPERMATIDS AND SPERM OF TOAD
(after Sharma and Dhindsa, 1955)

G- Golgi bodies M- Mitochondria

N- Nucleus M.P.- Middle piece

A- Acrosome

Dalcq (1921) concluded that the most probable cause in an imbalance in the mechanism of division and the distribution of chromosomes. The failures in gene distribution do not always lead to immediate cell death. Often their consequences lead to the phenomenon of polymorphism. The various stages of spermatogenesis at which cell death occurs are summarized in Table-8.

In recent years, germ cells' degeneration has been equated with apoptosis. Miething (1992), Sinhahikim (1997) and others opined that germ cell death during spermatogenesis is a classical example of apoptosis as found in other developmental events like organogenesis of liver, kidney, lymphocytes, nerves cells and others.

The sperm morphology has been evaluated in the context of serial and coordinated action of sperm specific genes and a number of mutations, artificial and normal have been found to be related with various morphological as well as functional forms of spermatozoa. Gene expression in the sperm is stage specific. The initiation of spermatogenesis during puberty in mammals is probably regulated by a gene called BMP8B which enhance the synthesis of these proteins by the spermatogonia. When BMP8B reaches a critical concentration, the spermatogonia differentiate into spermatids (Zhoe et.al.,1996). Mice lacking BMP8B do not initiate spermatogenesis. Similarly in man DA2 gene locatd on the long arm of Y-chromosome when deleted causes infertility. The DA2 gene is exclusively expressed in the gene cell line, specially in the spermatogonin and it appears to encode an RNA binding protein (Reijo et.al.,1995; Menke et.al.,1997). In *Drsophila,* similarly,two genes Rb97D and boule are essential for spermatogonial kinetics and

when mutated causes degeneration spermatogonia and the cells do not enter meiosis (Karsh-Mizrachi and Haynes,1993; Eberhart et.al.,1996). Some genes are also specific for male meiosis (Hoyle and Raff,1990; Nishioka et.al.,1990 etc..) In addition to gene transcription in diploid cells during meiotic prophase, certain genes are transcribed in the spermatids (Palmites et. al.,1984; Peschon et.al.,1997; Nantel et. al.,1996).

In the present investigation the frequency of atypical sperm is biologically significant. The typical sperms in these species are produced in large quantities in a comparable manner to fertilize huge number of eggs produced during the breeding seasons. But till date none has attempted to visualize the process along with the normal tadpoles vis-à-vis atypical tadpoles that are also produced and survive in the nature. The origin of such atypical forms of the same species may suggest a continual case of natural selection in action. The atypical toads may not survive in the existing situations but may be fit for a new condition in future. Rossen-Runge (1977) thus suggested "like the polymorphism of sperm structure by all appearances the effect is large and highly significant and deserves much more attention than it has received hitherto".