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Special Volume dedicated to
Professor P. Mohanty-Hejmadi

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On the cover page is the emblem of ***"NABAGUNJARA"*** a chimeric animal to Orissan art and literature. Literally meaning "Nine form" it is a common motif in Orissan paintings. This form has been described by poet Sarala Das in the oriya version of the epic Mahabharata . Apparently Lord Krishna appeared in "Nabagunjara" form consisting of the body of an elephant, a leg each of a horse, a deer and a tiger respectively; throat of a peacock, tail in the from of a serpent, waist of a lion, hump of a bull and head of a cock, to fool his friend Arjuna. The Chimera was holding a lotus flower in a human hand. Arjuna had never seen such a creature in his life and guessed that this could not be a real animal but a form assumed by Lord Krishna; and immediately bowed down at its feet. It is said that the human hand with the lotus provided the clue. In the paintings and sculptures however, the lotus is often replaced by the "Chakra" or the "stylized discuss" of Lord Krishna. Chimeric forms are encountered in literature and art of all over the world. However, as far as I know, a chimera of nine animals, is uniquely Orissan. Therefore, we thought that this will be an appropriate emblem for the journal of the Zoological Society of Orissa.

Dr. P. Mohanty-Hejmadi

Foreword

This special volume of the Journal of the Zoological Society of Orissa is dedicated to Prof. P. Mohanty-Hejmadi on her superannuation from the Department of Zoology, Utkal University, Orissa in November 1999. Prof. P. Mohanty-Hejmadi was associated with this Department from its inception. She was in the USA from 1963 to 1975. After her return from the USA in 1975, she was instrumental in starting a special course in Developmental Biology as a part of the curriculum for M.Sc. students. She also established a leading laboratory for research on Developmental Biology of amphibians and reptiles. Her work on vitamin A and regeneration studies started in the early 1980s, gained international recognition in 1992 due to the amazing discovery of Homeotic Transformation of tadpole tails to limbs after vitamin A treatment, the first of its kind in any vertebrate. She received the highest civilian award of the Government of India "Padmashri" from the Honourable President of India for her contributions in the field of Science in 1995.

The present volume is a humble effort to acknowledge her dedication towards the cause of Zoology and to the scientific society on the whole. The journal contains several reviews and research papers by distinguished workers both from India and abroad. There have also been some contributions by students of Prof. Mohanty-Hejmadi. I am grateful to all the authors who have tried to spare some of their valuable time by contributing many useful articles in the scheduled time frame. At the same time, I apologise for the delayed publication of this volume due to several unavoidable circumstances and undesirable shortcomings. But, I hope that this volume would serve as an essential reference book to many young and upcoming developmental biologists.

Kalyani Bohidar

Editor

LIFE SKETCH OF PADMASHRI PRIYAMBADA MOHANTY-HEJMADI

Born in an eminent aristocratic family in Cuttack, Orissa in 1939, Priyambada imbibed a sense of unique freedom and culture from her parents. Her father late Shri Bhagabat Charan Mohanty was one of the first Engineers from Orissa who relinquished from service only after six months as he did not want to serve under British government and had a successful career as a contractor and mine owner donating most of his earnings to the Freedom Movement. Her mother late Shrimati Nisamoni Devi was a freedom fighter and a social worker of eminence until her death. With encouragement and facilities provided by her parents, Priyambada chose a career as a Zoologist and is a scientist of International repute today.

Prof. P. Mohanty-Hejmadi obtained her B.Sc. Hons (Zoology) degree from Utkal University, M.Sc. from Lucknow University, M.S. and Ph.D. from University of Michigan, USA. Prof. Mohanty-Hejmadi joined the Orissa Educational Services in August, 1958 and then Utkal University as a lecturer in Zoology in 1961. She then went to the USA with Barbour Fellowship and Fulbright Travel Grant in 1963. She received her MS in 1964 and Ph.D. in 1970 under the guidance of Prof. G.W. Nace. She was a post-doctoral Fellow at the University of Michigan and Assistant Professor in East Carolina University.

After her return to Utkal University in 1975, Prof. Mohanty-Hejmadi built up one of the leading laboratories for research on endangered amphibians and reptiles of India. She was the Professor and Head of the Department of Zoology from 1982 to 1990 and Programme Coordinator, UGC Special Assistance Programme unitl July, 1995 and from 1998-2001. She was **Vice-Chancellor of Sambalpur University, the first woman to be appointed to this post from** 1995-1998.

Prof. Mohanty-Hejmadi has standardised techniques for "Rear and Release" for the endangered bull frogs of India. She has demonstrated that in the sea turtles and salt water crocodiles it is possible to produce males and females by manipulating the incubation temperature (TSD technique). By her TSD technique she was able to help the "Rear and Release" programme of estuarine crocodiles by hatching male yearlings to balance the shortage of males in nature. She was invited to give her expert opinion

on the impact of pesticides and fertilizers on the status of Amphibians in Indian context and the need for frog culture in 1986 under the auspices of MPEDA, Department of Commerce, the "Impact of chemcials on the herpetofauna of India" in the First World Congress of Herpetology in Canterbury, Kent, U.K. in 1989. She was also invited to give her expert opinion on environmental problems convened by the Department of Environment and Forest, Government of India and M.S. Swaminathan Foundation Trust, Madras in 1992. She conducted a highly successful Biodiversity Conservation Project of Chandaka Forest under the auspices of WWF-India. She is also associated with the National Project Management Committee for the ICEF-supported coastal wetlands project operating under M.S. Swaminathan Research Foundation.

Due to her dynamic activities in Science, Conservation efforts and Public Awareness Campaigns, recognitions have come to Prof.Mohanty-Hejmadi from far and wide. She was awarded **Padmashri** for her contribution to science by the Govt. of India in 1998. She received the **Thomas J.Dee Research Grant**, Field Museum of Natural History, Chicago (USA) in 1980, was **Professor Contratto** in the University of Naples, Italy in 1987 and **JSPS Fellow** (Japan) in 1995. She was invited to the Symposium on Transdifferentiation at Hiroshima University and Sendai University, Japan in 1992. In 1994 she was invited to the 14th Annual Sea Turtle Biology and Conservation Symposium in USA, gave guest lectures at different places in UK and the USA and was invited to the International Symposium on Regeneration at Okazaki, Japan. She also gave her overall view of 25 years of sea turtle research in India in the 19th Annual Sea Tuttle research and conservation in 1999 in South Padre Island, Texas, USA.

She was a member of the 19-member high power committee of the Marine Turtle Specialist Group of IUCN which drafted the Global Strategy for sea turtle conservation in the meeting in Mexico in June, 1994. She was the convener for sea turtle conservation programme in the Western Indian Conservation Workshop in Kenya in November, 1995. She was also the convener of the Northern Indian Ocean Sea Turtle Workshop in January, 1997 at Bhubaneswar under the auspices of IUCN Marine Turtle Specialist Group, UNEP/CMS and U.S. DOC/MNFS in which representatives from 17 different countries participated to draft out a strategy plan for the protection of sea turtles in the Indian Ocean. It is due to her initiative that the technology transfer programme for Turtle Excluder Device (TED) was carried out in November, 1996 in Paradeep, Orissa with representatives from the Department of Commerce, USA. In one

of the most prestigious assignments she **appeared at the WTO on behalf of the Government of India at the shrimp-turtle dispute hearing in Geneva** in September, 1997 and also before the **Apellate body of WTO** in August 1998. She was the expert for a special documentary on olive ridleys prepared by the Films Division, Government of India and the award winning film "Visitors from Sea" based on olive ridleys by SCERT, Orissa in 1993. In March, 1996 she attended the Heads of Commonwealth and Universities Meeting at Malta and contributed significantly to the deliberations on "gender imbalance in University Education System".

She has received laurels from all over the world for her "**breakthrough**" research in homeotic transformation of tadpole tails into legs after vitamin A treatment, published in **Nature** in January, 1992.

For her outstanding research on endangered species, she has been awarded the **Pitamber Pant National Environmental Fellowship Award for 1991**, from the Ministry of Environment and Forests, Government of India, **8th All India Congress of Zoology Gold Medal, 1994; Swami Pranavananda Award for 1994; Justice Raj Kishore Das Memorial Award, 1999; and Parija Award of Bijoygobinda trust 2000**. She is the recipient of **PADMA SHREE**, the highest civilian award from Government of India for her contributions to Science in 1998.

She is the **Chairman, Regional Museum of Natural History**, Bhubaneswar, established by the Ministry of Environment and Forests (MoE&F), Government of India; **Nominee** of the President of India (**Visitor**) of **Indian Institute of Technology** from August 1998; **Vice-Chairman** of the expert committee on **Marine Turtle Conservation** under MoE&F, Government of India; **Member,** for the **B.P. Pal Environmental Fellowship award** committee, MoEF, Govt. of India; **Member**, Task force for aquaculture and mariculture of **DBT**, Government of India; **Member, Panel Advisory Committee**, Department of Science and Technology, Government of India; **Member**, Research Coordination Committee on Eastern/Western Ghats, Ministry of Environment and Forests, Government of India; **U.G.C. Nominee Advisory Committee of CAS Program in Marine Biology** in Annamalai University. She is a **member** of the Governing Body of **Chilika Development Authority** and **Coastal Zone Development Authority, Orissa State Council of Science and Technology**, Govt. of Orissa. She is an **ex-nominee of the Board of Management of Central Institute of Fisheries**

Education, Bombay, Ministry of Agriculture, Govt. of India. She was the **UGC nominee for selection of Vice-Chancellor** for North Orissa University and Fakirmohan University.

Prof. Mohanty-Hejmadi has held many important posts including **President** of Section Zoology, Entomology and Fisheries of 80th Indian Science Congress (1993); **President** of Indian Developmental Biologists Society (1990-92); **President**, Zoological Society of Orissa; **Treasurer**, Zoological Society of India and **President**, Ladies Wing of Nature and Wildlife Conservation Society of Orissa.

She is a Fellow of **Sigma Xi (USA), Phi Sigma (USA), Zoological Society of India (Z.S.I.), Indian Academy of Sciences (F.A.Sc.)** and Association of Aquaculturists **(FAA)**.

Last but not the least, Prof. Mohanty-Hejmadi has received many awards for her contributions in the field of Odissi dance (Central Sangeet Natak Academy; Governor's Plaque, Orissa state council of culture; Sarang Dev Fellowship-Sur Singar Parishad, Bombay; Orissa Sanget Natak Academy Award etc.). She was a member of Central Sangeet Natak Academy (1998-93) and led a cultural delegation to South-east Asia under the auspices of the Indian Council of Cultural Relations in 1989-90. She was a delegate under the auspices of ICCR, Government of India to the International Odissi Festival held in Washington, USA in 2000; She was a member of the Central Film Censor Board, Orissa State Consumer's Forum and was a member of jury for Chetna Samman, 1992-93, Government of Orissa; Advisory Council for Jayadev State Museum, Orissa State Archives and Orissa State Archaeology; Selection Committee of Books for H.K. Mahatab State Library, 1994-96.

During the last few years Prof. Mohanty-Hejmadi has had the distinction of attending the following International Forums on invitation

19th International Symposium on Sea turtle Biology & Conservation	South Padre Island, USA	March 2-7, 1999

Appeared as an expert on sea turtles before the Appellate body on sea turtle-shrimp dispute hearing of WTO on behalf of Govt. of India	Geneva	August 15-16, 1998
Appeared as an expert on sea turtles before the dispute settlement panel of the WTO on behalf of the Govt. of India	Geneva	Sept. 15-16, 1997
Taniguchi Symposium on Developmental Biology	Hongkong	Jan.26-29, 1997
Northern Indian Ocean Sea Turtle Workshop	Bhubaneswar	Jan. 26-29, 1997
Indo-Japanese Seminar on "Morphogens, Genes and Development"	Mysore	Feb. 18-21, 1995. Also the Chairman for one session
19-member Committee for Global Strategic Planning on Conservation of Sea Turtles	Mexico	June 21-27, 1994
14th Annual Sea Turtle Symposium	South Carolina	March 1-7, 1994
International Symposium on "Approaches to the Cellular and Molecular Mechanism of Regeneration"	Okazaki	March 23-25, 1995
Genetic and Epigenetic Controls in Development	Bangalore	Feb.23, 1993
International Symposium on "Cellular and Molecular Aspects of Developmental Regulation"	Pune	Nov. 27–Dec.1 1993
Symposium on Mechanism of Formation & Transformation of Body Structure	Hiroshima	Dec. 3, 1992

At the same time she has also delivered several popular guest-lectures on different topics including "Regeneration and Homeotic Transformation"

The Riddle of Olive Ridley Sea turtle	Habitat Centre, New Delhi	July 7, 2001
Historical prospective of Odissi revival	Odissi Festival 2000, Washington	Oct.6-8, 2000
Origin, evolution and expansion of Odissi Dance	Nehru Centre, London	March 18, 1999
Keynote address, National Environment Day and 20th Anniversary of Foundation Day, National Museum of Natural History, Ministry of Environment & Forests, Govt of India	New Delhi	June 5, 1998
N.V. Modak Memorial Lecture at the 13th National Convention of Environmental Engineers and National Seminar on Environment-2025 A.D.	Bhubaneswar	Nov. 12, 1997
Platinum Jubilee Lecture, Indian Science Congress	New Delhi	Jan 5, 1997
Hokkaido University	Japan	Feb. 1996
Hiroshima University	Japan	Feb. 1996
NIST, Nara	Japan	Feb. 1996
Tsukuba	Japan	March, 1996
Mombasa	Kenya	Nov. 1995
University of Michigan	USA	Mar. 12-14, 1994
Hokkaido University	Japan	Dec. 5, 1992
Tohuku University	Japan	Dec. 7, 1992

Professor Mohanty-Hejmadi at work in her laboratory

Professor Mohanty-Hejmadi with 'Sundari',
a rare hawksbill sea turtle from Orissan coast.

Presiding over the Section of Zoology. Entomology and Fisheries at the 80th Indian Science Congress, held in Goa. To her left are Dr. S.Z. Qasim, the then Member, Planning Commission, Govt. of India and Prof. P.N. Srivastava, the then Vice-Chancellor. Jawaharlal Nehru University, New Delhi.

As Vice Chancellor,
Sambalpur University, Orissa

With Dr. David Owens,
President, 19th Annual Sea Turtle Symposium
Padra Island, USA

Receiving the highest civilian award 'Padmashri' for her contribution to Science
from President of India, Mr. K.R. Narayan

With her Japanese collaborators,
Mr.& Mrs. Chiaki Katagiri in Hokkaido

With Prof. A.P.J. Abdul Kalam (on her left), the then Scientific Advisor to Govt. of India at the inauguration of Northern Indian Ocean on Sea Turtle workshop held in Bhubaneswar and Mr. J.B.Patnaik the then Chief Minister of Orissa.

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THE SEARCH FOR MOLECULES RESPONSIBLE FOR SPERM BINDING AND FUSION TO THE EGG IN VERTEBRATES

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ABSTRACT

The sperm-egg interaction triggers egg activation that increases cytosolic Ca^{++}, increases pH and induces development. The acrosome reaction depends upon highly conserved signal transduction pathways similar to the regulated exocytosis of somatic cells. The fusion of gamete membranes is very much similar to viral function. The main characteristic of fertilization is that due to increase in cytosolic Ca^{++}, eggs are activated which in turn triggers development. Other than calcium, various biochemicals are also involved during fertilization such as PIP_2, IP_3, DAG, integrins, oscillin, fertilin etc. Integrins play a significant role during fertilization. The present paper aims to discuss the role of the above mentioned chemicals during fertilization and suggests a new model for the study of fertilization in the egg of *Discoglossus pictus*.

Key words: Fertilization, signal transduction, surface glycoprotein

INTRODUCTION

Sperm-egg interaction is comprised of distinct events. Sperm first passes through the outermost coats of the egg, then binds to the extracellular matrix that surrounds the egg, *i.e.* zona pellucida (ZP) in mammalian eggs and vitelline envelope (VE) or chorion in other eggs. This initial interaction (primary binding) with a ZP glycoprotein (*i.e.* ZP3) may trigger the exocytosis of the acrosomic enzyme content in the sperm that facilitates passage through the zona of the egg. Thanks to the subsequent exposure of the internal sperm membrane; secondary binding occurs between the inner acrosome membrane and a second zona pellucida glycoprotein (*i.e.* ZP2). This binding avoids removal and loss of the reacted sperm from the ZP. Similarly, fusion between sperm and egg membranes is accomplished in two stages: adhesion and fusion. This interaction triggers egg activation that provokes the increase of cytosolic Ca^{++}, pH changes and the onset of development.

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Mechanisms underlying these events are highly conserved in all cells, regardless of their origin, somatic or germinal. Indeed, the sperm-ZP interaction is comparable to adhesion between cell and extracellular matrix occurring during axon growth, lymphocyte migration etc. The acrosome reaction depends upon highly conserved signal transduction pathways similar to the regulated exocytosis of somatic cells such as neurons and mast cells. Furthermore, fusion between gamete membranes shares several features with viral fusion.

The main characteristics of the fertilization process are the high specialization of the gametes, and the ways the molecular basis of the mentioned biological processes are utilized. Eggs are activated by an increase in cytosolic calcium that triggers development. The mechanism that initiates such release is controversial and has been debated in several review articles (Jaffe, 1990; Nuccitelli, 1991; Foltz and Lennarz, 1993; Whitaker and Swann, 1993; Myles, 1993; Schultz and Kopf, 1993). There are two main hypotheses:1) sperm injection of a factor into the egg to trigger the Ca^{++} increase 2) occupation of an egg receptor by a sperm agonist and hydrolysis of phosphatidylinositol 4,5-bisphosphate [PIP_2]. In the first case Ca^{++} itself could be released from the Ca^{++}-rich sperm. In support of the first hypothesis, the experimental introduction of Ca^{++} into several kinds of eggs provoke an increase of inositol 1,4,5 trisphosphate [IP_3] and subsequent release of more Ca^{++} (Whitaker and Swann, 1993). Other data indicate that injected molecules from sperm homogenate are able to initiate egg development (Dale *et al.*, 1985). In particular, a 33 kDa sperm protein (Oscillin) releases calcium directly from calcium stores in mammalian egg cytoplasm (Parrington *et al.*, 1996). However, further data suggest that this is not the sperm molecule responsible for egg activation (Wolny, 1999).

According to the second hypothesis calcium release might be induced by the hydrolysis of PIP2 which generates IP_3 and DAG through the action of a phospholipase C [$PIP2^{PLC}$ IP3+DAG]. IP3 binds to specific receptors located in the endoplasmic reticulum, allowing propagative Ca^{++} release from this storage (Han *et al.*, 1992; Miyazaki *et al.*, 1993). In support of this hypothesis, it was found that IP3 injection into the egg provokes the release of Ca^{++} that activates the egg (Busa *et al.*, 1985 in *Xenopus*, Nuccitelli *et al.*, 1988 in *Discoglossus*). Also, a strong elevation of PIP2 and IP3 was found during fertilization (Snow *et al.*, 1996).

Lately, data coming from several experiments further strengthen the role of IP3 in fertilization both in mammals and amphibians, and sustain the presence of a receptor,

able to cause its release when occupied by a ligand. PIP2 hydrolysis may be activated along one of the following pathways:1) a sperm ligand activates a tyrosine kinase receptor that is phosphorylated and binds a phospholipase (PLCγ1) through its SH2 domains. As a consequence, PLCγ1 increase its hydrolytic activity on PIP2 (Jaffe, 1990; Creton and Jaffe, 1995; Snow *et al.*, 1996; Kline *et al.*, 1988; Foltz e Lennarz, 1993; Ohlendieck and Lennarz, 1995) as a result of which IP3 and DAG are produced. DAG activates protein kinase C that up to recently was thought to cause intracellular pH release in the sea urchin (Swann and Whitaker, 1985). 2) a sperm ligand activates the classical pathway of PIP2 hydrolysis through the involvement of G proteins. In *Xenopus*, Kline *et al.*, (1991) hypothesized the existence of a G protein typical of the egg and demonstrated that injection of a non-hydrolysable GTP analog triggers egg activation. The involvement of protein G in egg activation was shown in the starfish (Shilling *et al.*, 1994) and in the mouse (Moore *et al.*, 1994).

Integrins (α6β1, in mouse eggs) are possible receptors for spermatozoa, according to immunolocalization and fertilization inhibition assays with specific antibodies (Tarone *et al.*, 1993; Almeida *et al.*, 1995; Chen and Sampson, 1999). Furthermore, synthetic peptides that reproduce the active binding site of integrin ligands (disintegrins) inhibit fertilization (Bronson and Fusi, 1990a,b; Fusi *et al.*, 1992; Myles, 1994). The presence of disintegrins of the ADAM or MDC protein family was clearly demonstrated in mammal spermatozoa (Primakoff *et al.*, 1987; Blobel *et al.*, 1992). In mammalian sperm, fertilin, a heterodimeric molecule composed of α and β fertilin was found in the membrane of the post-acrosomal cap, *i.e.* the region that probably fuses first with the oolemma. Alfa-fertilin has molecular characteristics that may enable membrane fusion, while β-fertilin contains an integrin-binding domain (Primakoff *et al.*, 1987; Blobel *et al.*, 1992). In particular, it was hypothesized that β-fertilin and integrin may represent the functional ligand-receptor system. However, more recently it was found that β-fertilin knock-out mice are fertile, indicating that β-fertilin is not necessarily implicated in sperm egg binding and fusion (Cho *et al.*, 1998). Indeed, a second disintegrin ciritestin was cloned in mouse spermatozoa with a localization in the sperm similar to that of β-fertilin (Yuan *et al.*, 1997). Synthetic peptides that reproduce its disintegrinic domain are able to inhibit fertilization.

Recently, Zuccotti *et al.*, (1998) demonstrated that integrin α6 and β1 gene expression starts in primordial germ cells in female embryos following the first two weeks of life and goes up to the end of oogenesis. The heterodimer α6β1 was localized at the surface of 25-30 μm oocytes.

However, integrins are present on the egg membrane also after fertilization in contrast to the current knowledge that receptors used by the sperms are inactivated after fertilization. Indeed, it was shown that integrin $\alpha 6\beta 1$ is involved in the trophoectoderm migration, suggesting a role of this molecule in embryogenesis and shadowing its possible function in fertilization.

Among lower vertebrates, anurans are a useful model for biochemical studies of fertilization because of the ease in manipulation of their numerous and large eggs and the accumulating knowledge of the activation process in the egg. Injection of IP_3 into the egg induces egg activation in *Xenopus* and *Discoglossus* (Busa and Nuccitelli, 1985; Nuccitelli *et al.*, 1988; Nuccitelli *et al.*, 1993). In *Xenopus,* sperms cause hydrolysis of PIP_2 to produce IP_3 (Stith *et al.*, 1993, which has been found to increase after fertilization (Snow *et al.*, 1996) and trigger a transient increase of cytosolic Ca^{++} (Busa and Nuccitelli, 1985; Kline, 1988). These data suggest the involvement of receptors in the sperm-mediated activation signal. According to Yim *et al.*, (1994) tyrosine kinase or G-proteins (Kline *et al.*, 1991) may be implicated in this process. In amphibians, integrins may play a significant role during fertilization. It has been shown that a molecule of the MDC family (MDC16) is present at the surface of the posteriormost head region *i.e.* a region which is however, not competent for initial sperm-egg fusion. Synthetic peptides, comprehensive of the disintegrin domain of MDC16 or of a similar disintegrin domain (RGD) are able to depolarize the egg membrane and induce intracellular Ca^{++} release (Iwao and Fujimura, 1996; Shilling *et al.*, 1997,1998). In *Xenopus*, Sato *et al.*, (1999) showed the role of a non-receptor tyrosine kinase of the (Src) family (p57) for egg activation. It was observed that p57 increases in concentration and is phosphorylated following egg activation by RGD peptide. It has been hypothesized that p57 activity is connected to integrinic receptor in signal transmission. Indeed micro-injection of synthetic inhibitors of p57 inhibits egg activation by RGD. However, up to date, integrins have been not found at the egg surface, indicating that many open questions still exist in a possible model implicating a disintegrin-integrin system active at fertilization.

Further research on sperm proteases indicate that *Cynops pyrrogaster* sperm extract containing a trypsin-like protease is able to activate the *Xenopus* egg. *Xenopus* sperm extracts have a proteolytic activity whose function in fertilization is unclear. Interestingly, it has been hypothesized that the protease itself may contain a ligand domain for egg receptors or that the protease may hydrolyze the egg receptor exposing an agonistic peptide in the receptor (Mizote *et al.*, 1999). Altogether, these data reveal the complexity of the fertilization process. The study of sperm-egg interaction is flourishing with new data, yet deserve many more integrative experiments.

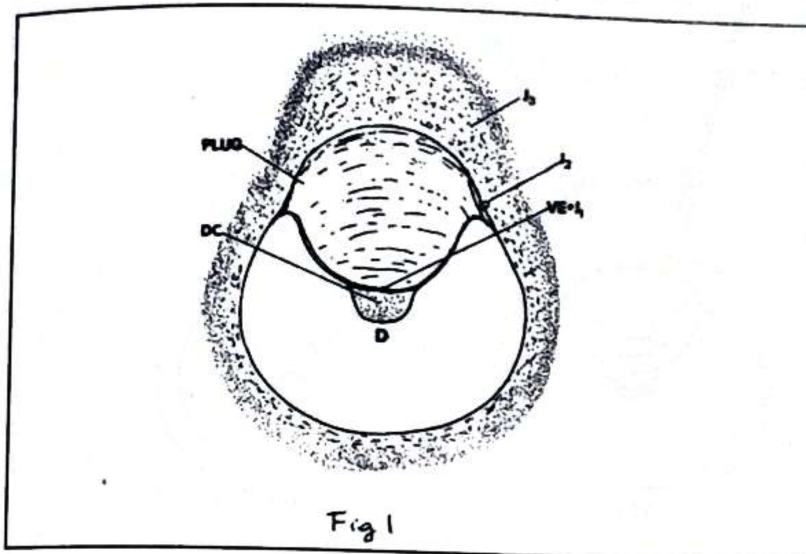
Introducing a model for the study of fertilization, *Discoglossus pictus*

In anuran eggs, 2 main territories are present, the whitish vegetal hemisphere and the pigmented animal hemisphere where fertilization occurs. This remarkable compartmentalization is extreme in the genus *Discoglossus* where the predetermined site of fertilization is further concentrated in the centre of the animal half. Indeed, in the egg of *Discoglossus pictus*, fertilization takes place in the dimple (D) while in the rest of the egg, sperm cannot activate development (Fig.1). *D.pictus* has several advantages over other species for studies aiming at the detection of the surface molecules that take part in the fertilization process. First, experimental evidence indicates that the dimple is a site where the animal hemisphere molecules are highly concentrated, thus simplifying biochemical studies (Talevi *et al.*, 1985; Talevi and Campanella, 1988; Nuccitelli *et al.*, 1988; Gualtieri *et al.*, 1989; Tatone *et al.*, 1993). Second, in the dimple, glycoprotein content keeps the D surface apart from the vitelline envelope (VE) for a depth of about 300 µm. This makes it possible to pinch off the VE manually and to proceed for biochemical analysis of D versus Dimple-less-egg (DLE) cleared of all the extracellular component of the egg. Third, the presence of terminal fucose has been histologically determined only at the surface of the dimple center, D_1. This natural marker can be utilized for the identification of glycoconjugates typical of this region which are probably involved in the fertilization process as they settle on the D_1 surface during dimple formation (Denis Donini and Campanella, 1977). Fourth, the acrosome reaction of *D. pictus* sperm (Figs. 2,3) has been studied in its natural environment and the membranes interacting and fusing with the oolemma have been determined (Campanella *et al.*, 1997). Sperms arrive at the oolemma (Fig. 4) at the initial stage of acrosome reaction, *i.e.* covered with hybrid vesicles (plasma membrane+outer acrosome membrane) and intragametic fusion occurs between the inner acrosome membrane and the oolemma in a narrow funnel located at the centre of the fertilization cone (Campanella and Gabbiani, 1979; Campanella *et al.*, 1997).

Further data render this egg an interesting model for the study of fertilization. Electrophysiological experiments have shown that while the jelly coat and particularly the *plug*, is essential for sperm convergence on the dimple, the dimple itself is endowed with the specific ability to generate typical fertilization potentials (FP) upon insemination followed by the formation of the fertilization cone and the onset of development. More precisely, this property is possessed only by D_1, whereas elsewhere in the dimple, initial sperm penetration occurs but this event does not evoke either typical FP or the onset of development (Talevi and Campanella, 1988).

In D_1, calcium release into the cytosol is triggered as a consequence of either sperm penetration or IP3 injection (Talevi *et al.*, 1985; Campanella *et al.*, 1988; Nuccitelli *et al.*, 1988; Talevi and Campanella, 1988). In fact, only in D_1, the endoplasmic reticulum reaches the critical concentration that permits the calcium elevation event to occur (Campanella *et al.*, 1988; Gualtieri *et al.*, 1992). A privileged localization of specific molecules in D_1 is supported by the finding that in D_1, the concentration and distribution of intramembrane particles is clearly different from that of DLE (Gualtieri *et al.*, 1989). In this regard, electrophysiological measurements and specific staining showed that Cl^- channels are located only in D_1; these are the ionic channels which are depolarized at activation in amphibians and are calcium activated (Nuccitelli *et al.*, 1988 and Talevi *et al.*, 1992). Recently, Maturi *et al.*, (1998) have determined the presence of four glycoproteins of apparent molecular weight of 200,230,260 and 270 kDa on the surface of the egg and precisely only at the dimple territory. Gp200 and gp270/260 are the major glycoconjugates. As these molecules are fucosylated, it is supposed that they constitute the main glycoproteins responsible for the reactivity to UEA I (a lectin binding to terminal fucose), histologically localized at the D_1 surface. Indeed, in ultrastructural and lectin-fluorescence studies, the affinity with UEA I and LTA, a lectin that similarly binds α-L-fucose, is high in D_1 and drastically decreases towards the dimple walls (Denis Donini and Campanella, 1977; Gualtieri and Andreuccetti, 1996). Therefore, the ability to bind UEA I was considered as a marker for determining which molecule among those located at the dimple plasma membrane surface is highly concentrated in the D_1 domain (Maturi *et al.*, 1998).

Several studies have indicated that among the glycoconjugates involved in sperm-egg interaction, fucose is present particularly in its sulfated form, although it is not yet clear whether a specific role exists for this sugar (Bowell *et al.*, 1979 and Stafford *et al.*, 1992, in *Fucus*; Focarelli *et al.*, 1988, 1995 in *Unio;* Segall and Lennarz, 1979, Glabe *et al.*, 1982, De Angelis and Glabe, 1987,1988; Foltz and Lennarz, 1993; Dhume *et al.*, 1996; Rosati and De Santis, 1978, 1980; De Santis *et al.*, 1983 in *Ciona*; Ruttenberg-Barnum and Brown, 1983 in *Limulus*; Ahuja 1982; Dravland and Mortimer, 1988; Jones *et al.*, 1988; O'Rand *et al.*, 1988 in mammals). However, these studies are mainly concerned with glycoproteins located in extracellular coats with some exceptions such as *Fucus* (Bowell *et al.*, 1979; Stafford *et al.*, 1992) and Hamster (Dravland and Mortimer, 1988) whose egg does not have external investments. The fucosylated glycoproteins of D_1 are located in the glycocalyx and/or in the plasma membrane. They are not part of the dimple content, as shown by SDS PAGE pattern on this matrix (Maturi *et al.*, 1998).



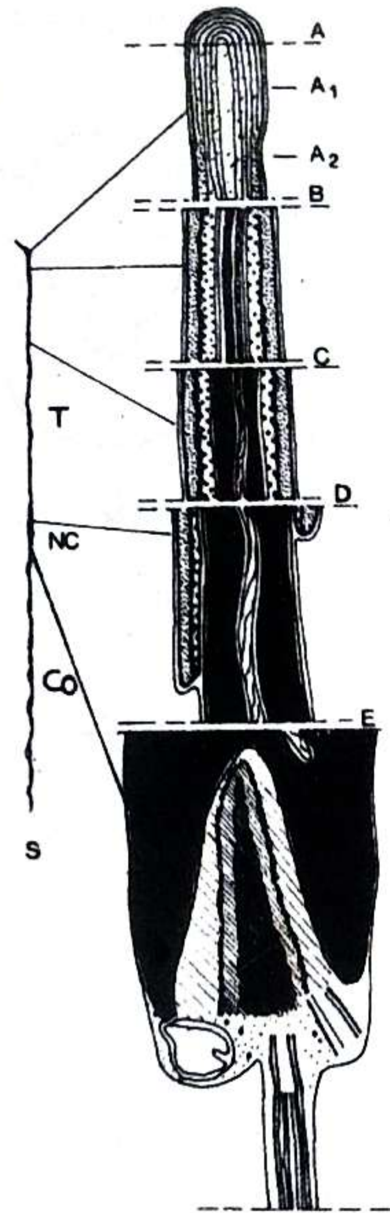


Fig. 2

Fig. 3

Fig. 1 : Schematic drawing of a longitudinal section of *D. pictus* egg. D,dimple; DC,dimple content; J3,Jelly 3; J2,Jelly2. J1,jelly1;VE,vitelline envelope. The *plug* is sitting at the center of the animal hemisphere, in the concavity, which is typical of the eggs belonging to the genus *Discoglossus* ; **Fig. 2 :** Diagram of a single *D. pictus* sperm (S); T,head; NC,neck; Co,Tail. *D. pictus* sperm head is about 1mm long. Remarkable is the acrosome that covers the whole sperm nucleus except for (A) a short posterior segment (level E). It has an anteriormost specialization, the *apical rod,* containing several membrane-like structures (A1-A2). The rest of the acrosome cap has two kinds of granular content (levels B-D).
Fig. 3: Sperm bundles. Sperms (arrow) are ejaculated in bundles of 20. Smear stained with propidium iodide and PSA. X25.

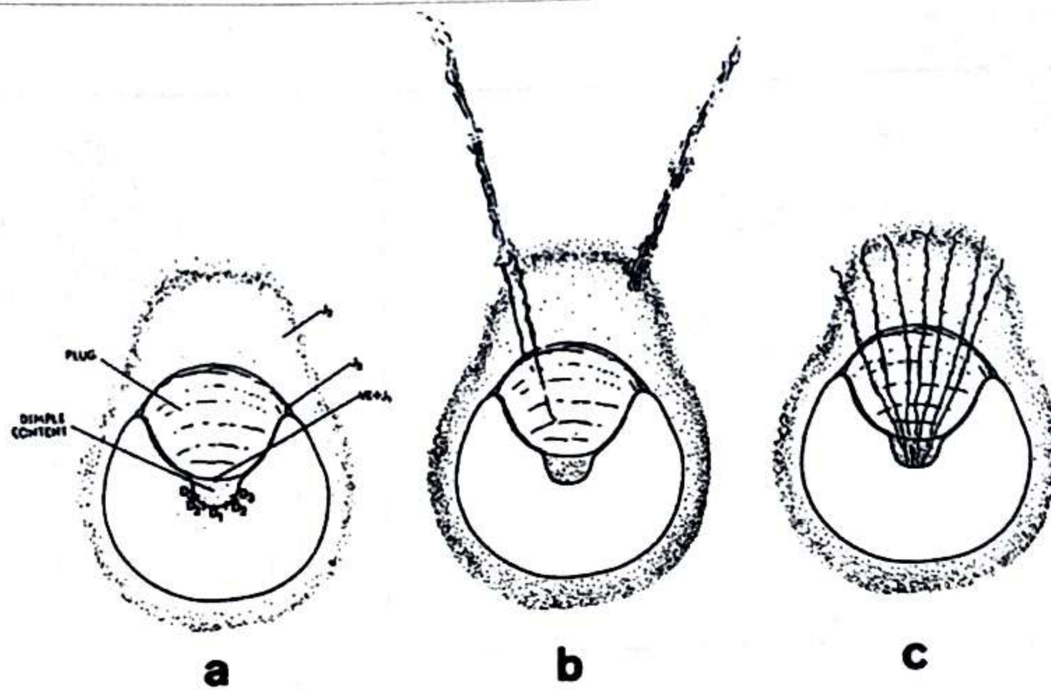


Fig. 4

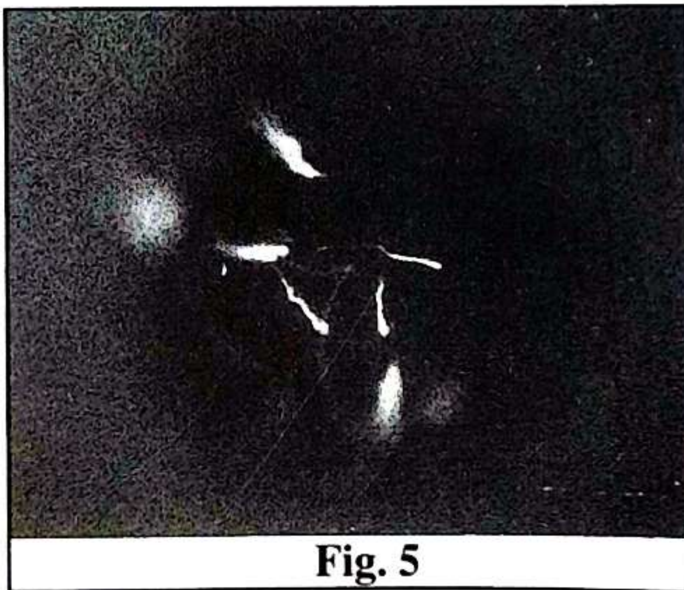


Fig. 5

Fig. 6

Fig. 4 : Schematic diagram of *D. pictus* egg before and in two stages of interaction with sperm. **a** Dimple regions D1,D2, and D3 are indicated. D1 is the only site where fertilization can occurr. **b**,at insemination sperms are ejected from their bundle into the jelly. **c**,sperms become embedded along their whole length in the jelly layers ; **Fig. 5 :** Sperm heads bound on a polystyrene beads where gp 200 was absorbed. DAPI staining. X250 ; **Fig.6:** Thin section of a sperm at initial stage of penetration. Showing plasma membrane-acrosome vesiculation at the surface of the sperm head (arrow); Sperm content released during the acrosome reaction (asterisk); Oolemma glycocalyx (arrowhead). X 66,000.

Gps 200 and gp 270/260 were excised out of polyacrylamide gels, electrocuted and adsorbed to polystyrene beads. Sperms bind in an *in vitro* assay to the gps-coated beads (Fig. 5) thus discriminating between unreacted sperms and sperms having undergone acrosome reaction following treatment with Ca-ionophore. Sperms stuck to gp200 beads before but not after Ca-ionophore treatment. Conversely, for gp270/260 beads, binding occurred after ionophore treatment. Beads stained with DAPI indicate that sperms bind the beads through their heads, thus rendering rather remote the hypothesis that binding might occur in sites where the acrosome is absent (Fig. 5). In contrast, lipovitellin and BSA coated beads, used as controls did not bind the sperms. Two types of molecular interaction may occur between sperm and egg, one mediated by gp200 and the other mediated by gp270/260. In its natural environment, the acrosome reaction is triggered when sperms come into contact with the jelly layer. In a few seconds, they become embedded in the thick jelly plug which sits in a large concavity of the animal hemisphere, and converge through this jelly coat and the VE into the dimple (Fig. 4). Penetration of egg investments take about 15 sec. When sperms arrive at the dimple surface, they are still at a very early stage of hybrid vesicles formation as documented by ultrastructural studies (Campanella *et al.*, 1977) (Fig. 6). The first contact occurs between sperm plasma membrane in the process of vesiculation and the D_1 glycocalyx while fusion occurs between the inner acrosome membrane and the oolemma deprived of the antennular glycocalyx (ultrastructural observations), when a release of acrosome content is observed at the site of gamete interaction (Campanella *et al.*, 1997) (Fig. 6). This suggests that acrosomal enzymes may clear the glycocalyx after binding has occurred, thus permitting a second interaction and fusion to occur (Maturi *et al.*, 1998; De Santis *et al.*, 1992; Sato *et al.*, 1999). In this connection unpublished observations reveal that, following Ca-ionophore treatment, *D.pictus* sperm release chymotrypsin and trypsin-like proteases (Infante and Amirante, unpublished) similar to *Cynops* (Sato *et al.*, 1999) and *Bufo* (Yamasaki *et al.*, 1988). These data can be compared to the ultrastructurally determined heterogeneous acrosomal content of *D. pictus* sperm (Campanella *et al.*, 1997).

Presently, the research is concentrated to molecular micro-sequencing of *D. pictus* gp200, the most prominent among the D_1 glycoconjugates. Therefore, *D. pictus* model is ready to offer new data on the identification and separation of a molecule related to the oolemma binding *in vitro* homologous sperm. In *Xenopus*, where information is available on MDC16 (Shilling *et al.*, 1997, 1998), it is not clear however, sperm perform an acrosomal reaction. Similarly, the characteristics of this egg surface render practically impossible for the study of surface molecules implicated in sperm-egg interaction. In this regard, it will be interesting to know the possible relationship between *D. pictus* gp200 and integrin-like molecules.

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THE MECHANISMS OF LIMB REGENERATION IN URODELES AND ANURANS

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ABSTRACT

The amphibians provide excellent model systems to study the remodelling of the body plan. As well known, urodele amphibians have prominent ability to regenerate their limbs throughout their life, whereas anurans lose this ability during metamorphosis. This apparent difference has attracted biologists because these two species of animals are phylogenetically close to each other and grouped into the same class in taxonomy. This review summarizes the current status of understanding of the molecular mechanism of amphibian limb regeneration placing an emphasis on the difference in the regenerative ability between urodeles and anurans. Urodeles can reorganize the morphogenetic field in the blastemas of regenerating limbs as in limb buds, but anurans lose the expression of signalling molecules for pattern formation during metamorphosis. Synthesis and degradation of extracelluar matrices during limb regeneration probably proceed in different ways in the two species. Origin and properties of blastemal mesenchyme, and the potential for the transdifferentiation of stump tissues are also different. When we thoroughly reveal the difference in the mechanism of limb regeneration at the molecular level between the two, it might be possible to produce genetically-engineered frogs which regenerate their limbs throughout their life.

Key words : Limb regeneration, urodeles, anurans

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INTRODUCTION

The amphibians, urodeles and anurans, have several advantages to study the remodelling the body. Regeneration as well as metamorphosis is a biological phenomenon unique to amphibians (Yoshizato, 1989). Although regeneration can be observed commonly among the animal kingdom, amphibians are outstanding in their regenerative ability. The regeneration of large parts of the adult body is not a rare event among invertebrates. However, the urodeles such as newts and axolotls are the only vertebrates that can regenerate large parts of the body in adulthood. Urodeles can regenerate lens, limbs, tails and jaws (Brockes, 1997) as well as internal organs such as heart and intestine (Stocum, 1995). Interestingly, anurans which are phylogenetically close to urodeles, cannot retain the regenerative ability throughout their lives. *Xenopus laevis* larvae are able to regenerate lens from the outer cornea and this capacity decreases gradually between stages 50 and 58 (Filoni *et al.*, 1997) contrasting adult urodeles which can regenerate lens from pigmented iris epithelial cells. Regeneration of limbs is also observed in anuran larvae. However, this ability is lost during metamorphosis. Dent *et al.*, (1962) reported detailed descriptions about the decline in the regenerative capacity of *Xenopus* hind limbs. At stage 51, *Xenopus* larvae regenerate complete limbs as in normal development. However, as the animals grow, the extent of heteromorphosis increases. Amputation at stages 53 and 55 induces incomplete limbs with 4 and 3 digits, respectively.

In this review, comparing the differences in limb regeneration between urodeles and anuran, we address the following questions on the basis of current understandings on amphibian limb regeneration at the molecular level. How can we explain the regenerative ability in urodele limbs? What are the reasons which might explain the lack of the ability of complete regeneration in adult anuran limbs? We also discuss a possible method to endow adult anurans with the ability of limb regeneration.

Pattern formation

Pattern formation is a common event in both development and regeneration of limbs, and is thought to utilize a similar molecular program in both processes. Grafting of a regenerating blastema into a developing limb produced a chimeric limb which had a well-organized morphology (Muneoka and Bryant, 1982), suggesting that the signalling molecules are shared in both processes. In the developing limb buds, the zone of polarizing activity (ZPA) was identified as the mesenchymal region of a posterior margin that can induce mirror-image digit duplications when grafted to the anterior margin of a host limb bud (Saunders and Gasseling, 1968; Tickle *et al.*, 1975). Thus, ZPA is

regarded as the centre for the anterior-posterior patterning. *Sonic hedgehog (shh)* gene was cloned as a vertebrate homolog of *Drosophila* segment polarity gene *hedgehog (hh)*. The expression of *shh* is restricted to ZPA. The application of retinoic acid (RA), a molecule known to alter the positional value and cause digit duplication, induced an ectopic expression of *shh* in anterior cells. The implantation of *shh*-expressing cells mimicked the graft of ZPA (Riddle *et al.*, 1993). The results indicate that *shh* is a candidate gene responsible for the activity of ZPA. The expression of *shh* gene was also observed in mesenchymal cells of a posterior region in the blastema of regenerating limbs (Imokawa and Yoshizato, 1997, 1998), which suggested that ZPA is formed in regenerating newt limbs as in the developing limb bud. The experiments with reversed antero-posterior and dorso-ventral grafts also supported this notion. We demonstrated the ectopic expression of *shh* in a new posterior region in addition to the original region of the grafts (Fig.1). In the regenerating axolotl limbs, a similar expression pattern of *shh* was observed and exposure to RA induced an ectopic expression of *shh* in anterior cells in regenerating blastema as in developing limb buds (Torok *et al.*, 1999).

Products of *Hox* genes have been shown to be involved in the pattern formation of limbs during vertebrate embryonic development. Genes of *HoxA and HoxD* are suggested to be related to the specification of proximal-distal and anterior-posterior axes, respectively (Yokouchi *et al.*, 1991). In addition, the results of the study on a forced expression of these genes in the limb buds suggested that the products of Hox gene determine the positional value in the developing limbs. The over-expression of *HoxD11* in chick leg buds causes the transformation of digit 1 to more posterior digit 2 (Morgan *et al.*, 1992). An ectopic expression of *HoxA*13 induces a homeotic transformation of the zeugopod cartilage to the more distal cartilage (carpus or tarsus) (Yokouchi *et al.*, 1995). As in developing limbs, *Hox* genes are also expressed in the blastema of regenerating limbs. Expression of *HoxA and HoxD* is not observed in normal unamputated axolotl limbs. Thus, it can be said that these genes are re-expressed in the blastema. The expression patterns of *HoxA and HoxD* complexes during limb development of axolotl resemble those of mouse and chick (Gardiner *et al.*, 1995; Torok *et al.*, 1998). Comparison of the temporal expression pattern between regeneration and development in axolotl limbs showed that *HoxD* genes were expressed in a similar time course in both cases (Torok *et al.*, 1998). However, expression patterns of *HoxA* genes were different between blastemas and limb buds. In the developing limb bud, HoxA genes are expressed in a temporally and spatially regulated manner. A more 3' gene, *HoxA9*, was expressed at an earlier stage than

HoxA13, a more 5' gene. In addition, the region expressing *HoxA9* was located more proximal than the region expressing *HoxA13* (Gardiner *et al.*, 1995). On the contrary, in the regenerating limb, *HoxA9* and *HoxA13* were expressed simultaneously and the typical spatial pattern observed in developing limbs was established at a late stage of regeneration (Gardiner *et al.*, 1995). These results obtained from the studies of *shh* and *Hox* genes suggest that regenerating and developing limbs utilize mostly the same genes for the patterning.

As described above, the pattern formation in regenerating limbs recapitulates that in developing limb bud at the level of gene expression. Therefore, it can be said that urodeles possess some mechanism to reactivate the genes at the time of limb amputation which have been expressed in limb development, and to reorganize a proper morphogenetic field for the pattern re-formation. Adult anurans apparently do not have such mechanisms because they only regenerate spike-like limbs without any patterns. In fact, Endo *et al.*, (1997) observed a declined expression of *shh* in regenerating limbs which had been amputated at advanced stages in *Xenopus* larvae. However *fgf-8*, a gene related to the formation of apical ectodermal ridge (AER) during limb development, was expressed in the regenerating limbs which had been amputated at stage 55 (Christen and Slack, 1997) or young adult stage (T.Endo– personal communication), implying the formation of the functional apical epidermal cap (AEC) in anuran blastemas which are not able to produce the regenerates with the complete limb pattern. The AEC is regarded as the region in the blastema homologous to AER in the developing limb bud (Stocum, 1995). The AEC supports the proliferation and inhibition of precocious differentiation of blastemal mesenchymal cells as AER does (Globus *et al.*, 1980). The experiments of grafting frog skin to axolotl suggest that the AEC produced by anuran is functional (Carlson, 1982). Dedifferentiation of amputated stump tissues and formation of blastema proceed normally in the axolotl limbs covered by the wound epidermis in *Rana pipiens* (Carson, 1982). From these studies, the decline in regenerative ability during metamorphosis in anurans might be reasoned by a loss of signalling molecules for the re-patterning of the blastemal mesenchymal cell. However, D'Jamoos *et al.*, (1998) suggested the difference in the expression of fibroblast growth factor receptors (FGFR) in the AEC of regenerating (stage 53) and non-regenerating limbs of *Xenopus* froglets. FGFR-1 and -2 expressed in both AEC and mesenchyme in blastema of regenerating limbs but are absent from AEC of the non-regenerating blastema. At present, the relation between the expression of FGFR in AEC and the regenerative ability is not clear, because FGFR-1 is absent from newt AEC (Poulin *et al.*, 1993).

Savard *et al.*, (1988) reported that newt *HoxC6* (formerly *NvHbox 1*) was expressed in the unamputated normal limbs, whereas the *Xenopus* homolog of *HoxC6* (formerly *HlHbox 1*) is not expressed in the adult limbs. This study suggests the pre-existence of morphogenetic field in unamputated normal limbs in adult urodele but not in adult anuran.

Remodelling of Extracelluar Matrix

Synthesis and degradation of extracelluar matrix (ECM) are thought to be essential processes in regeneration (Stocum, 1995). The collagenolytic activity of amputated newt limbs was first described by Grillo *et al.*, (1968). The highest collagenolytic activity was observed in the stump tissue near the amputation site rather than a blastema. The activity was highest at 15 d after amputation when dedifferentiation continues, and decreased as redifferentiation proceeded, suggesting some correlations of ECM degradation and dedifferentiation taking place in the stump tissues. The collagenolytic activity found in limb regeneration of urodeles was biochemically characterized and identified as matrix metalloproteinases (MMPs) (Yang and Bryant, 1994). The enzymes require Ca^{++} and Zn^{++} for their activity and are secreted as latent forms. Yang and Bryant (1994) performed a zymographic analysis on the newt MMPs and found a 90-kDa gelatinase/collagenase in regenerating limbs. The time course of the activity of this enzyme showed a good agreement with the collagenolytic activity reported by Grillo *et al.*, (1968). Interestingly, the suppression of this enzymatic activity at the palette stage was inhibited by the denervation or the prevention of AEC formation. The down-regulation of MMP activity at the palette stage seems to be quite important for the complete pattern formation of regenerating limbs because at this stage blastemas start redifferentiation and pattern formation and concommitantly, the stump tissue ceases to redifferentiate (Iten and Bryant, 1973)

Miyazaki *et al.*, (1996) cloned four cDNAs of newt MMPs (nMMP9, nMMP3/10–a, nMMP3/10-b and nMMP13) and concluded that nMMP9 was a 90-kDa gelatinase as reported by Yang and Bryant (1994) after comparing the molecular weights, substrate specificity, and time course of their expression during limb regeneration. Three newt MMPs-MMP9, MMP3/10-a, and MMP3/10-b were not expressed in normal unamputated limbs but were up-regulated in regenerating limbs (Miyazaki *et al.*, 1996; Fig. 2). *In situ* hybridization analysis of MMP9 and MMP3/10-b suggested interesting functions of MMP family during limb regeneration. Expression of MMP9 showed a bi-phasic pattern in regenerating limbs of axolotl (Yang *et al.*, 1999). In the first phase, MMP9 was detected only in the wound epidermis at 2 h after amputation but not in the normal skin. The expression of MMP9 in wound epidermis continued for 2 d. Then MMP9 was again

detected in the mesenchyme at early bud stage and in the amputated site of the bone at medium bud stage. This second phase expression is probably related to the removal of damaged cartilage matrix. The epidermal expression of MMP9 in the first phase might be a cause for the retarded reformation of the basal lamina (BL) and probably facilitates the interaction of epidermis and mesenchyme of the blastema. We observed a similar expression pattern of MMP3/10-b in the wound epidermis (Miyazaki *et al.*, manuscript in preparation). The interaction of epidermis and mesenchyme in the blastema during limb regeneration is thought to be important for regeneration. It is well known that denervation inhibits the early stages of regeneration (Singer, 1952). Bryant *et al.*, (1971) observed the premature formation of BL, which was termed the adepidermal membrane in the cited paper, in denervated regenerates. Interestingly, denervation delays the onset of MMP9 expression in the regenerates, suggesting the role of MMP9 in preventing the formation of BL prematurely and signifying the importance of epidermal-mesenchymal interactions in the early phase of regeneration.

Also in anurans, there is a correlation between the formation of BL and regenerative ability. Dent (1962) reported that heteromorphic limb regeneration was first noticed at stage 53 in *Xenopus*. By stage 52, BL was not evident subadjacently to the apical tip of wounding epidermis in the regenerates, but present in the regenerates of stage 53 to 55 tadpoles (Khan and Liversage, 1990). Furthermore, Neufeld and Day (1996) suggested that delayed closure of BL is a feature of regenerating appendages distinguishable from non-regenerating ones. The continued direct epithelial-mesenchymal interaction is essential for the accumulation of a critical mass of blastemal cells. From this context, it will be interesting to see the expression pattern of MMP9 and MMP3/10 in the adult anuran limb regeneration.

Formation and Maintenance of Blastema

Production of blastemal mesenchyme or blastema-like undifferentiated mesenchymal cell population is necessary for regeneration. The relationship between the regenerative ability and the formation of blastema has been reported in many regeneration models. For example, fetal and newborn mice can regenerate the digit tips when amputated at the distal region but not at proximal region (Reginelli *et al.*, 1995). They observed the accumulation of undifferentiated cells expressing *Msx1* in the regenerates produced by distal amputation of the digits. However, proximal amputation did not induce such cell accumulation.

Adult anurans that only regenerate heteromorphic spike-like limbs also produce blastema-like structures, which consist of undifferentiated mesenchymal cells and AEC

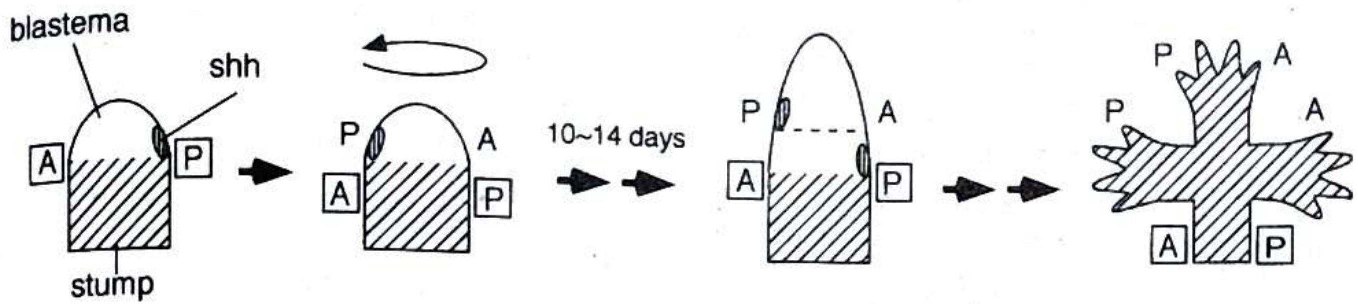

Fig. 1

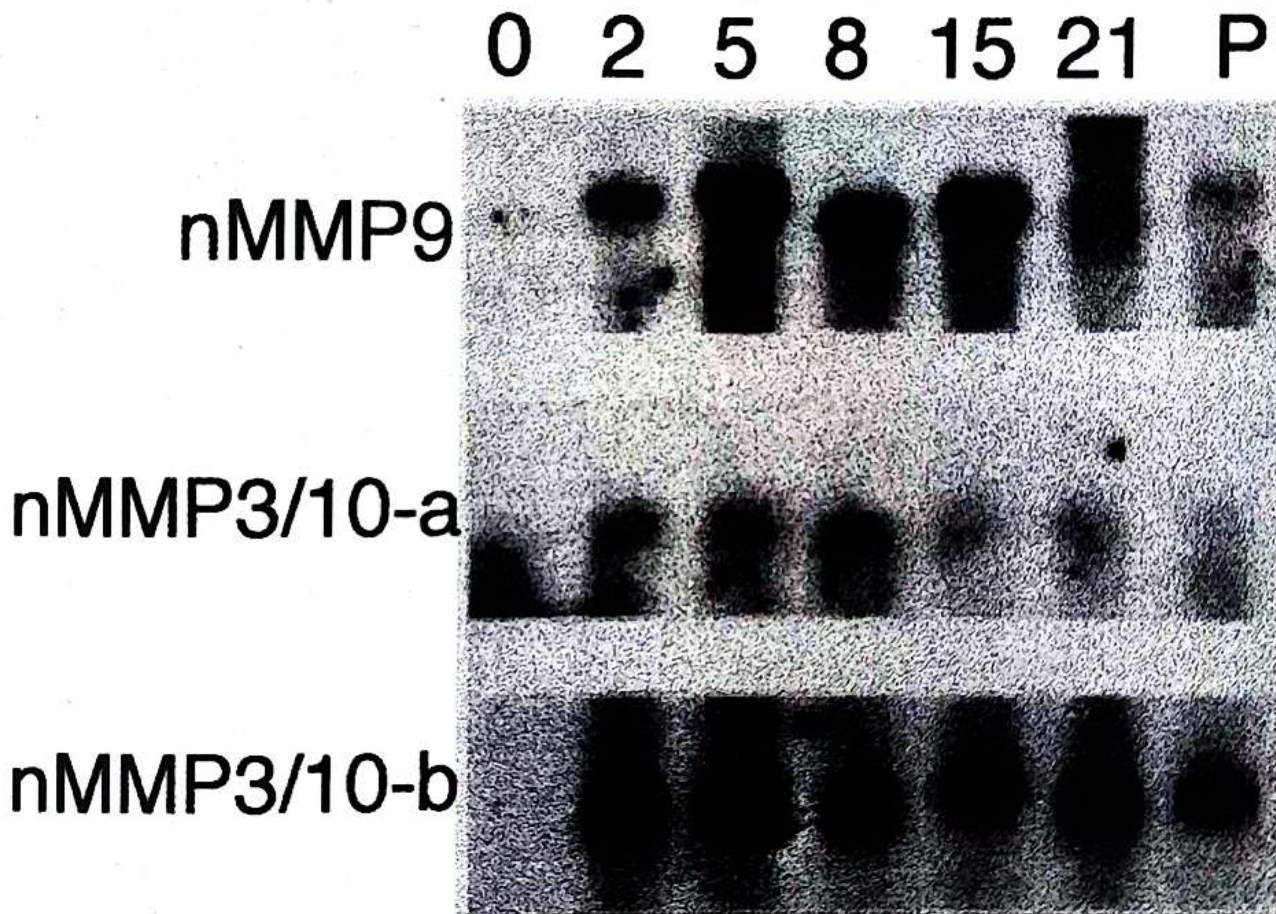

Fig. 2

Fig.1 : Schematic drawings of the axial reversal graft and *shh* expression. Blastemas at medium bud stage are cut and rotated 180°. Resulting blastemas express *shh* in a new posterior region in addition to the original site at 10-14 days after grafting. Different positional values are newly formed by these two *shh*-expressing regions. Therefore, three limbs were regenerated by this operation. A-anterior; P-posterior. From Imokawa and Yoshizato (1998) ; **Fig.2 :** Expression pattern of newt MMPs during regeneration. Expression of MMPs were examined in normal limbs (0), regenerating limbs at the indicated days after amputation (2,5,8,15,21), and palette-stage limbs(P). Probes used for hybridization are indicated on the left side of each panel. From Miyazaki *et al.*, (1996).

(Dent, 1962; T. Endo–personal communication). We attempted to characterize the state of differentiation in the blastemal mesenchyme of both urodeles and anurans at the molecular level, by determining the expression of *Id* ("inhibitor of DNA binding" or " inhibitor of differentiation") genes (Shimizu-Nishikawa *et al.*, 1999). The *id* proteins belong to the members of helix-loop-helix (HLH) type transcriptional regulators (Benezra *et al.*, 1990). *Id* proteins can form heterodimers with other HLH proteins, such as MyoD or E protein through the HLH region but the heterodimer thus formed cannot bind DNA elements, such as E box because *Id* proteins lack the basic region responsible for DNA binding (Benezra *et al.*, 1990). Therefore, the transcription of tissue-specific genes such as muscle creatin kinase gene which is transcribed with the heterodimers of MyoD and E protein, is inhibited by *Id* protein. In addition, over-expression of *Id* protein inhibits the differentiation of the C2C12 muscle cell line (Jen *et al.*, 1992). Thus, we hypothesized that the blastema, a mass of undifferentiated cells, expresses *Id* genes at a high level. Furthermore, we speculated that their expression might be different between urodeles and anurans. As we expected, the expression of two *Id* genes, *Id2* and *Id3*, was not detected in the normal unamputated limbs and increased in the blastema of both urodeles and anurans and was not detected in the normal unamputated limbs. The expression pattern of the two *Id* genes were very similar at early medium-bud stage blastema of both species. *Id2* was expressed predominantly in the AEC and *Id3* was expressed at a high level in the precartilage condensation (Fig. 3). However, the expression of *Id3* was differently regulated in the late stage of regeneration in these two animal species. As shown in Fig. 4A, the expression of *Id2* and *Id3* remained high at the digits stage when redifferentiation and pattern formation proceeded in urodeles. On the other hand, anuran spike-like regenerates, which correspond to urodele regenerates at the palette-digits stage, also expressed *Id2* at a high level, but interestingly the expression level of *Id3* decreased to the level of normal limbs (Fig. 4B). The earlier decrease of *Id3* expression is probably related to the precocious redifferentiation of cartilage and results in incomplete limb regeneration in adult anurans. In contrast, the continued high level expression of this gene in urodeles is related to the fact that urodele regenerates contain undifferentiated cells even at a late stage of regeneration, which may be necessary for the pattern formation.

The origin of blastemal mesenchyme is different between urodeles and anurans. Dent (1962) suggested the origin of blastema of stage 60 *Xenopus* tadpoles which only regenerate spike-like structure. These are the chondrocytes and the fibroblasts. In amputated limbs of larvae, muscles contract (Dent, 1962) and show no signs of dedifferentiation (Khan and Liversage, 1990). Spike-like regenerates of adult *Xenopus*

mostly consist of cartilage. Muscle tissue are hardly seen in the regenerates (Dent, 1962; Kurabuchi and Inoue, 1983). This observation shows that blastemal mesenchymes of adult anurans do not contain muscle progenitor cells. There are experimental data which suggest that the urodele blastema is formed by cells derived from cartilage, dermis and muscle (Namenwirth, 1974; Dunis and Namenwirth, 1977; Muneoka *et al.*, 1986). Especially, the muscle is believed to produce all types of mesodermally-derived tissues in the regenerates, such as muscle, connective tissue and cartilage but the cartilage scarcely gives rise to the muscle (Namenwirth, 1974). This suggestion was obtained from experiments of grafting of the muscle from triploid axolotls to X-irradiated diploid host. There is a possibility that the grafted muscle included several types of cells. Thus, the multipotency of muscle cannot be concluded from this experiment. To examine the multipotency of muscle, Lo *et al.*, (1993) used the combination of cell culture system and grafting. They cultured myoblasts from newts and induced muscle differentiation *in vitro* by lowering the concentration of serum. Differentiated multinucleate myotubes were selectively labelled by the injection of rhodamine-conjugated dextran and were implanted into the regenerating blastema. After implantation, the labelled mononucleate cells were found in the blastema and in the cartilage of regenerate, suggesting that the myotubes dedifferentiate and transdifferentiate into chondrocytes. Taking this into consideration, it can be concluded that the urodele muscle can dedifferentiate into the multipotent blastemal mesenchyme. In contrast, the anuran muscle cannot dedifferentiate and therefore, do not give rise to blastemal cells that differentiate into muscle. Thus, the dedifferentiation of muscle is an important event for the complete regeneration.

Possible methods to improve regeneration in adult anuran

In this review, we have discussed about the difference of limb regeneration in urodeles and anurans. Based on these discussions, we propose possible methods to induce "an improved regeneration" in adult anurans. As described in the section on pattern formation, regenerating limbs of adult anurans lose the ability to organize a proper morphogenetic field. Therefore, if we can produce such a field in a blastema by the ectopic expression of appropriate genes, adult anurans can regenerate limbs with the normal pattern. One candidate is *shh* gene, because its expression is gradually lost in the regenerating limbs during *Xenopus* metamorphosis (Endo *et al.*, 1997). However, there is a possibility that the ectopic expression of *shh* is insufficient for the pattern re-formation of adult anuran regenerates if the anuran tissues cannot respond to the *shh* signalling. Our observations (Shimizu-Nishikawa *et al.*, 1999) have suggested that mesenchymal cells in the blastema are different between urodeles and anurans. The anuran blastema cannot retain undifferentiated states at a late stage of regeneration

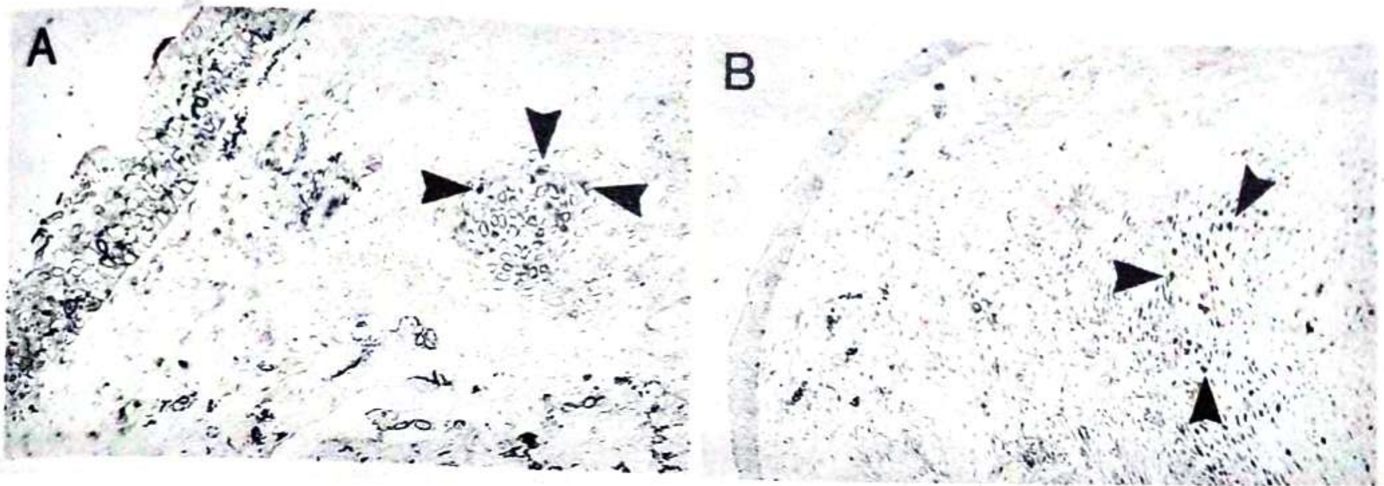

Fig. 3

newt

Xenopus

normal early bud medium bud late bud palette digits

normal blastema spike

Id2

Id3

Fig. 4

Fig.3 : High *Id3* expressed in the precartilage condensation. *In situ* hybridization was performed with sections of late bud stage of newt blastema (A) and early-medium bud stage of *Xenopus laevis* blastema (B). Arrowheads indicate *Id3*-expressing cells in the precartilage condensation. From Shimizu-Nishikawa *et al.* (1999) ; **Fig.4 :** Expression of *Id* genes. The expression was examined during limb regeneration in urodeles (A) and anurans (B). Stages of samples and probes are indicated on the top of the panels and left side of the panels, respectively. From Shimizu-Nishikawa *et al.*, (1999).

suggesting the difference of the response to the signalling molecules. One factor which is necessary to prevent the precocious differentiation of cartilage in the urodele blastema is AEC. Removal of the AEC induces an earlier differentiation (Globus *et al.*, 1980). However, AEC of adult anuran was shown to be functional; it induced the formation of normal blastemal mesenchymes in urodeles as described previously (Carlson, 1982). Irrespective of the formation of functional AEC in adult anurans, anuran blastemal mesenchymes apparently cannot retain undifferentiated state. This discrepancy could be explained by the fact that the adult anuran blastema prematurely forms BL which is thought to block continuous epidermal-mesenchymal interactions (Khan and Liversage, 1990; Neufeld and Day, 1996). Several experiments were carried out to cause trauma and irritation repeatedly on the amputation surface of adult anurans. These treatments retarded the formation of BL. Rose (1944) and Kurabuchi and Inoue (1982) reported that repeated treatment with NaCl solution enhanced regeneration. A similar result was obtained by treatment of the amputated adult anuran limbs with dimethyl sulfoxide (Cecil and Tassava, 1986). Neufeld (1980) performed a similar experiment on adult mice. Adult mouse toes were amputated at the proximal phalanx. The wound epidermis was removed repeatedly and also treated with NaCl solution repeatedly. Interestingly, these treatments induced the accumulation of blastema-like mesenchymes under the wound epidermis, but did not induce limb pattern formation (Neufeld, 1980). Although limb regeneration was stimulated by these treatments, the complete regeneration of adult anurans has not been accomplished yet; especially, the induction of limb re-patterning is a difficult task. It appears that in order to induce the complete limb regeneration in adult anurans, we will need appropriate and efficient ectopic expression methods to produce a morphogenetic field in the blastema as in limb development.

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PROTEASOMES IN MELANOMA CELLS-REGULATION IN MELANIN BIOSYNTHESIS, CELL DIFFERENTIATION AND CELL TRANSFORMATION-FEW FACTS AND SPECULATIONS

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ABSTRACT

Proteasomes, the multicatalytic proteases, are involved in the non-lysosomal, ubiquitin-mediated protein degradation pathway. They are multisubunit ubiquitous proteins and distributed both in the cytoplasm and in the nucleus. They are also implicated in multiple other functions like antigen presentation, cell cycle regulation and embryogenesis and possibly in cell differentiation. Recent reports on their possible role in melanin biosynthesis in melanoma cells, attest further their involvement in the regulation of tissue-specific gene expression and possibly in cell differentiation. In this article, I have tried to review previous research work of others and our current work on this area with an emphasis on the future research and their potential use in human applications.

Key words: Multicatalytic proteases, mouse melanoma cells, tyrosinase activity, differentiation, transformation

INTRODUCTION

Proteasomes: Proteasomes are multi-subunit protein complexes which are involved in ubiquitin-mediated, non-lysosomal intracellular protein degradation pathway. They have been reported from a variety of organisms and have been described in the literature by various names, namely, prosomes, multicatalytic proteases (MCPs) or proteinases, high molecular weight proteases, depending on their co-purification with other particles and prospective functions (reviewed in Scherrer and Bey, 1994). However, recent reports on their identity with the MCPs (Arrigo *et al.*, 1988; Falkenburg *et al.*, 1988; Pal and Murakami, 1988, 1995) have established their proteolytic activity

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This paper is dedicated to Prof.P.Mohanty-Hejmadi on her 60th birthday for her pioneering work on homeotic transformation in frogs and for her consistent and valuable contribution in teaching and research in Developmental Biology.

as the principal function; they were therefore renamed as proteasomes. Since then there has been a boost in the research activity on these high molecular weight proteases that are involved in intracellular protein turnover pathway (Hilt *et al.*, 1993). In this major pathway of selective protein degradation in eukaryotic cells, ubiquitin is used as a marker that targets cytosolic and nuclear proteins for rapid proteolysis by proteasomes (Cooper, 1997). It is now well established that the proteasomes (MCPs) exist in two molecular forms, 26S and 20S proteasomes.

26S and 20S proteasomes : The 20S proteasome, a barrel-shaped structure as seen in the electron microscope (Fig.1A) is composed of four rings each containing seven subunits (α7, β7, β7, α7). Based on sequence similarity, all 20S proteasomal subunit sequences may be classified into two groups, alpha and beta, each group having distinct structural and functional roles. The seven alpha-subunits comprise the outer rings and the seven beta-subunits the inner rings of the 20S proteasome. Each subunit is located in a unique position within the alpha or beta-rings. The molecular weight of the subunits ranges between 21kDa and 36kDa. A subfraction of 20S proteasomes (prosomes) contains in addition, a small RNA of 50 to 150 nucleotides long which in case of mammalian prosomes, turns out to be a reverse primer of the retroviral tRNA type (Nothwang *et al.*, 1992a). The estimated size of 20S proteasome is 720 kDa (Coux *et al.*, 1992; Tanaka *et al.*, 1992). These complexes have been characterized from a variety of organisms from archeobacteria to man and are found to be evolutionarily conserved (Scherrer and Bey, 1994). Fig. 1B shows the typical protein pattern of 20S proteasomes analysed by SDS PAGE.

The 26S proteasome consists of the 20S proteasome as the catalytic core and over twenty additional proteins, ranging in molecular weight from 25 to 10 kDa, located in a distinct complex called the `PA700 proteasome activator' or the 19S factors (inhibitors, activators) including ubiquitin. The 26S proteasome may be involved in ATP-dependent, ubiquitin-mediated protein degradation (Li and Etlinger, 1992; Goldberg and Rock, 1992), except in case of ornithine decarboxylase degradation which is ubiquitin-independent (Murakami *et al.*, 1992).

Unique characteristics of proteasomes : The unique, highly compact and cylinder-shaped structure makes the proteasomes, the 20S proteasomes in particular, one of the most stable complexes in the cell. They are resistant to non-ionic detergents, low concentrations of SDS, desoxycholate, salt solutions of high ionic strengths and up to 1% sarkosyl (Schmid *et al.*,1984; Pal *et al.*, 1994). Based on their sarkosyl resistance,

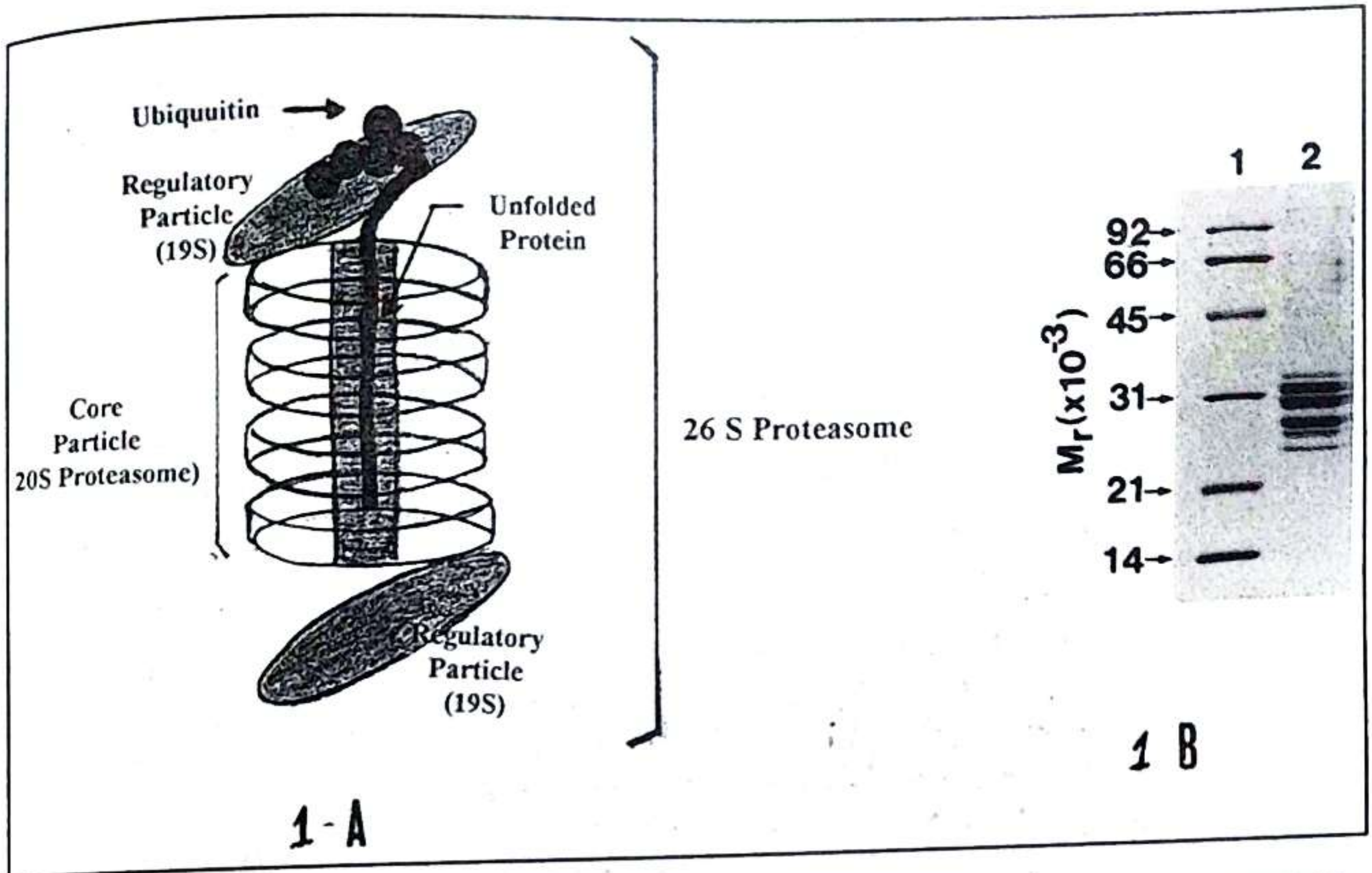


Fig 1 : (A) Schematic representation of proteasome structure; **(B):** Protein Profile of proteasomes analysed by SDS PAGE. A set of 8-10 polypeptides of small size are part of proteasome (20S), seen in lane 2; lane 1 contains molecular weight marker proteins.

20S proteasomes have been purified by a single-step procedure as intact 20S particles from oocytes and embryos (Pal *et al.*, 1994). Interestingly, however, two bivalent metal ions, Zn^{++} and Cu^{++} (0.01 to 1.0 mM) inactivate the MCP (proteasomes) instantaneously and dissociate the complexes into their subunits (Nothwang *et al.*, 1992b). This property may eventually be responsible for *in vivo* dissociation as well as reassociation of the subunits of this complex. It has been established that the 20S proteasome-mediated protein degradation is ATP-independent.

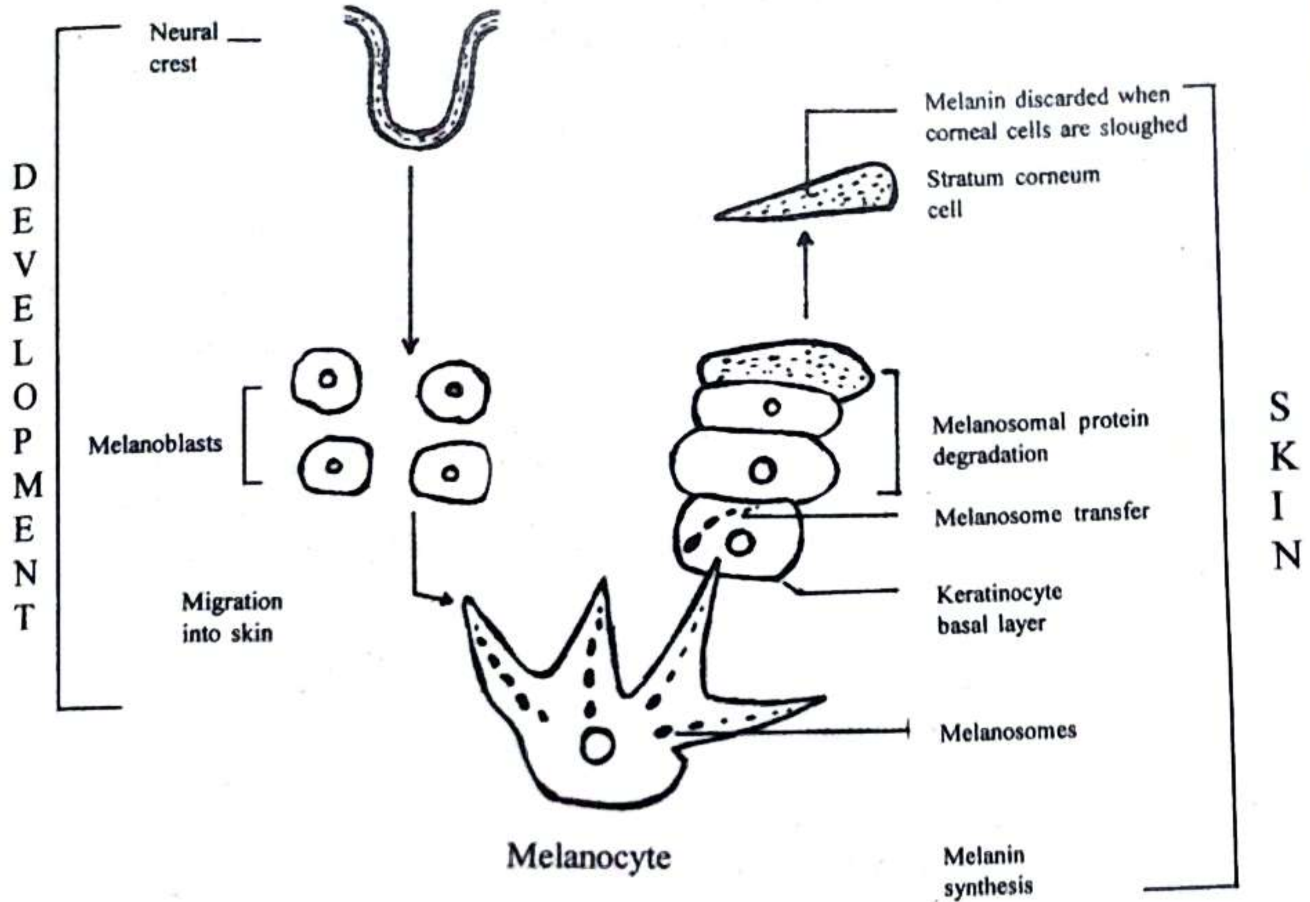
Proteasome substrates : A number of proteins, namely, N-myc, c-myc, c-fos, P53 and E1A have been identified as natural substrates of the MCPs or proteasomes. More recently, involvement of proteasomes in regulating degradation of cyclin and thus regulating cell cycle have also been proposed (Kawahara and Yokosawa, 1992; Amsterdam *et al.*, 1993). Furthermore, proteasome depletion and inhibition studies in cell extracts, and *in vivo*, using yeast 20S proteasome mutants defective in proteolysis have demonstrated that proteasomes are not only responsible for degradation of proteins, but are also required for activation of proteins by processing of inactive precursors (Palombella *et al.*, 1994). Interestingly, two of the proteasome polypeptides are encoded within the MHC class II gene cluster suggesting their role in the processing of intracellular antigens for cytolytic immune responses (Goldberg and Rock, 1992). Thus, due to their association with many such functionally unrelated structures and also due to their ubiquitous distribution, a number of laboratories working on unrelated areas of research have undertaken investigations on these seemingly important cellular particles.

The vastness of proteasomes as multicatalytic protein : The ubiquitin-proteasome pathway is responsible for the degradation of the vast majority of cellular proteins, including not only short lived regulatory proteins, such as cyclins, cyclin inhibitors and various oncogene products, but also the bulk of long-lived cellular proteins. The 19S cap complexes contain subunits with ATPase activity and subunits able to bind and recognize polyubiquitin chains as well as putative unfold assessor inverse chaperonins which most probably unfold protein chain and translocate them through the central channel of the core 20S proteasome to the inner chamber, where catalytically active threonines of at least three different beta-subunits reside. Biochemical studies of the 20S proteasome revealed at least three different proteolytic activities, namely, chymotrypsin-like (ChTL), trypsin-like (TL) and peptidylglutamylpeptide hydrolizing (PGPH), and two minor activities, namely, branched amino acid-preferring and small neutral amino-acid preferring. Among all these activities, ChTL activity seems to be most important, since the use of specific inhibitors of this activity leads to the rapid accumulation of ubiquitin-protein conjugates, cell vacuolization, cell cycle block and apoptosis.

Proteasomes in gametes and embryos : Previous studies including our own have shown that oocytes and mature eggs are rich sources of proteasomes and that there is differential synthesis of proteasomes in chick embryos during development (Akhayat *et al.*, 1987; Pal *et al.*, 1988; Gautier *et al.*, 1988; Pal, 1991; Pal *et al.*, 1994). Furthermore, in embryonic and somatic cells, cytolocalization of proteasomes changes as a function of development and differentiation (Grossi de Sa *et al.*, 1988; Gautier *et al.*, 1988; Pal *et al.*, 1988). In general, it appears that during differentiation, there is downregulation of proteasomes in eukaryotic cells (Scherrer and Bey, 1994).

Melanocytes - development, differentiation and melanin biosynthesis : Melanocytes are derived from the neural crest cells and they contain arborising nerve-cell dendrites. They are melanin pigment producing cells and are distributed in CNS, eye, skin and hair. In the skin, melanocytes are located in the basal layer and they project their dendrites into the malphigian layer of the epidermis where they transfer malanosomes to keratinocytes (Fig. 2A). Melanocytes possess the metabolic machinery for the synthesis of a copper-containing enzyme, tyrosinase, the key enzyme in melanogenesis. Tyrosinase is involved in the conversion of tyrosine to brown coloured polymer, the eumelanin and a reddish-yellow polymer, the pheomelanin (Jimbow *et al.*, 1993). The detailed chemical reaction steps are shown in Fig. 2B. The pigments are synthesized in round to ellipsoidal organelles known as melanosomes. Melanocytes which reside on the dermis of the skin transport the melanosomes to keratinocytes and thus impart colour to the skin.

Melanoma : Melanoma occurs when melanocytes undergo transformation and become malignant. Most pigment cells are in the skin and melanoma of the skin is called cutaneous melanoma. Besides, melanoma may also occur in the eye (ocular or introcular melanoma) and rarely in the meninges, digestive tract, lymph nodes. Melanoma may occur on any skin surface. However, in men, it is often found on the trunk or the head and neck, and in women, it develops on the lower legs. Melanoma is rare in people with dark skin; however, when it develop in them, it occurs under the fingernails or toenails, or on the palms or soles. The occurrence of this disease increases which age, in general. People whose immune system is weakened by certain cancers, by drugs used after organ transplants or by AIDS, are at increased risk of developing melanoma. It is generally believed that the worldwide increase in melanoma is related to an increase in the amount of time people get exposed to the sun. The UV radiation in the sun is primarily involved in the initiation of this disease.



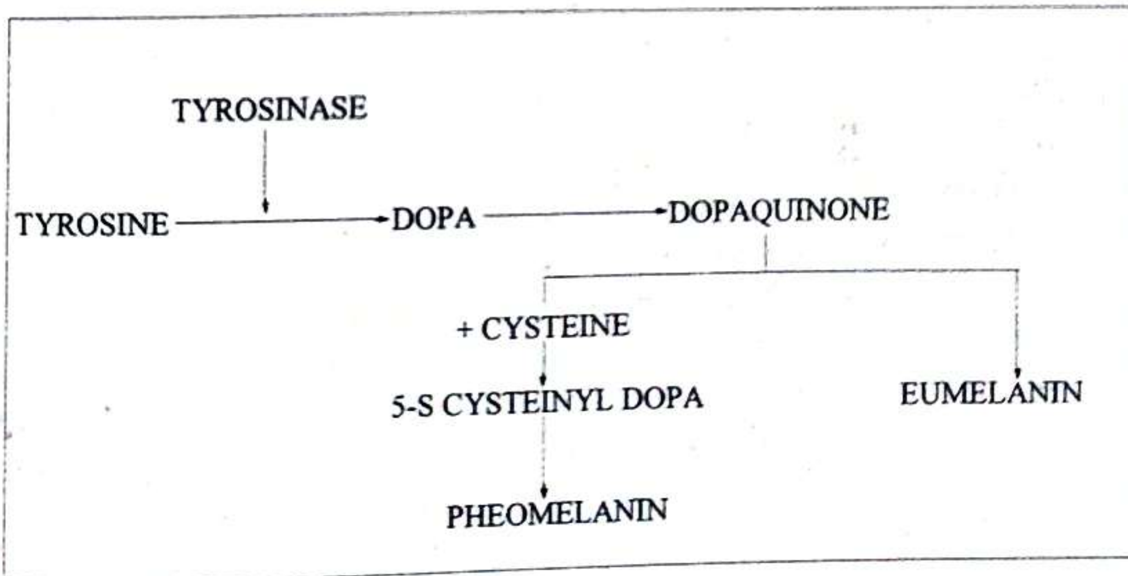


Fig.2 : (A) Origin and differentiation of melanocytes and formation of melanosomes. The process of differentiation of melanocytes, formation of melanosomes and their transport are schematically shown; **(B)** Melanin biosynthesis and the role of tyrosinase. Tyrosinase converts tyrosine or DOPA into Eumelanin or Pheomelanin.

Proteasome-melanocyte/melanoma link : While investigating on the distribution and cytolocalization of proteasome subunits, it was observed that in a newt *Pleurodeles waltli*, some of the subunits were localised in a few specific cell types (Fig. 3) during development. Some among these cells appeared to be melanocytes (Pal *et al.*, 1988). It was difficult at that time to determine any functional correlation between proteasomes and melanocytes. Subsequently there has been a long gap in research in this area Halaban *et al.*, (1997) demonstrated that in amelanotic melanoma cells, lack of pigment formation is related to the degradation of tyrosinase, an enzyme involved in melanin biosynthesis, by proteasomes. They showed that proteasome inhibitors induce the accumulation of tyrosinase and in absence of such inhibitors, a portion of newly synthesized tyrosinase is diverted from the endoplasmic reticulum to degradation by proteasome complex. This process occurs normally in melanocytes, but it is accelerated in melanoma cells. From this study, however, detailed information regarding the type of proteasomes involved in this process quantitative as well as qualitative distribution of proteasomes and their effect on melanin biosynthesis are not available. We have therefore undertaken an investigation on the role of proteasomes during cell differentiation with a particular interest on melanocyte cell differentiation in mice. Some of our preliminary observations (Pal *et al.*, 2000) are included here.

As melanocytes rarely proliferate *in vivo* and are difficult to maintain *in vitro*, melanoma cells have been used as a model system for various studies in the regulation of melanin synthesis. Mouse melanoma cells, B-16, are routinely used for various *in vitro* studies. Interestingly, B16 mouse melanoma cells show a fluctuation and exist in the following phenotypes: (a) highly melanotic cells, b) partially melanotic cells, and c) amelanotic cells (Fig. 4).These characteristics make them suitable also for understanding the mechanism of melanin biosynthesis. It has been recently shown that these stages represent oscillations between an embryonic neural crest derived premelanoblasts to committed, non terminally differentiated melanocytes. In the newly committed melanocytes high molecular weight, heat sensitive trypsin resistant tyrosinase doublet are expressed (Sharma *et al.*, 1998). It has also been shown by RT-PCR that mRNA of tyrosinase, dopa chrome tautomerase and DHICA oxidase are transcribed at all stages of the melanocyte lineage differentiation programme but as proteins these are present only in the committed melanocytes. Thus, only melanocytes manifest tyrosinase activity and tyrosinase protein detected by western blotting (Jagadisan *et al.*, 1999; Mojamdar, 1999). Therefore, it is likely that proteasomes are involved in removing the newly translated tyrosinase protein and keeping the melanocyte in the blastic condition.

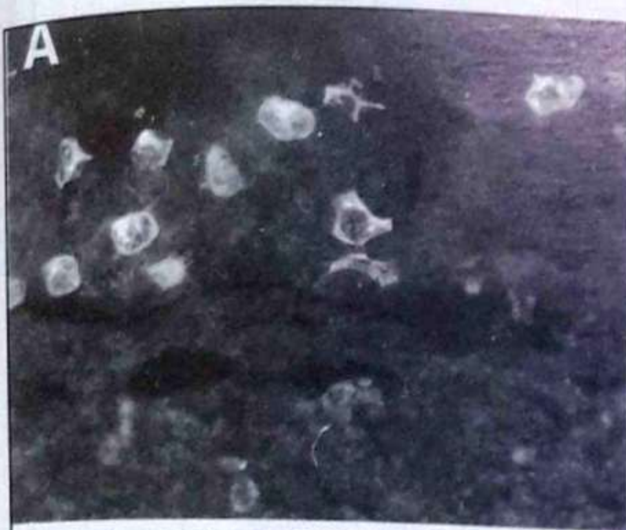
Proteasomes also appear to play a major role in metastasis. An extracelluar proteasome-like structure has been identified in the media of cultured astrocytoma cells (Vaithilingam *et al.*,1995). Thus, it is quite possible that melanoma metastasis which involves the free movement of melanoblast-like cells may be mediated by the extracellularly secreted proteasomes. The implications of such an hypothesis are varied and will have great bearing on pigmentary disorders like vitiligo (common in India) and melanomas (prevalent in western countries). Therefore, determining a possible role of proteasomes in the manifestation of the different phenotypes in B-16 melanoma cells becomes very important.

OUR CURRENT RESEARCH

Expression of proteasomes in B-16 mouse melanoma cells : Expression of proteasomes and their various subunits in B-16 melanoma cells was determined by Dot/Western blot analysis of the protein extracts both under native and denaturing conditions (Kampasi, 1998; Shukla, 1999). Five different monoclonal antibodies corresponding to mammalian proteasome subunit antigens, namely, p23K, p27K, p31K, p30-33K and p33K were used for detecting the proteasome antigens in mouse melanoma cells of various phenotypes (*e.g.*, melanotic, partially melanotic, induced melanotic and amelanotic cells.).

In Dot blot analysis, under non-denaturing condition, it was observed that melanotic melanoma cells were positive for all the four antigens tested,namely, p23K, p31K, p30-33K, and p33K (Fig. 5A); results of p31K are not shown. However, their quantity varied from highest to lowest in the following order : p31K >p33K>p30-33K>p23K. Interestingly, however, when the melanoma protein extract (antigens) was denatured prior to dot blot, the reactivity of all the antigens except that of p23K were reduced substantially (Fig. 5B). These results indicated that the proteasome subunits are more reactive to the corresponding antibodies under native condition than under denaturing condition.

In Western blot analysis, among the four antigens tested by their respective antibodies, two (p27K and p30-33K) were found to be present in considerable amount in melanotic melanoma cells (Fig. 5C); results with other two antibodies, anti-p23K and anti-p33K are not shown. Therefore, in the subsequent experiments, extracts from melanoma cells of various phenotypes, namely, melanotic, partially melanotic induced melanotic and amelanotic were probed with antibodies to p27K and p30-33K antigens. As seen in Fig. 6, both the antigens were most intense in the melanotic melanoma cells, and they were



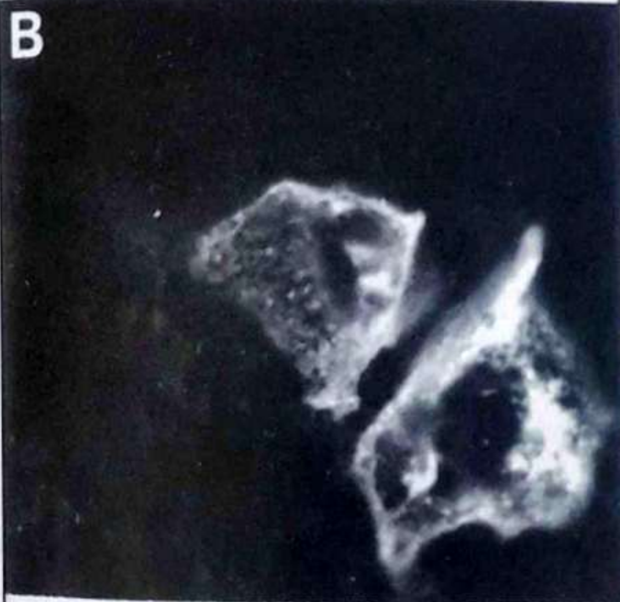


Fig. 3

Fig. 4A

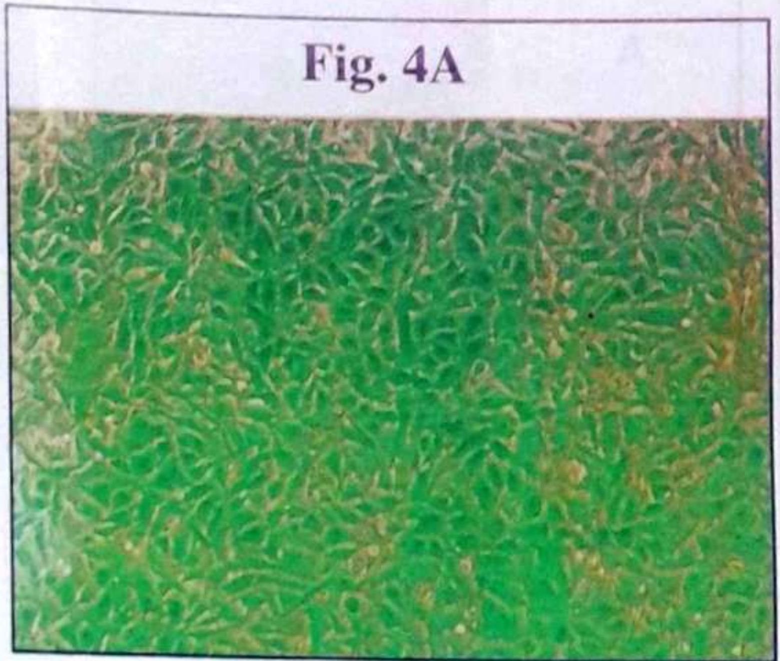
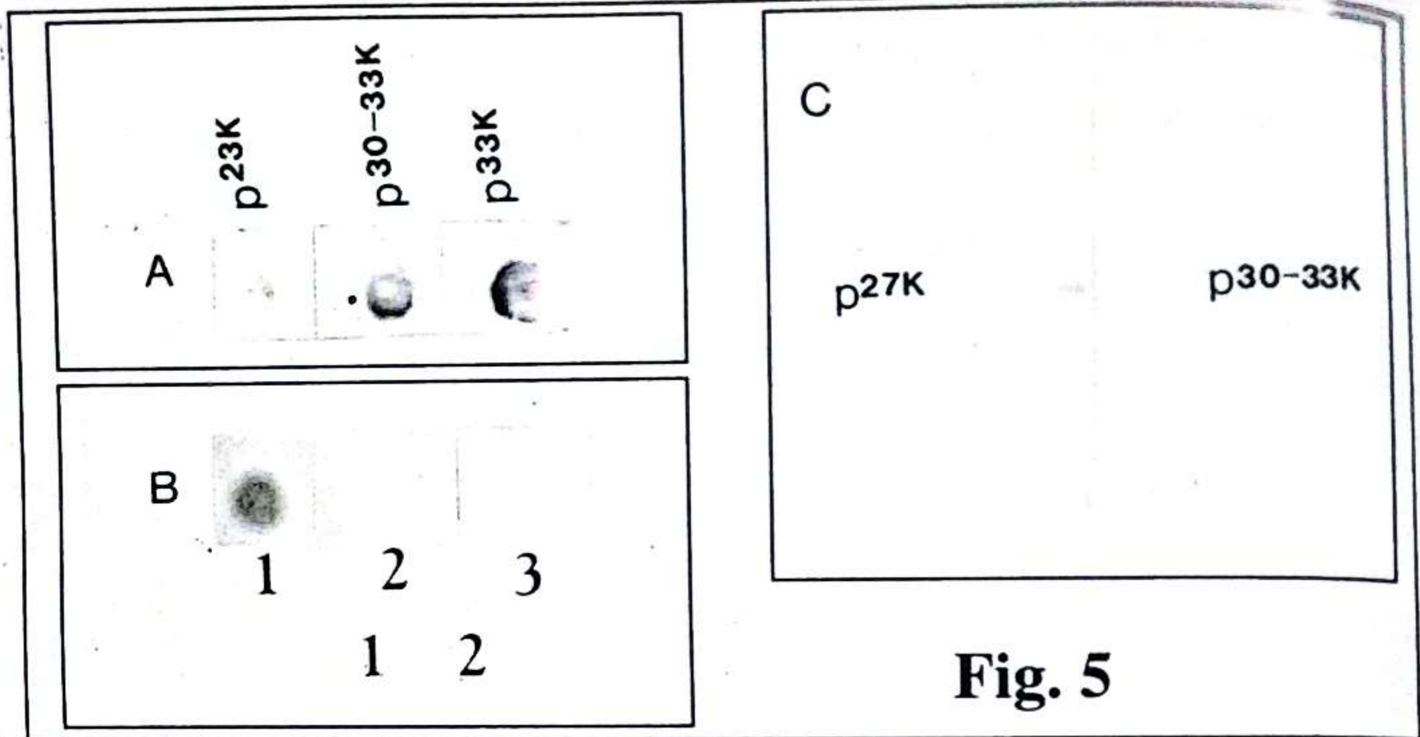


Fig. 4B

Fig. 3: Immunofluorescence localisation of proteasomes in various cell types in the skin of *Pleurodeles* (sagittal sections), A (X 190); B (X 750) ; **Fig. 4 : (A)** Mouse melanoma cells in culture. A photograph of the confluent monolayer of B-16 mouse melanoma cells cultured *in vitro* in Minimum Essential Medium (MEM) containing 10% Foetal Calf Serum (FCS); **(B)** Different phenotypes of mouse melanoma cells. Eppendorf tubes containing cell pellets showing the colour of (1) melanotic , (2) partially melanotic and (3) amelanotic melanoma cells.



MW Marker (kDa)
Melanotic
Partially melanotic
Induced melanotic
Amelanotic

66
45
34
24
18

MW Marker (kDa)
Melanotic
Partially melanotic
Induced melanotic
Amelanotic

A p27K

Fig. 6

B p30-33K

Fig 5: Expression of proteasomes in melanoma cells. Dot blots of native proteins **(A)** and denatured proteins **(B)**. Same quantity (25μg) of total protein (melanoma extract) was used for dot blot experiment with (1) anti-p23K, (2) anti-p30-33K and (3) anti-p33K antibodies **(C)** Western blot of melanoma extract with anti-p27K and anti-p30-33K antibodies. In each lane same quantity of total protein (100 μg) was loaded ; **Fig 6:** Expression of proteasomes in melanoma cells. Western blots of protein extracts from melanoma cells of four different phenotypes, namely, melanotic, partially melanotic, induced melanotic and amelanotic using **(A)** anti-p27K and **(B)** anti p30-33K antibodies.

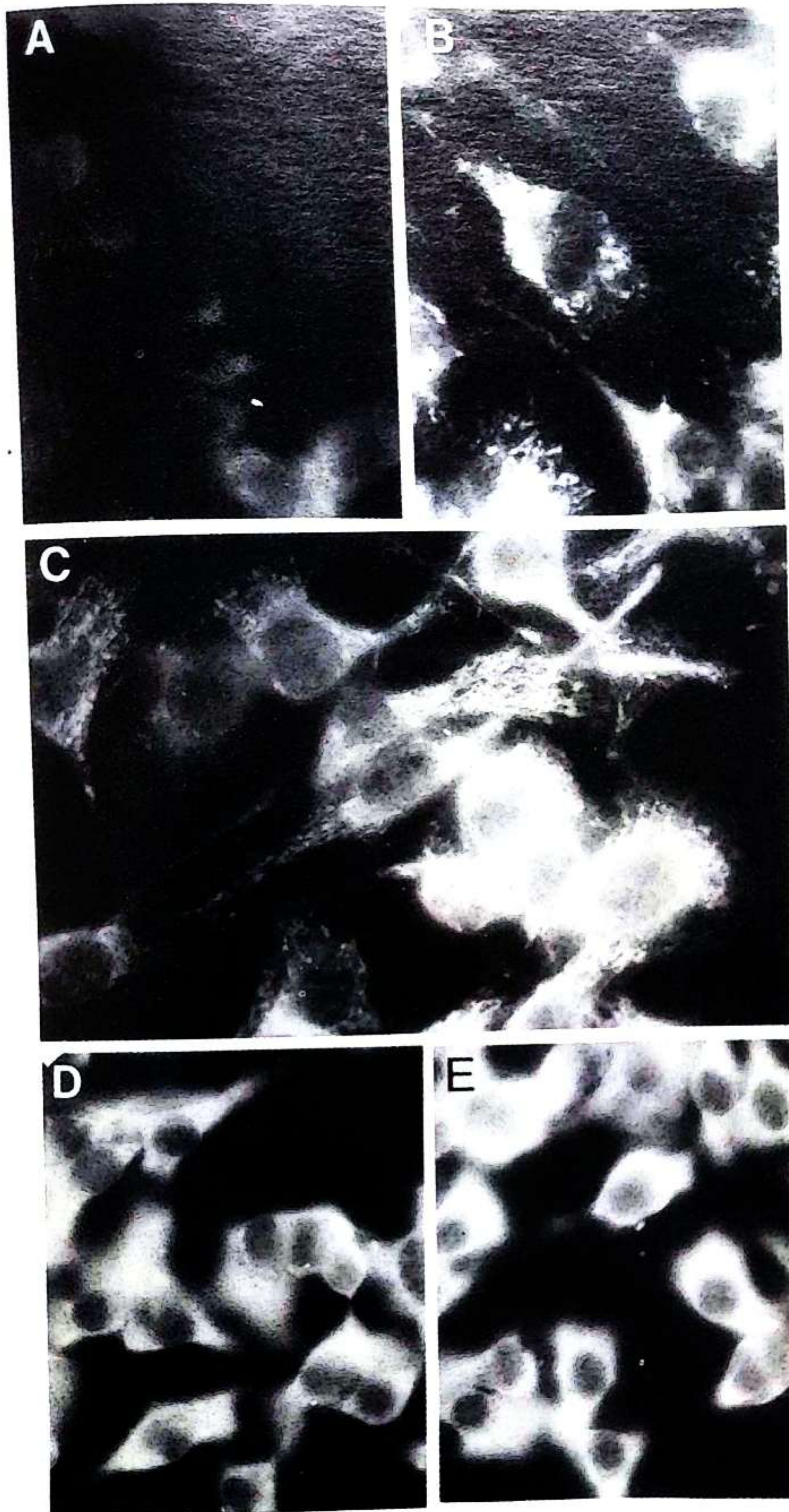


Fig. 7

Fig 7: Immunofluorescence localisation of proteasomes in mouse melanoma cells. **(A)** Control (- first antibody), **(B)** and **(C)**, anti-proteasome antibody (anti-p31K) fluorescence, **(D)** and **(E)** positive control with anti-actin (B) and anti-hsp90 (E) antibodies. A,D and E (X 300); B and C (X 480).

less intense in other cell phenotypes. Within them, the quantity of antigens decreased in the following order: partially melanotic> induced melanotic> amelanotic.

Cytolocalization of proteasome antigens in B-16 mouse melanoma cells : Cytolocalization of proteasomes in melanoma cells were carried out by immunofluorescence using anti-p31K antibody. Cells were also labelled for anti-actin and anti-hsp90 antibodies as positive control. As seen in Fig. 7, cells were brightly labelled with fluorescence at the cytoplasm, indicating cytoplasmic distribution of proteasomes (Fig. 7B); the negative control (without any first antibody, Fig. 7A) did not show any fluorescence. As compared to the fluorescence observed with anti-actin (Fig. 7D) and anti-hsp90 (Fig. 7E) antibodies, which were also cytoplasmic, proteasome fluorescence was seen spreading out in the cytoplasmic processes that connect two cells, giving it a distinct milky-way appearance in the cytoplasmic connections(Figs. 7B,C)

Tyrosinase enzyme activity assay in melanoma cells : Tyrosinase activity assay was carried out in the extracts of melanoma cells of four different phenotypes, namely, melanotic, partially melanotic, amelanotic and induced melanotic melanoma cells. The results are presented in Table 1. Tyrosinase activity was found to be highest in the melanotic melanoma cells and lowest in the induced melanotic cells. The low tyrosinase activity in the induced melanotic melanoma cells is perhaps due to high melanin content which is known to inhibit tyrosinase activity.

CONCLUSION AND FUTURE RESEARCH

The preliminary results on the expression of proteasomes in melanoma cells of various phenotypes as determined by western blot analysis, indicated that among the five proteasome antigens tested, p31K, p27K and p30-33K are more abundant in melanoma cells. Further, it is apparent that the concentration of proteasomes are dependent on the melanogenic status of the phenotypes: melanotic phenotypes being enriched with proteasome and amelanotic being deficient. This data correlates well with the tyrosinase activity of the cell phenotypes. These results are in partial agreement with the results reported by Halaban *et al.*, (1997), wherein, they have suggested that proteasomes are involved in the regulation of melanogenesis during melanocyte differentiation.

Exclusive localisation of proteasomes in the cytoplasm as determined by immunofluorescence experiments, is also in support of their contention that the

proteasomes might be involved in degradation of tyrosinase activity (Halaban *et al.*,1997), which occur during their synthesis in the endoplasmic reticulum in the cytoplasm. However, further experiments are required to determine the mechanism of proteasome-mediated regulation of melanin biosynthesis.

As it is evident from the discussion above, it is premature to derive any significant conclusion with reference to the role of proteasome in melanoma cell differentiation as well as transformation. Proteasomes, although involved primarily in protein breakdown, are known and speculated to have multiple functions (reviewed in Scherrer and Bey, 1994). However, their direct involvement in cell differentiation either *in situ* during embryonic development or *in vitro* has not been demonstrated so far. Although a number of studies including our own (reviewed in Pal, 1991) have indicated that eggs and embryos are enriched with proteasomes and they are synthesized and localised as a function of differentiation (Pal *et al.*, 1994), no conclusive data is yet available on their role in cell differentiation. Therefore, further studies on their expression during development and differentiation of melanocytes in mouse embryos, will be of importance to determine their precise role in cell differentiation. Similarly, it will be of interest to determine if proteasomes are also involved in regulating melanocyte phenotypes. These information will be of immense importance in understanding the mechanism of the disease, *vitiligo*, which is prevalent in our country, and also of *melanoma*, a cancer prevalent in the western countries. It may perhaps be possible to correct some of these and other related disorders, by manipulating the system/cell types with anti-proteasome antibodies as therapeutic reagents in humans. Work in progress in our laboratories is directed towards seeking answers to some of the above questions.

Table 1: Tyrosinase activity in melanoma cells of different phenotypes

Serial. No.	Melanogenic state of melanoma cells	Specific activity (µmol/min/mg protein)
1	Melanotic	0.0321
2	Partially melanotic	0.0195
3	Amelanotic	0.0132
4	Induced melanotic	0;0099

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GLYCOSYLATION INHIBITION BY TUNICAMYCIN LEADS TO ABNORMAL MORPHOGENESIS IN FROG (*MICROHYLA ORNATA*) AND CHICK (*GALLUS DOMESTICUS*) EMBRYOS

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ABSTRACT

Early morphogenesis in the vertebrate embryo is driven by continuous interactions between cells and the extracelluar matrix. Cell surface glycoconjugates are thought to play an important role in various cell-cell and cell-matrix interactions necessary for cell adhesion and cell migration. In the present study, we have assessed the effects of tunicamycin (Tn), an inhibitor of N-glycosylation, on early morphogenesis of vertebrate embryos. Embryos of the frog *Microhyla ornata* and chicken *Gallus domesticus* were used as models for these studies. Tn adversely affected morphogenesis in both gastrulating frog embryos and chick embryo explants cultured *in vitro* in a concentration-dependent manner. Tn, in a concentration range of 0.90 to 7.14 μM, inhibited morphogenetic movements in *Microhyla* embryos leading to shortening and/or bending of the embryonic axis. At concentrations of 3.57 and 7.14μM, Tn arrested neurulation and overall embryonic development. Scanning electron microscopic studies of control and treated frog embryos revealed abnormal cellular architecture in Tn-treated embryos which appeared to have adversely affected normal progression of development. Chick embryo explants cultured *in vitro* responded in a more or less similar manner to Tn treatment. While treatment with 11.9 μM Tn led to detachment of embryos from the vitelline membranes resulting in mortality, Tn at 1.19 μM produced several abnormalities in the neural tube, head fold, heart, somites and body axis in developing chick embryos. The results show that Tn, due to its glycosylation inhibitory activity, interferes with embryonic cell-cell interactions leading to abnormal morphogenesis. Since glycoconjugates participate in a large number of embryonic cellular interactions, effects of Tn are not restricted to any particular organ or system but occur globally.

Key words: Tunicamycin, frog embryo, chick embryo, cell interactions, morphogenesis

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INTRODUCTION

Early morphogenesis of the vertebrate embryo involves multiple interactions between different types of embryonic cells and between the cells and the extracelluar matrix (ECM). Cell adhesion and cell migration are two of the most important cellular processes which drive morphogenesis (reviewed in Gilbert, 1997). Cell interactions of most kinds involve glycoconjugates present on the cell surface and in the ECM (Muramatsu, 1990). Glycoconjugates also play a crucial role in cell-cell recognition, an essential attribute of embryonic cells for normal morphogenesis and organogenesis (Edelman, 1984; Thiery *et al.*, 1985; Ekblom *et al.*, 1986; Bourrillon and Aubery, 1989; Shur, 1989). Here we have studied the role of glycoproteins in early morphogenesis of gastrulating frog embryos and chick embryo explants cultured *in vitro* by experimentally inhibiting the glycosylation of proteins. Tunicamycin (Tn), an inhibitor of N-glycosylation, was used for this purpose. We find that inhibition of glycosylation in developing frog and chick embryos leads to abnormal morphogenesis.

MATERIALS AND METHODS

Frog embryos

Naturally fertilized embryos of the frog *Microhyla ornata* were collected from ponds in and around Pune. The embryos were dejellyed with 2% cysteine, pH 8.9. Early blastula (stage 8; Padhye and Ghate, 1989) and early gastrula (stage 10; Padhye and Ghate, 1989) were used in the present study.

Chick embryos

Freshly laid White leghorn chicken eggs were obtained from a local poultry and allowed to develop at 37.5C in the laboratory till the desired stage of development. The embryos were staged according to the morphological criteria described by Hamburger and Hamilton (1951).

Treatment of frog embryos

De-jellyed frog embryos were treated with 0.90, 1.79, 3.57 or 7.14 μM Tn in filtered and autoclaved pond water. The control embryos received equivalent volume of dimethyl sulfoxide (DMSO) vehicle used for Tn. The embryos were allowed to develop at room temperature (22°C-28°C) for upto 24 h. Deviation in normal development, if any, was studied and embryos were photographed as required. Thirty embryos were used for each of the control and treatment groups and the experiments were carried out in triplicate.

Tunicamycin solution

Tunicamycin (Sigma Chemical Co., USA) was dissolved in dimethyl sulphoxide (DMSO) at the concentration of 1 mg/ml.

Scanning electron microscopy of *Microhyla* embryos

In order to assess the effects of Tn at ultrastructural level, scanning electron microscopic (SEM) studies were undertaken on treated and control embryos. The embryos were processed as described before (Ghaskadbi, 1994). They were fixed in 2.5% glutaraldehyde (Sigma, USA) in 0.2 M phosphate buffer, pH 7.4 for 24 h at 4-6C. Embryos were repeatedly washed to remove all the fixative, dehydrated in a graded series of ethanol and critical point dried from CO_2 in E3000 critical point drier (Bio-Rad). The embryos were roughly cut into halves with a sharp blade before mounting on the stubs, coated with gold in a E5200 automatic sputter coater (Bio-Rad), studied with a Steroscan S120 scanning electron microscope (Cambridge) and photographed on a 35 mm black and white negative film.

Culture and treatment of chick embryos

Gastrulating blastoderms were explanted at Hamburger Hamilton (HH) stage 4 and cultured according to the single ring technique of New (1955). The cultures were treated with Tn as described before (Ghaskadbi *et al.*, 1994; Patwardhan *et al.*, 1996). Briefly, 100 µl Pannet Compton (PC) saline (New, 1966) containing desired concentration of Tn (final concentration 11.9 or 1.19 µM) was carefully placed inside the ring on top of the blastoderm. Control embryos received comparable quantity of DMSO. The embryos were left at room temperature for 30 min to allow proper diffusion of Tn and then incubated at 37.5C for 18 h. After this the live embryos were studied carefully and deviation from normal development, if any, was recorded. The blastoderms were fixed in ethanol:acetic acid (3:1), dehydrated and stained with haematoxylin and eosin for permanent preparation and detailed examination. The experiments were replicated thrice with a total of 75 (34 for controls, 16 for 11.9 µM Tn and 25 for 1.19 µM Tn) cultures.

RESULTS

Effect of tunicamycin treatment on *Microhyla* development

Tn was found to adversely affect the development of frog embryos. With increasing concentration of Tn, there was a significant decrease in the proportion of normal embryos (Table 1). The most common abnormality induced was bent body axis. This was often associated with shortening of the length of the body axis (Table 1). At concentrations of 3.57 and 7.14 µM, Tn arrested the neurulation in about 16% of the

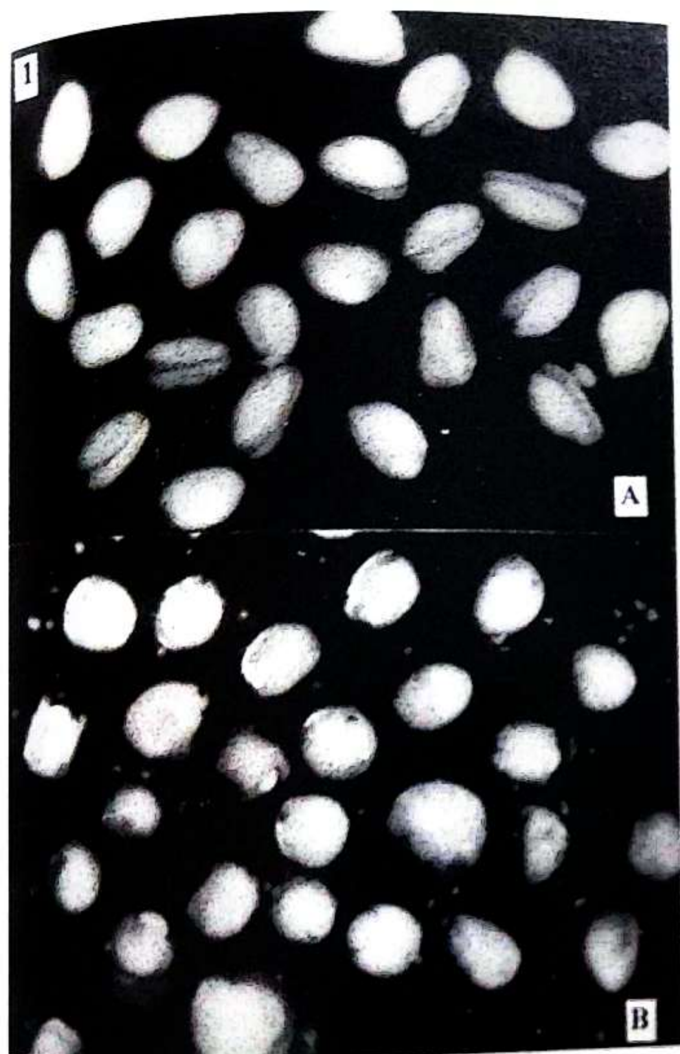
treated embryos (Table 1; Figs. 1A,B). At the highest concentration of 7.14 µM used in the present study, Tn arrested the development in 80% of the embryos within a few minutes as evidenced by complete inhibition of morphogenetic movements. The effects of Tn were concentration dependent; the proportion of abnormal embryos went on increasing considerably with increasing concentration of Tn. At lower concentrations of Tn, the embryos continued to develop, albeit abnormally. Major abnormalities were noticed in the body axis which was either curved or short, or both (Fig. 1C). Underutilization of yolk was also of common occurrence.

Effects of Tn treatment on cell surface ultrastructure:
SEM studies of cut open control (Fig. 2A) and treated (Fig. 2B) *Microhyla* embryos revealed inhibition of formation of the blastocoel. The cells of the Tn-treated embryos appeared very loosely organized as compared to those of the control embryo. There was also an apparent decrease in the amount of the ECM in treated embryos (Fig. 2B). No obvious differences in the size of cells from control and treated embryos were noticeable. At higher magnifications, cell surfaces in Tn-treated embryos appeared considerably smoother as compared to those from the controls.

Effects of tunicamycin treatment on chick development
All the 34 chick embryo explants cultured with New's single ring technique in the presence of 100 µl PC saline developed normally upto 18 h post-culturing (Fig. 3A). Embryos developing in medium containing a comparatively low concentration of Tn (1.19 µM) continued to develop upto 18 h post-treatment. However, all of the treated embryos exhibited developmental abnormalities (Figs. 3B,C). These included shortened anteroposterior body axis, inhibition of neural tube closure, fewer and improperly located somites, microcephaly, inhibition of fusion of heart primordia and, in some cases, inhibition of looping of the heart. Most of the embryos treated with the high concentration of 11.9 µM Tn got detached from the vitelline membrane and hence could not develop further. The embryonic tissue persisted on the vitelline membrane as a button-shaped structure (Fig. 3D).

DISCUSSION

Cell surface, and ECM glycoconjugates are thought to play crucial roles in embryogenesis (Hay, 1991). Over the years, several approaches have been adopted to identify the role of these molecules in early development. Studies on the structure and function of cell adhesion molecules (CAMs) (Cunningham *et al.*, 1983,1987; Edelman, 1984) and endogenous animal lectins (Roberson and Armstrong, 1980; Zalik



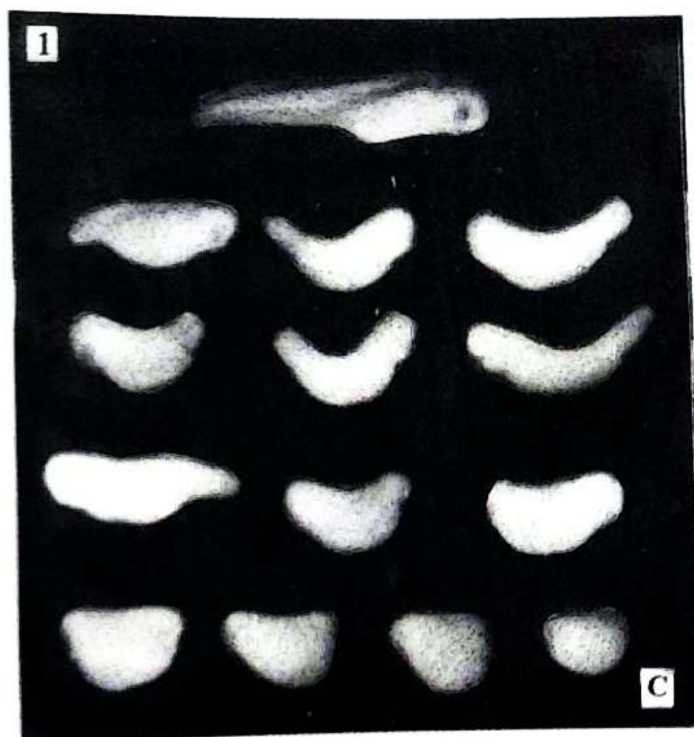
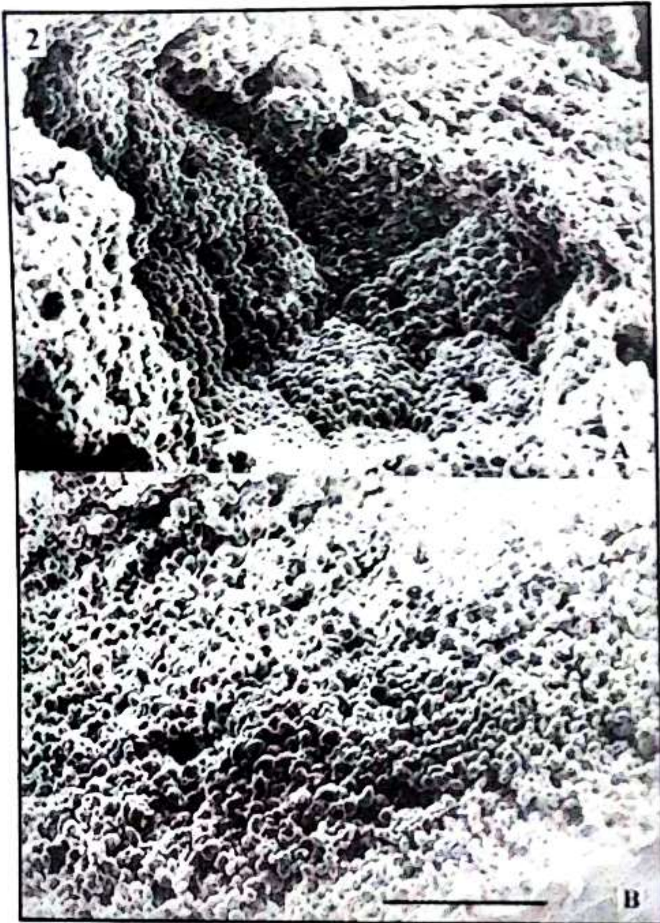


Fig.1 : Frog embryos grown in the presence of either DMSO **(A)** or tunicamycin for 3h **(B)** or 18h **(C)**. a. Control embryos showing normal neurulation after 3 h. **(B)**. Embryos treated with 7.14 µM Tn showing complete arrest of development accompanied by collection of fluid in the perivitelline space within 3 h. **(C)** Tadpoles resulting from embryos treated with 1.79 µM Tn for 18 h. Note various abnormalities in the treated tadpoles as compared to the control tadpole at the top.



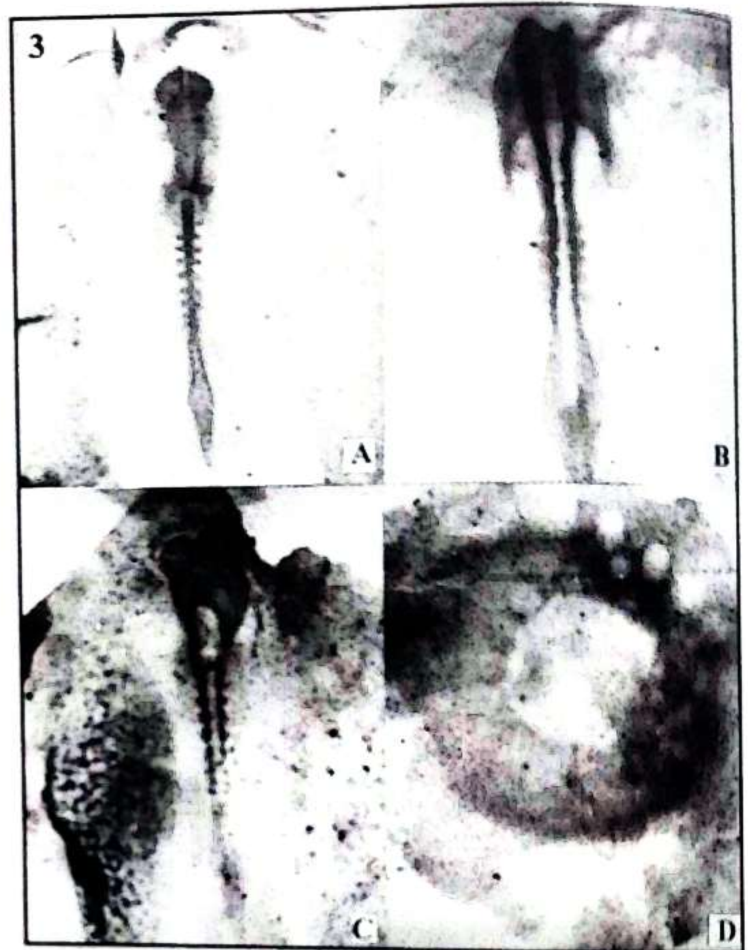


Fig.2 : Scanning electron micrographs of frog embryos treated either with DMSO **(A)** or 3.57 µM tunicamycin **(B)** for 8 h and cut roughly in the centre, **(A)** Normal cellular architecture of the control embryo, **(B)** Loosely attached cells and loss of cellular architecture of Tn-treated embryo. Note apparent loss of extracelluar matrix leading to defective cell adhesion and migration. Scale bar=50 µm ; **Fig.3** : Chick embryo explants cultured *in vitro* and grown in the presence of either DMSO (A) or tunicamycin (B-D) for 18 h. **(A)** Control embryo grown in presence of DMSO. Note normal development. **(B,C)** Embryos grown in presence of 1.19 µM Tn for 18 h. Note inhibition of neural tube closure, defective brain formation, dispersed and incompletely formed somites. **(D)** Embryo grown in presence of 11.9 µM Tn for 18 h. Note button-shaped embryonic tissue, apparently detached from the underlying vitelline membrane.

and Milos, 1986) have been two of the more popular approaches. One could also address this question by inhibiting glycosylation experimentally and then studying the embryonic development. Under such conditions, the carbohydrate moieties which are attached to the proteins and participate in cell recognition and adhesion are absent. In the present study we have used Tn, a nucleoside antibiotic from the bacterium *Streptomyces lysosuperificus*, as an inhibitor of glycosylation. Tn inhibits N-glycosylation by interfering with the very first step of the process, involving GlcNAc-1-P transferase (reviewed in Elbein, 1993). It happens to be the most widely used and most valuable inhibitor of N-linked glycosylation (Elbein, 1993). We have grown gastrulating frog and chick embryos in a medium containing various concentrations of Tn and studied the effects of this experimentally created inhibition of glycosylation on morphogenesis.

Development of both frog and chick embryos was significantly affected by Tn-treatment. While treated frog embryos exhibited abnormalities in the body axis, overall retardation of development and underutilization of yolk, chick embryos showed abnormalities in almost all the developing organs and systems. SEM studies in frog embryos demonstrated the direct effects of inhibition of glycosylation on cell adhesion and migration, as a result of which normal cellular architecture was significantly affected leading to abnormal development. Using New's single ring culture technique, it was possible to directly visualize the morphogenesis of vital embryonic tissues, such as the neural tube, brain, heart and somites. Similar studies are not possible with intact frog embryos. The essential role of glycoconjugates became evident from these observations on frog and chick embryos. The two model systems complemented each other in the present study; frog embryos were easier to handle for SEM analysis while cultured chick embryos allowed direct observations of different tissues and organs. Effects of Tn-treatment on the development of very early (stage X and XIII of Eyal-Giladi and Kochav) chick embryos have been studied and the formation of primitive streak in such embryos was found to be inhibited (Zagris and Pangopoulou, 1992). Our studies emphasize the important role of glycoconjugates throughout gastrulation and early morphogenesis.

At 11.9 μM concentration of Tn, blastoderms got detached from the vitelline membranes. This probably led to the arrest of development since the vitelline membrane is essential for proper expansion, growth and development of the embryo (New, 1959, 1966; Spratt, 1963). Fibronectin has been strongly implicated in the attachment and migration of cells on the vitelline membrane (Lash *et al.*, 1990). The ability of fibronectin to recognize matrix components such as collagen as well as the cell surface (Muramatsu, 1990) is probably vital for this function. In such a scenario, one would expect the binding of fibronectin to the vitelline membrane as well as to the cell

surface to be drastically hampered because of inhibition of glycosylation brought about by Tn. At the higher concentration, therefore, Tn did not seem to allow the expansion, and consequently, the growth and development of the chick embryo.

The effects of Tn observed by us were not restricted to any particular germ line, organ or system but were global. This is not surprising since Tn is expected to inhibit glycosylation of a whole range of proteins (Cook and Keynes, 1992). Another potential drawback in using Tn for functional studies is that it may inhibit protein synthesis (Elbein, 1993). It will be necessary to employ specific detection techniques to assess differences in the quantity and distribution of ECM components in control and treated embryos. In any case, the present study clearly brings out the importance of glycoconjugates in early morphogenesis of the vertebrate embryo.

Table 1: Effect of tunicamycin on developing frog embryos

Dose (μM)	%Normal embryos	% Embryos with bent axis	% Embryos with short and bent axis	% Embryos arrested at neurulation	% Embryos instantly arrested
Control (DMSO)	100 (90)	0(0)	0(0)	0(0)	0(0)
0.90	35.56(32)	55.56(50)	8.89(8)	0(0)	0(0)
1.79	4.44(4)	81.11(73)	14.44(13)	0(0)	0(0)
3.57	4.44(4)	28.89(26)	42.22(38)	16.67(15)	7.78(7)
7.14	0(0)	0(0)	3.33(3)	16.67(15)	80.00(72)

Figures in parentheses indicate corresponding number of embryos.

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DELAYS IN EMBRYONIC DEVELOPMENT IN CHIROPTERA - A REVIEW

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ABSTRACT

One of the reproductive strategies employed by bats is the phenomenon of embryonic delays during development. Twenty species of bats belonging to 6 families: Pteropodidae, Emballonuridae, Rhinolophidae, Phyllostomidae, Natalidae and Vespertilionidae exhibit delays which are of 2 types (i) Delayed implantation in which the fertilized ovum develops in a normal manner upto the bilaminar blastocyst stage, enters into a period of developmental quiescence and remains in an unimplanted condition for a variable length of time in the uterine lumen. Delayed implantation has been reported in *Eidolon helvum, Rhinolophus rouxi, Miniopterus australis, Miniopterus schreibersii* from France and Africa, *Miniopterus minor* from Kenya, *Miniopterus schreibersii natalensis, Miniopterus frateculus, Corynorhinus rafinesquei, Rhinolophus landeri* and *Scotophilus borbonicus* from South Africa (ii) Retarded embryonic development in which the blastocyst implants on schedule but after implantation, the embryonic development slows for a period of time. *Haplonycteris fischeri, Cynopterus sphinx, Taphozous longimanus, Hiposideros caffer caffer, Hipposideros ruber, Hipposideros lankadiva lankadiva, Macrotus californicus, Artibeus jamaicensis, Carollia perspicillata* and *Natalus stramineus* exhibit retarded embryonic development after implantation. Both delayed implantation and retarded embryonic development after implantation occur in *Miniopterus schreibersii* (Japanese and Australian population). In bats which exhibit either or both phenomena there is a prolongation of the period of gestation. The physiological mechanisms which control the fascinating phenomena are exceedingly different in the different species of bats which employ this reproductive strategy.

Key Words : Development, chiroptera, review

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INTRODUCTION

One of the reproductive strategies employed by bats is the phenomenon of embryonic delay during development. Two types of embryonic delays occur in bats:

1. **Delayed implantation**-the fertilized ovum after it develops in a normal manner upto the bilaminar blastocyst stage, gets into a period of developmental quiescence and remains in an unimplanted condition for a variable length of time in the uterine lumen. This is a common type of embryonic delay occurring in mammals. Apart from Order chiroptera it occurs in six distantly related Orders of Mammals *viz.* Marsupilia, Insectivora, Edentata, Carnivora, Rodentia and Artiodactyla (Enders, 1963; Daniel, 1970; Wimsatt, 1975; Renfree and Calaby, 1981; Mead, 1993).

2. **Retarded Embryonic Development**-the blastocyst implants on schedule but after implantation the embryonic development slows for a period of time. Post implantation embryonic delay or retarded embryonic development after implantation is uncommon and is reported to occur only in bats from both suborders-Mega and Microchiroptera (Heideman, 1989; Karim and Khan, 1996). In bats which exhibit either or both phenomena there is a prolongation of the period of gestation-the longest gestation period of 11.5 months is reported for the Fischer's pygmy fruit bat, *Haplonycteris fischeri* (Heideman, 1989). Twenty species of bats from six different families of Chiroptera (Koopman, 1994) *viz.* Pteropodidae, Emballonuridae, Rhinolophidae, Phyllostomidae, Natalidae and Vespertilionidae exhibit embryonic delays (Table 1). The physiological mechanisms which control this fascinating phenomena are exceedingly different in the different species of bats which employ this reproductive strategy.

a. **Births to occur at the peak of rainfall creating favourable conditions for maximum survival of young bats when weaned** (Obligate Delay) *e.g. Eidolon helvum* (Mutere, 1965,1967); Fayenuwo and Halstead, 1974), *Rhinolophus rouxi* (Ramakrishna and Rao, 1977); and *Macrotus californicus* (Bradshaw, 1962). The pregnancy cycle of the African fruit bat, *Eidolon helvum* in Uganda (O° 20'N) is characterised by a three month period of delayed implantation (Mutere, 1965, 1967). Mating takes place from April to June. Free uterine blastocysts are observed through July to September. Implanting blastocysts are in evidence in October-November. Births takes place in

Table 1: Embryonic Delays in Chiroptera

Family (Species)	Latitude	Delay Strategy	Duration of Delay (Months)	Gestation Periods (Months)	References
1	2	3	4	5	6
PTEROPODIDAE					
(*E. helvum*), Uganda	0°20'N	DI	3	7	Mutere (1967), Fayenuwo & Halstead (1974)
Nigeria	7°24'N	DI	1	4	Funmilayo (1979)
Nigeria	7°N	DI	5	9	Bernard & Cummings (1997)
(*H. fishcheri*), Phillipines	9° 11'N 121°-123'E	RD	8	11.5	Heideman (1989)
(*C. sphinx*), India		RD	1	4	Krishna & Dominic (1983)
EMBALLONURIDAE					
(*T. longimanus*)		RD	-	1st preg. (105 d) 2nd preg. (86 d)	Krishna & Dominic (1982)
RHINOLOPHIDAE					
(*R. landeri*), N. Nigeria	10.5°N	DI	2	5	Menzies (1973)
(*R. rouxi*), Khandala, India	18°52'	DI	1.5	5	Ramakrishna & Rao (1977)
(*H. caffer*), Natal, Africa	~29°S	RD	-	7.3	Bernard & Meester (1982)
Torpor, N. Nigeria	10.5°N	DI	2	5	Menzies (1973)
(*H. ruberi*), Tanzania, Africa	7°S	RD	2	6.5	Howell (1976)
(*H. lankadiva*) Mandu, India Semi-torpor	-	RD	5	9	Khan (unpublishd)
PHYLLOSTOMIDAE					
(*M. californicus*), USA	-	RD	4.5	9	Bradshaw (1962); Burns *et al.* (1972); Bleier (1975 a,b); Burns & Easley (1977)
(*A. jamaicensis*), Panama	-	RD	2.5	7	Fleming (1971)

Contd...

(*C. perspicillata*) French Guiana	–	RD	–	–	Cosson & Pascal (1994)
Trinidad	–	RD	44–50 d (Wild Population)	Variable (see text)	Rasweiler & Badwaik (1997)
NATALIDAE					
(*N. stramineus*)	–	RD	–	8	Mitchell (1965); Wimsatt (1975)
VESPERTILIONIDAE					
(*M. schreibersii*), France	45°N	DI	5	105	Peyre & Herlant (1963, 1967)
Japan	32.5°N	DI,RD	2, 3	8.5–8.7	Kimura & Uchida(1983);Kimura *et al.*(1987)
Hibernating Australia	28°S	DI,RD	3,1.5	8	Richardson (1977), Wallace (1978) Crichton *et al.* (1989)
Africa	33°S	DI	3.5	7	Bernard (1994)
	29°S	DI	4	7.5–8	Bernard (1980)
	26°S	DI	4	8	van der Merwe (1979,1986)
	18°S	DI	3	6–7	Bernard *et al.* (1996)
	11°S	DI	3	6–7	Anciaux de Faveaux (1977)
(*M. minor*), Kenya, Africa	4°S	DI	3 Weeks	4.5	Mc Williams (1988)
(*M. s. natalensis*), Pretoria	25°48'S	DI	–	–	van der Merwe (1980); Bernard (1980)
	28°01'E	DI	4±	8±	
Uitkomst	25°44'S 25°46'E	DI	–	–	
(*M. fraterculus*) Natal, S. Africa	29–30°15'S	DI	2.5	–	Bernard (1980)
(*M. australis*), Australia	28°S	DI	1	4.5	Dwyer (1968); Richardson (1977)
(*P. (Corynorhinus) rafinesqui*)	–	DI	–	(56–100d) 1.8–3.3	Pearson *et al.* (1952)
(*S. borbonicus*)	22°S	DI	3.5	7.5	van der Merwe *et al.* (1988)

DI: Delayed implantation; RD: Retarded development after implantation.

February-March just before the onset of rainfall, presumably creating favourable conditions for the maximum survival of the young bats when weaned. The observations of Fayenuwo and Halstead (1974) on *Eidolon helvum* studied somewhat further north 7°24'N (Nigeria) and at higher altitude differ from those of Mutere (1967) in that the reproductive sequence was restarted by a month and implantation occurred at the beginning of the winter dry period and births at the beginning of the spring wet season.

Studies on two reproductive synchronized Indian populations of the rhinolophid bat, *Rhinolophus rouxi* (Ramakrishna and Rao, 1977) indicates that early development normally proceeds slowly at Bangalore while there is a period of embryonic diapause at Khandala. At Bangalore tubal ova were observed on 27th November, early uterine morulae on 6-8 December while unimplanted blastocyst on 21st December, while at Khandala, ovulation and fertilization occur during the first week of January, late uterine morulae being found between 7th and 23rd January, unimplanted blastocysts between 1st-2nd February and early implanting blastocysts on February 23. This led Ramakrishna and Rao (1977) to conclude that embryos remain free in the uterii of bats at Khandala for 40-45 d. The gestation period in both the places is 150±8 d. The delivery of the young at both places occurs just before the onset of monsoon which is apparently an adaptation to the environment to ensure that mothers in lactation and the weaned young have plentiful supply of insects which are abundant during the rainy months.

In *Macrotus californicus* (Bradshaw, 1962) implantation occurs in October-November in South Western United States (Arizona) but the primitive streak stage is not attained by the embryos until 4-5 months later. The rate of development accelerates in March and parturition takes places in June. Bradshaw (1962) suggested that the delay might be caused by low winter food supplies. However, Burns *et al.*, (1972) observed that the delay is independent of temperature and food conditions and showed that the rate of embryonic development in females maintained under constant temperature (24°C) does not exceed that of specimens in the field. Field observations also suggest that *Macrotus* feeds daily during the winter, deposit fat, but does not hibernate or migrate (Bleier, 1975a).

Retarded embryonic development after the initiation of implantation has been reported in the captive as well as a reproductively synchronized wild population of the short-tailed fruit bat, *Carollia perspicillata* (Rasweiler and Badwaik, 1997). Gestation periods for females which successfully reared their young varied as follows : females breeding first year in captivity (105-178 d), mated at post partum estrus during their first year in captivity (110-158 d), mated during their second year in captivity (113-169 d); females

born and mated in captivity (113-159 d). Most females in the group had a gestation period of 113-119 d which may represent the normal (non delayed) period for the species. According to the authors, stress rather than age was responsible for the prolongation of pregnancy. Many wild caught females successfully gave birth at 160-229 d after being isolated from breeding males in captivity. In the wild caught females the prolongation of gestation has also been attributed to stressful situations (*e.g.* lack of sufficient food or roosting sites) and it has been postulated that the ability to delay pregnancies would be of considerable adaptive value in *Carollia*.

b. Related to post partum pregnancy *e.g. Artibeus jamaicensis* (Fleming, 1971), *Cynopterus sphinx* (Krishna and Dominic, 1983) and *Haplonycteris fischeri* (Heideman, 1989).

The first pregnancy of each year in the neotropical fruit bat, *Artibeus jamaicensis* (Fleming, 1971) lasts about 4 months and proceeds without embryonic delay. The second pregnancy (post partum) of each year is of 7 months duration and the post implantation embryonic development is extended by 2.5 months. The retarded embryonic development after implantation of the blastocyst occurs during the height of the rainy season and is not caused by lactation. Development does not cease completely but proceeds at a slow rate. Although no variation in size of corpus luteum (CL) was observed in the two pregnancies the luteal cells were significantly smaller in size in the CL of post partum pregnancy with retarded embryonic development. According to Fleming (1971), CL is functional but low amounts of luteotrophic hormone may be operating indirectly to slow embryonic development after implantation. A possible post implantation delay has been reported in one of the two annual pregnancies in *Cynopterus sphix* (Krishna and Dominic, 1983). However, the nature of the delay is not clear as it was demonstrated using weight changes of whole uterii, and the results are also compatible with delayed implantation accompanied by a uterine weight increase due to a progestational reaction at the beginning of the delay.

In *Haplonycteris fischeri* (Heidemen, 1989) the parous female are impregnated in June within a few weeks of parturition. Shortly after implantation the rate of embryonic development slows tremendously for up to 8 months. During the period of delay, the mean length of the embryoblast increased only from 280 μm to 580 μm. In the following year during March, the development rate increased and the embryos completed development in the next 3 months. The 8 month delay gives this bat a gestation period of 11.5 months, the longest known in bats. Most nulliparous females became pregnant at an age of 3-5 months, and the embryos entered a similar delay that terminated in

March or April, after 2-6 months of delay. No clear changes in the luteal cells over the period were observed but according to the author this does not preclude the possibility that there are changes in steroid production during the delay. The author emphasized the need for a detailed study to determine the mechanism and control of the delay.

c. Related to hibernation-torpor/semitorpor: Eisentraut (1937) was the first to draw attention to the effect of variations of environmental temperature on the fetal development in heterothermic bats. In *Pipistrellus pipistrellus, Myotis myotis* and *Corynorhinus rafinesquei*, reduced environmental temperature significantly lengthens gestation by slow embryonic development (Eisentraut, 1937; Pearson *et al.*, 1952). In *Pipistrellus pistrellus* (Racey, 1973, 1980; Racey and Swift, 1981) the length of gestation varies with temperature and food supply.

Differential rates of fetal growth in two successive pregnancies have been reported in *Taphozous longimanus* (Krishna and Dominic, 1982). The authors observed slow rate of growth of the embryo during the first pregnancy which they attributed to the adaptive hypothermia during the height of winter. Uchida, *et al.* (1984) have shown that *Miniopterus schreibersii fuliginosus* when kept in captivity without hibernation at elevated temperatures (23°C-25°C) in winter, the embryonic growth rate was accelerated and parturition was advanced by a period equivalent to the of exclusion from hibernation as compared with that in the wild population. Also the CL became active indicated by an increase of `light' lutein cells with a 15 mm embryo but it was less active with more `dark' cells in two hibernating control bats with an implanting blastocyst.

Several authors (Dwyer, 1963; Wimsatt, 1969; Richardson, 1977; Bernard, 1980, Van der Merwe, 1980; Racey, 1982; Bernard *et al.,* 1996) have noted that the duration of delayed implantation decreases with decreasing latitudes. It is interesting to note that no delay strategy occurs in the miniopterine bats, *Miniopterus schreibersii fuliginosus* from India (Gopalkrishna *et al.*, 1985 a,b), *Miniopterus australis* from Australia 21°S (Sanborn and Nicholson, 1950), 15°S (Baker and Bird, 1936), 4°N (Medway, 1971) and *Miniopterus inflatus* from Gabon, Africa 0°4'N (Brosset and Saint Girons, 1980). Bernard *et al.,* (1996) suggested that the studies of the relationship between latitude and the duration of embryonic delay should be concentrated on populations of a single species from different latitudes in the same continent.

In *Hipposideros caffer caffer* from Natal, South Africa (~29°S) (Bernard and Meester, 1982) ovulation occured in late April (early winter), CL was extruded from ovary and disappeared within one month after ovulation. The gestation period was estimated at 220 d of which the period of zygote accounted for 21 d or 9.5%, the period of embryo 123 d or 55.9% and the period of fetus 76 d or 34.5%. According to the authors, development from fertilization to implantation was normal but embryonic development was retarded during winter. They further observed that *Hipposideros caffer caffer* does not hibernate throughout winter but entered short periods of torpor during cold spells. In *Hipposideros l. lankadiva* (Khan, unpulished) preimplantation stages of embryo were noticed until the second week of September after which the blastocyst implants (14th September). By the end of October the bats enter into a phase of semi-torpor in winter for about 5 months until mid-March. The implanted bilaminar blastocyst remains in the embryonic disc stage throughout semi-torpor exhibiting retarded embryonic development. After arousal from semi-torpor in mid-March the development is accelerated. The primitive streak develops after arousal 7 months post-coitum and deliveries take place during end of May or early June. The CL persists for 8 months and regresses at the late limb bud stage of development.

d. Related to Hormones: Recent research work on delays in embryonic development in bats is directed to establishing the role of hormones of the thyroid, CL (progesterone) and those of the anterior pituitary. Mead (1993) summed up information on *Macrotus californicus* which is as follows: maternal blood levels of thyroxine are depressed during retarded embryonic development in *Macrotus californicus*, but increase when embryonic development is renewed. Maternal triiodothyronine is only slightly elevated during winter months and then appear to decline when embryonic development resumes (Burns, 1981). Repeated injections of thyroxine for 20 d fail to increase the rate of embryonic development. Gonadal steroids are secreted in a biphasic pattern during pregnancy. An early rise in estrogen and progesterone secretion occurs at the time of implantation and placental development (October-December). However, the precise stage of development was not confirmed in these animals. This is followed by a period of marked reduction in both estrogen and progesterone, which coincides with mid-delay period (January-March). Secretion of both hormones again increased significantly during the more rapid and final stages of embryonic development (April-May). Thereafter, progesterone levels rapidly declined prior to parturition (Burns and Wallace, 1975; Burns and Easley, 1977). Unilateral removal of the right functional ovary bearing the CL at any stage of pregnancy, consistently resulted in abortion whereas sham ovariectomy was without effect (Burns, 1981). Changes in ultrastructure of luteal cells of this bat are correlated with changes in plasma levels of progesterone (Bleier, 1975b; Crichton *et al.*,

1990). These observations suggest that reduced luteal function is somehow linked to the retardation of embryonic development. Richardson (1979, 1981) reported an increase in the number of prolactin (PRL) cells prior to renewed embryonic development. This led him to hypothesize PRL was somehow implicated in the process of retarded embryonic development. Prolactin might be essential to stimulate increased luteal function. Alternately, PRL might somehow influence uterine function or growth of the embryo.

In *Miniopterus schreibersii*, Peyre and Herlant (1967) observed that the CL is poorly developed throughout delayed implantation of 5 months which coincided with hibernation. An arrest in LH secretion after ovulation together with insufficient PRL is responsible for delayed implantation. In the Japanese long-fingered bat, *Miniopterus schreibersii fuliginosus* (Kimura *et al.*, 1987) the plasma progesterone concentration was not significantly elevated during development after the implantation which occurs during hibernation and rose significantly during the rapid embryogenesis that occurs after arousal from hibernation. Changes in CL volume corresponded closely with those of plasma progesterone values. Van der Merwe and van Aarde (1989) have observed that in *Miniopterus schreibersii natalensis* the plasma progesterone concentration increased significantly during the period of delayed implantation. Values peaked at implantation but decreased thereafter. Concentrations remained low (60 ng/ml) during initial period of fetal development and attained peak values (85.6-181.3 ng/ml) 216-222 d after fertilization which coincided with significant increase in placental weight.

Bernard *et al.*, (1991) and Bernard and Bojarski (1994) have studied in detail the factors responsible for delayed implantation in *Miniopterus schreibersii.* Their studies have shown that in *Miniopterus schreibersii,* torpor may be responsible for suppression of luteal activity and consequently delay implantation. During this period plasma progesterone and LH concentrations were also found to be low. Treatment of pregnant bats with exogenous PRL for 10 d in early delayed implantation resulted in activation of luteal activity, high plasma progesterone concentration and the initiation of implantation. Treatment of bats with human chorionic gonadotrophin (hCG) during this period also includes similar changes in luteal activity and plasma progesterone concentration but failed to initiate implantation. Treatment with exogenous progesterone had no influence on the luteal activity as well as the initiation of implantation. Therefore, they concluded that PRL is the most important pituitary hormone in the control of delayed implantation in *Miniopterus schreibersii.*

Khan (unpublished) has observed that the CL in *Hipposideros lankadiva lankadiva* is well developed during the preimplantation stages of development of the embryo and during implantation of the blastocyst and the plasma progesterone level is high 34.66 ng/ml and 40.9 ng/ml, respectively. There is slight reduction in the size of the CL during retarded embryonic development and the plasma progesterone level decreased to 12.6 ng/ml, respectively. By mid-March the arousal period of bats from semi-torpor there is no appreciable increase in the size of the CL but the level of progesterone increases, the value being 41.6 ng/ml. After arousal, the embryonic development is accelerated, and by 2nd April advanced stages of development were observed. The chorioallantoic placenta becomes fully established at the late limb bud stage and the CL regresses. At advanced pregnancy no CL is present but the level of progesterone is high reading 46.0 ng/ml which is due to a well developed and functional chorioallantoic placenta. At term, the level of progesterone decreases and reads 12.0 ng/ml.

e. Evolution of Reproductive Patterns and Delays: With regard to African bats, Bernard and Cummings (1997) have proposed that in fruit bats (Megachiroptera) and non-molossid Microchiroptera there is a trend from either seasonal or seasonal polyestry, with prolonged or continuous spermatogenesis in the tropics, towards a seasonal monoestry and seasonal spermatogenesis at more temperate latitudes. Reproductive delays (sperm storage. delayed implantation and delayed development) are rare in tropical latitudes, but are the norm in the non molossid Microchiroptera away from the tropics. The molossids are mostly polyestrous at tropical and temperate latitudes although the duration of the reproductive season decreases with increasing latitude. The molossids appear to have escaped the constraint that affect reproduction of the other Microchiroptera. According to them flight capabilities and foraging behaviour of molossids gave them access to year-round food, and to the thermal characteristics of their roosts. They suggested that the ancestral reproductive pattern of the Chiropteran was probably seasonal or a seasonal polyestry as seen in extant tropical species, and therefore their reproductive cycles have evolved from the polyestrous to the monestrous condition. They observed short period of reproductive delays in some species of tropical bats and suggested that reproductive delays originally were not adaptations to temperate latitude but rather to the long dry season, which is characteristic of African tropical latitudes. With the move away from the tropics, selective pressures, acting on the timing of lactation and spermatogenesis, would have ensured that the process continued to occur in the warm, wet season, and that the length of the reproductive delay increased. This model accommodates the probable evolutionary origin of bats and links the evolution and development of reproductive delays to the differences in climates that occur with changes in the latitude. According

to them there is evidence that mate choice and sperm competition may be important to modern bats, but believed that they need not be invoked as casual factors in the evolution of reproductive delays, which according to them can be adequately explained using purely energetic arguments.

To sum up, we emphasize that the secretion of ovarian (CL) hormone is controlled by the pituitary which in turn is under the influence of the hypothalamus. Although some cues regarding factors controlling delayed implantation in some species of bats are known, we agree with Mead (1993) that the phenomenon of embryonic delays in Chiropteran still holds mysteries for scientists to solve.

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HOMEOTIC TRANSFORMATION IN ANURANS

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ABSTRACT

In this paper we report for the first time normal looking tail regeneration along with homeotic transformation of tail to limbs following vitamin A treatment in *Microhyla ornata*. We also present a comparative account of the effects of vitamin A including homeotic transformation of the tail amputated tadpoles of four anurans namely *Polypedates maculatus, Microhyla ornata, Uperodon systoma* and *Bufo melanostictus*. Further, uptodate report on homeotic transformation is analyzed in the present context.

Key words: Homeotic transformation, vitamin A, regeneration, anurans

INTRODUCTION

There are several reports on the effect of vitamin A and its derivatives, the retinoids, in different systems of the developing embryo. Vitamin A also has remarkable effect on limb development and regeneration (Maden, 1983; Scadding and Maden, 1986a,b; Ludolph *et al.*, 1990). Local application of retinoic acid (RA) to the anterior side of the developing chick limbs cause duplication in the anteroposterior (AP) axis of limb (Tickle *et al.*, 1982; Summerbell, 1983). In regenerating amphibian limbs, retinoids can lead to pattern duplication in the proximodistal (PD), AP and dorsoventral (DV) axes (Maden, 1982,1983; Niazi and Ratnasamy, 1984; Maden *et al.*, 1985; Ludolph *et al.*, 1990). The inhibitory and modifying effect of vitamin A on tail regeneration of anurans was observed by Niazi and Saxena (1968) for the first time in the tadpoles of *Bufo andersonii.* Similar inhibitory effects have also been reported in *Notopthalmus viridescens, Ambystoma mexicanum* and *Xenopus laevis* (Scadding, 1987), However, in addition to inhibition of tail regeneration, limbs were generated at the site of tail amputation in *Uperodon systoma* (Mohanty-Hejmadi *et al.*,1992), *Polypedates maculatus* (Mohapatra and Mohanty Hejmadi, 1994); *Rana temporaria* (Maden, 1993);

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Rana ridibunda (Muller *et al.*, 1994), *Bufo melanostictus, Microhyla ornata* and *Hoplobatrachus tigerinus* (Das and Dutta, 1996). In this paper we report a comparative account of the effect of vitamin A on the tail amputated tadpoles of four anurans namely *P. maculatus, M.ornata, U. systoma* and *B. melanostictus*. We also report the development of ectopic limbs along with normal looking tail after regeneration in the same tadpole of *M. ornata* for the first time.

MATERIALS AND METHODS

Tail amputation and rearing of tadpoles were followed as per the details given earlier (Mahapatra and Mohanty-Hejmadi, 1994). The experimental tadpoles were exposed to vitamin A 10IU/ml for different time periods, *i.e.* 24 h (set I), 48 h (set II), 72 h (set III), 96 h (set IV), 120 h (set V) and 144 h (set VI), respectively. The tadpoles of *B. melanostictus* were exposed upto 72 h since they did not survive beyond this. In all the species except for *B. melanostictus* the regenerated tail tip of the control and experimental tadpoles (vitamin A 10IU/ml treatment for 144 h) were processed for histology. Serial transverse sections were cut at 10µm and stained in Mallory's triple stain for examination under the light microscope.

RESULTS

MORTALITY

Vitamin A had a toxic effect on the survival of the tadpoles (Table 1). In *P. maculatus* a positive relaionship between mortality and duration of exposure has been reported (Mahapatra and Mohanty-Hejmadi, 1994). Similar toxic effects were also observed in the tadpoles of *M. ornata*, since 60-100% died prior to the emergence of forelimbs. There was 80% death in each from set II (48 h) and set III (72 h) which was reduced to 70% each in set IV (96 h) and set V(120 h). The percentage of death increased to 100% in set VI (144 h). However, the correlation coefficient (r) was 0.82 showing a positive correlation between the duration of exposure and mortality. In case of *U. systoma*, 30-70% died before the emergence of forelimbs (Mohanty-Hejmadi *et al.*, 1992). The percentage of death was the highest, *i.e.* 70% in set III (72 h) which decreased to 30% in set IV (96 h). There was again increase in the percentage of death, *i.e.* 50% and 60% from set V (120 h) and set VI (14 h), respectively. The correlation coefficient (r) was 0.53 showing a positive correlation between exposure period and mortality rate. In *B. melanostictus* 10% death occurred in set I (24 h) which increased to 30% and 60% respectively after 48 h (set II) and 72 h (set III) treatment. Beyond 72 h of treatment the tadpoles did not survive. The correlation coefficient (r) was 0.97 showing a positive

correlation between exposure period and mortality. The most puzzling result was in *M. ornata* and *U. systoma.* The mortality rate was sometimes more in the sets where the tadpoles were treated for a shorter period which might have been due to individual tadpole's resistance to vitamin A.

TAIL REGENERATION

As reported earlier, in *P. maculatus,* vitamin A treatment inhibited normal tail regeneration (Mahapatra and Mohanty-Hejmadi, 1994). There was direct correlation between the abnormal tail and duration of exposure (Table 2). With 24 h exposure, 60% developed abnormal tail which increased to 100% after 96 h of treatment and beyond, indicating that the continuous exposure suppressed the process of regeneration. Tail abnormality included bulbular mass formation at the tail tip, upwardly, downwardly or laterally curved axial tissue and supression of ventral or both tail fins (Figs. 1E,F) in addition to ectopic hindlimb (EHL) formation (Fig. 2A) (Mahapatra and Mohanty-Hejmadi, 1994).

In *M. ornata*, 24 h treatment had no adverse effect on tail regeneration. With 48 h and 72 h treatment, 60% and 80% tadpoles regenerated abnormal tail, respectively. Beyond 72 h, tail regeneration was abnormal in 100% tadpoles. Tail abnormality included bulbular mass formation of axial tissue at the cut end of tail, upwardly curved axial tissue (Fig. 1C), downwardly curved axial tissue, suppression of ventral tail fin or both tail fins and EHL development from tail tip (Fig. 2B). In one tadpole (10%) from set III (72 h) there was development of two EHLs along with normal tail regeneration (Fig. 3).

As reported earlier in *U. systoma* (Mohanty-Hejmadi *et al.*, 1992) vitamin A treatment for 24 h inhibited tail regeneration in 90% tadpoles and in the remaining 10%, tail regeneration was normal. In all the tadpoles from 48 h to 144 h tail regeneration was abnormal. Tail abnormality included bulbular mass at distal end of the axial tissue, upwardly curved axial tissue, downwardly curved axial tissue (Fig. 1D) and EHL development from tail tip (Fig. 2C). In this species, suppression of tail fin was not observed. In *B. melanostictus* tail regeneration was abnormal in all the experimental tadpoles. Tail abnormality included bulbular mass at the distal end of tail (Fig.1B), upwardly curved and downwardly curved axial tissue, suppression of ventral tail fin including ectopic hindlimb development from the tail tip (Figs. 2C,D).

A comparative account of different types of tail abnormalities (Table 3) indicate that bulbular mass formation is the highest in *P. maculatus* and lowest in *M. ornata.* Upwardly curved axial tissue was the highest in *P. maculatus* and lowest in *M. ornata.*

Downward curvature of axial tissue was maximum in *P. maculatus* and laterally curved axial tissue was seen only in *P. maculatus.* Suppression of ventral tail fin was observed in three species excluding *U. systoma.* Total tail fin suppression was observed in *P. maculatus* and *M. ornata* but did not occur in *U. systoma* or in *B. melanostictus.* In all the four species, the experimental tadpoles regenerated a blunt tail tip within 2 d of amputation. The occurrence of blunt tips was the highest in the tadpoles of *U. systoma* and lowest in *B. melanostictus* as some tadpoles of the three species except *P. maculatus* died within 2 d of amputation. Another tail abnormality was development of a pouch like structure at the tips of all the experimental tadpoles after 3 d of amputation which persisted up to 2 d post amputation. The occurrence of such pouches was the highest in *U. systoma* and lowest in *B. melanostictus* as some tadpoles of the three species except *P. maculatus* died within 3 to 10 d postamputation. Beyond 10 d the pouches were converted to different types of abnormal tails as described previously.

METAMORPHOSIS

Metamorphosis was delayed after vitamin A treatment in all the four species (Table 4). In *P. maculatus* the delay increased on exposure upto 72 h, but was relatively constant on exposure for 96 h to 144 h (Mahapatra and Mohanty-Hejmadi, 1994). In *U. systoma* there was delay in metamorphosis and maximum time was taken on exposure for 120 h (Mohanty-Hejmadi *et al.*, 1992). In *M. ornata* also there was delay in metamorphosis in the experimental tadpoles. There was gradual increase upto 48 h exposure which decreased on 72 h exposure. Beyond 72 h there was again increase in the mean days for the onset of metamorphosis. Maximum time was taken by the tadpoles from 120 h exposure group. In *B. melanostictus* there was also delay in the onset of metamorphosis. Maximum days were taken by the tadpoles from 72 h exposure group.

There was reduction in the S-T (snout to tail tip) length at the onset of metamorphosis in the experimental tadpoles (Table 5). The S-T length was always the greatest in the tadpoles of the control group. In *P. maculatus* and *U. systoma* S-T length was the lowest in the 144 h treated group. (Mohanty-Hejmadi *et al.*, 1992; Mahapatra and Mohanty-Hejmadi, 1994). In *B. melanostictus* the lowest S-T length was seen in the 72 h treated tadpoles. However, in *M. ornata* there was gradual reduction in the S-T length upto 96 h treated group and a marginal increase in set V (120 h treated) tadpoles.

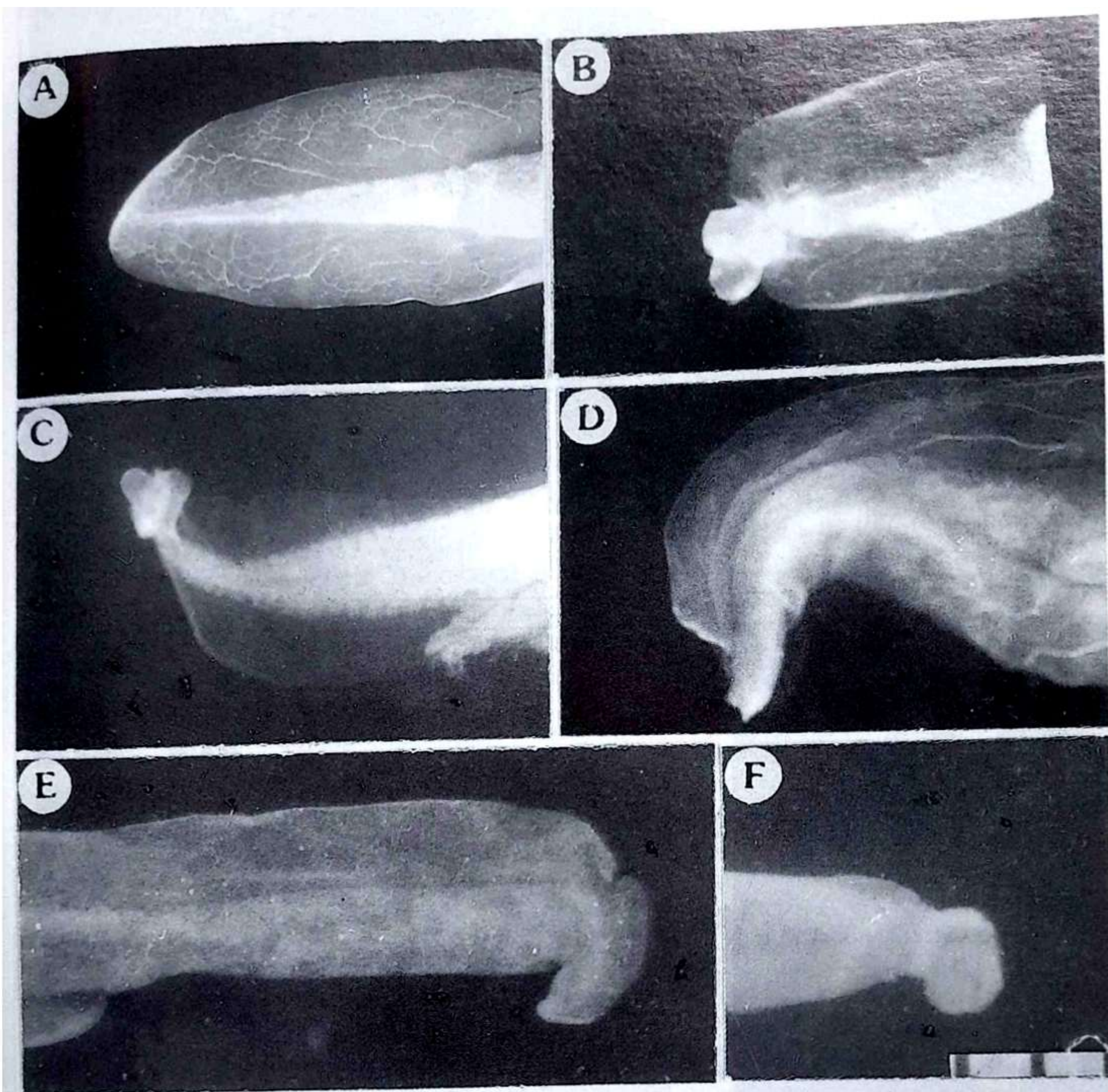
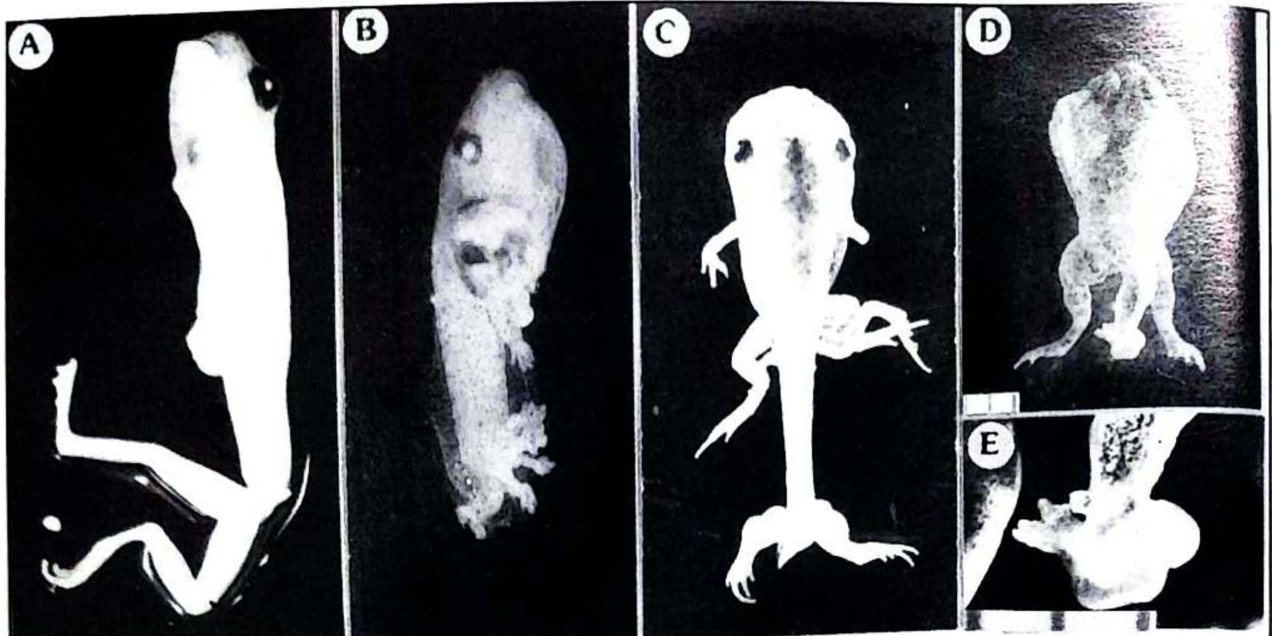
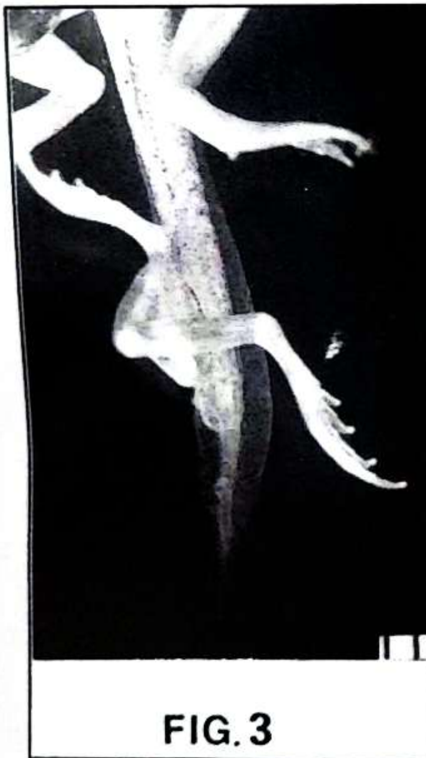


Fig. 1

Fig.1 : Abnormal tail regeneration in the experimental tadpoles ;
A. normal tail in *B. melanostictus* (control)
B. bulbular mass formation at the tail tip in *B. melanostictus*,
C. upwardly curved axial tissue in *M. ornata*,
D. downwardlly curved axial tissue in *U. systoma*,
E. suppression of ventral tail fin in *P. maculatus*
F. supression of both tail fins in *P. maculatus*.
Figs. **A-F**, scale bar represents 2 mm.

Fig. 1





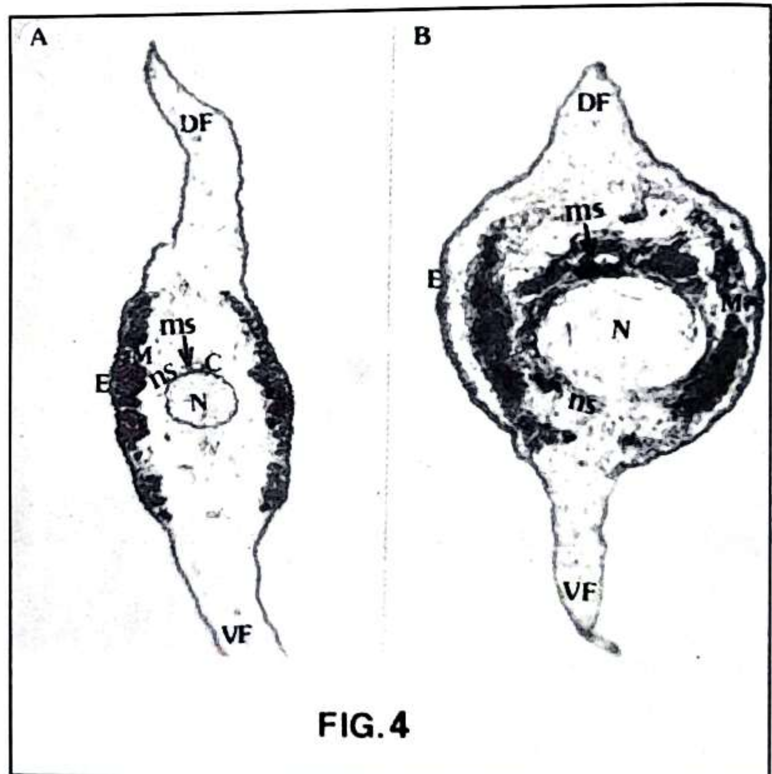


Fig. 2 : Ectopic hindlimb development

A. a pair of ectopic limb in *P. maculatus*, both pairs of normal limbs suppressed. **B.** four ectopic limbs in *M. ornata*, **C.** a pair of ectopic limb with duplicate hindlimbs in *U. Systoma*, **D.** two small ectopic limbs in *B. melanostictus*, **E.** enlarged view of the same ectopic limb (arrow) in *B. melanostictus*.

Fig. 3 A pair of ectopic hindlimbs in *M. ornata*. A normal looking tail regenerated beyond the ectopic limbs. Scale bar represents 2mm.

Fig. 4. **A.** Transverse section of the regenerated tail tip of *P. maculatus* (control) 144h post ampuation, showing a thin epidermal layer (E), muscles (M) in the form of bundles, normal looking notochord (N) covered by a thin notochordal sheath (ns) and nerve cord (C) surrounded by a thin myllin sheath (ms). X 100.

B. Transverse section of the regenerated tip of *P.maculatus* (experimental, vitamin A 10IU/ml treatment for 144h) 144hr post amputation showing a thick epidermal layer (E), unorganized and clumped muscles (M), enlarged notochord (N) with a thick notochordal sheath (ns) and an enlarged nerve cord (C) surrounded by a thick myelin sheath (ms). X 1000.

ECTOPIC HINDLIMBS

Ectopic hindlimb (EHL) formation occurred in all the species with the highest in *U. systoma* and lowest in *B. melanostictus*. Thus, although EHL formation occurred in all the species there was variation in the percentage as well as the type of abnormal tail regeneration. The EHLs were always hindlimbs with distinct thigh, shank, ankle and digits. Generally the size of EHLs were smaller than the normal hindlimbs. There was differentiation of bone and cartilage in the EHLs of *P. maculatus* (Mahapatra and Mohanty-Hejmadi, 1994). In *P. maculatus* out of the 7 tadpoles which developed EHLs, pelvic girdle developed in 5 (71.4%). In *U. systoma*, the skeletal elements were cartilaginous (Mohanty-Hemadi *et al.*, 1992). In *U. systoma*, 8 (61.5%) tadpoles developed pelvic girdle out of the total 13 which developed EHL. In *M. ornata* EHL developed in 3 tadpoles. In 1, pelvic girdle developed and there was differentiation of bone and cartilage in the EHLs. The other 2 tadpoles (Fig. 2B) died early and they were too fragile to be processed for the study of skeletal elements. In *B. melanostictus* EHL developed in only 2 tadpoles and it was beyond the scope to process the skeletal elements as the EHLs were very delicate and fragile.

The EHLs were not always oriented along the AP axes in all the four species. They were oriented laterally in 4 (57.1%) out of 7 tadpoles in *P. maculatus*. In case of *U. systoma* in 2 (15.3%) out of the 13 tadpoles the EHLs were oriented laterally. However, there was lateral orientation in all the EHLs of *B. melanostictus*. In *M. ornata*, 2 (66.3%) out of the 3 showed lateral orientation of limbs (Fig. 2B) and in the other (33.3%) there was reversal in the orientation of 1 EHL along the AP axes (Fig. 3).

LIMB ABNORMALITY

a. Supression of limb development

Vitamin A had an inhibitory effect on limb development (Table 6). As repoted earlier (Mahapatra and Mohanty-Hejmadi, 1994) in *P. maculatus* limb supression ranged from partial to total (Fig. 2A). Maximum suppression was seen in the tadpoles with 72 h treatment.

In tadpoles of *M. ornata, U, systoma* and *B. melanostictus* only partial limb suppression was observed. In *M. ornata* 10% tadpoles from set III (72 h) showed partial limb suppression. In *U. systoma* 10%, 20% and 10% tadpoles showed partial limb suppression from set II (48 h), set III (72 h) and set V (120 h), respectively. However, in *B. melanostictus* there was partial suppression of limbs in 10% tadpoles each from set I (24 h) and set III (72 h). Thus, treatment period of 72 h caused limb suppression

in all the species, the supppression being more severe in *P. maculatus* and less in the other 3 species. If one considers frequency, the suppression was highest (48.6%) in *P. maculatus*. The incidence of limb suppression decreased to 6.6% both in *U. systoma* and *B. melanostictus* whereas it was only 1.6% in *M. ornata*. This indicates species specificity of limb-bud tissue to vitamin A.

b. Limb duplication

Development of duplicate limbs occurred in the tadpoles of *B. melanostictus* and *U. systoma* (Table 7). In *U. systoma* (Fig. 2C) only 10% tadpoles from set IV (144 h) developed duplicate limbs, bilaterally (Mohanty-Hejmadi *et al.*, 1992). In *B. melanostictus* 50% each from set I (24 h) and set II (48 h) and 20% from set III (72h), respectively, developed duplicate limbs. There was unilateral or bilateral hindlimb or forelimb duplication which sometimes extended to both pairs of limbs.

HISTOLOGY:

Histological analysis of the regenerated tail tip revealed some remarkable difference between the control and experimental tadpoles in all the four species. In the control group of *P. maculatus* (Fig. 4A) the epithelial layer was thin. Muscles were arranged in bundles on lateral sides just beneath the epithelium. The notochord was circular with a thin layer of sheath and the nerve cord was normal with a thin myelin sheath. However, in the experimental tadpoles (Fig. 4B) the epithelial layer was multilayered. The muscles were not arranged in their respective bundles on lateral sides. There was enlargement of the notochord and a thick notochordal sheath encircled it. The nerve cord also became enlarged with a thick myelin sheath around it.

DISCUSSION

The above results (Table 1) show a positive relationship between mortality and duration of exposure in the tadpoles of *P. maculatus* (Mahapatra and Mohanty-Hejmadi, 1994), *U. systoma* (Mohanty-Hejmadi *et al.*, 1992), *B. melanostictus* and *M. ornata* since the correlation coefficient showed a positive value. In case of *U. systoma* and *M. ornata* there were some surprising results showing relatively more death under less hour of treatment. This can only be explained as individual tadpole's resistance to vitamin A. Similar exposure-related mortality has been reported in *B. andersonii* (Niazi and Saxena, 1968; Saxena and Niazi, 1977); *R. cyanophlyctis* (Niazi and Saxena, 1972); *R. temporaria* (Maden, 1993); *B. melanostictus, M. ornata* and *H. tigerina* (Das and Dutta, 1996).

In all the four species there was delay in the onset of metamorphosis in the experimental tadpoles. However, in *P. maculatus*, set III (72 h) tadpoles took the longest time for the onset of metamorphosis (Table 4). In *U. systoma* the time taken for the onset of metamorphosis decreased in the tadpoles from set II (48 h) and set III (72 h) which further increased in set IV (96 h). The set V (120 h) tadpoles took the longest period (Table 4) for the onset of metamorphosis. These unusal results may be due to individual tadpole's response to vitamin A. But when compared with the controls all the treated tadpoles took a longer period for the onset of metamorphosis. Niazi and Saxena (1972) also reported prolongation of life cycle in *Rana cyanophlyctis* on exposure to vitamin A. Das and Dutta (1996) have reported no metamorphosis in *B. melanostictus* and delayed metamorphosis in *M. ornata*. Further, Niazi and Saxena (1972) have related this delay to decreased thyroid gland development and reported that the thyroid gland became progressively smaller on exposure of the tadpoles to higher concentration of vitamin A. Fewer follicle cells were seen in the histological sections of the thyroid gland of the tadpoles treated with vitamin A. The present finding might also be due to suppression of thyroid gland development as explained earlier.

Reduction in S-T length at the onset of metamorphosis was a common phenomenon in all the four species (Table 5). The extent of reduction was directly related to the period of exposure except *M. ornata* where S-T length in set I tadpoles was smaller than the set II tadpoles. There was gradual decrease upto set IV and in set V, a marginal increase in S-T length was observed. However, the S-T length of all the experimental tadpoles were always shorter than the control tadpoles. This deviation may be due to species specific resistance to vitamin A. These results are consistent with our earlier reports on *P. maculatus* (Mahapatra and Mohanty Hejmadi, 1994), *B. andersonii* (Niazi and Saxena, 1968) and *R. cyanophlyctis* (Niazi and Saxena, 1972).

Tail regeneration was abnormal in 100% experimental tadpoles of *B. melanostictus*. Abnormality was reduced to 98.3% and 91.7% in the tadpoles of *P. maculatus* and *U. systoma*, respectively (Mahapatra and Mohanty-Hejmadi, 1994; Mohanty-Hejmadi *et al.*, 1992). Tail abnormality was the lowest (73.3%) in *M. ornata*. The variation in tail abnormality was due to species-specific resistance to vitamin A. Similar inhibition of tail regeneration after vitamin A treatment has been noted in *B. andersonii* (Saxena and Niazi, 1977); *X. laevis, N. viridescens* and *A. mexicanum* (Scadding, 1987); *R. temporaria* (Maden, 1993); *B. melanostictus, M. ornata* and *H. tigerinus* (Das and Dutta, 1996).

Histological studies in *B. melanostictus* have shown that vitamin A adversely affects all the tail tissues (Das and Mohanty-Hejmadi, 2000). In *P. maculatus*, the epidermis becomes multilayered confirming the observation of Fell and Mellanby (1953) in chick that vitamin A causes metaplasia. Redifferentiation of the muscles leading to bundle formation was inhibited. The muscles remained clumped and disorganised. Vitamin A also caused enlargement of notochord. Similar notochordal enlargement has also been reported in *R. temporaria* by Maden (1993). There was development of a constriction at the site of amputation. The notochordal sheath was thicker in comparison to that of control. The regenerated nerve cord in the experimental tadpoles was always encircled by a very thick myelin sheath. In all the species both notochord and nerve cord redifferentiated earlier than muscles and this finding confirms the observation of Niazi and Saxena (1979).

Vitamin A induced EHL development from the amputated tail stumps in all the four species. The incidence was the highest (21.6%) in *U. systoma* tadpoles (Mohanty-Hejmadi *et al.*, 1992). In the tadpoles of *P. maculatus*, EHLs developed in 11.6% experimental tadpoles (Mahapatra and Mohanty-Hejmadi, 1994). It was further reduced in the tadpoles of *B. melanostictus* (6.6%) and *M. ornata* (5%). Das and Dutta (1996) have reported in *B. melanostictus* a total of 2.3% EHL formation with 10IU to 15IU vitamin A treatment for 24-144 h. If one takes only 10IU dosage which is comparable to our work, in 12.5% tadpoles EHLs developed and the percentage is higher in comparision to the present study. In *M. ornata* they have reported a total of 3.3% EHL formation with a higher dosage than that of the present study. Even then the percentage of EHL formation is higher in the present study. Similar induction of EHL has also been observed in *R. temporaria* (Maden, 1993); *R. ridibunda* (Muller *et al.*, 1994) and *H. tigerinus* (Das and Dutta,1996). As indicated earlier (Mohanty-Hejmadi *et al.*, 1992), in addition to normal morphogenetic field for limb formation, other areas including the tail also have the capacity to form limbs. This indicates a broad morphogenetic field at the limb bud stage which probably becomes localised in the limbs later on. The present results demonstrate a possible morphogenetic action of vitamin A on the blastemal cells of the tail as well as the cells of the limb bud by some mechanism.

According to Bryant and Gardiner (1992), the apparent homeotic change of the tail to limbs in regenerating frogs can be understood in terms of the effect of RA in changing pattern formation competent cells to a posterior-ventral-proximal (*i.e.* flank) positional value, followed by interaction along the rostral-caudal axis of the body to generate two additional pairs of hindlimb sites on the tail. It is difficult to determine whether during homeotic transformation the tail tissue is directly converted to limbs or transformed into

flank tissue first, as suggested by Bryant and Gardiner (1992). There is circumstantial evidence in favour of the latter possibility (Mahapatra and Mohanty-Hejmadi, 1994). Condensation of the tissue takes place first in the posterior-anterior and lateral to the amputated tail tip and then the EHL develops. The EHL grows out of this mass usually in a ventro-lateral direction. However, in some cases there is lateral and at times total reversal along the antero-posterior axes. The EHL development occurs usually in pairs. However, the size of EHLs are generally smaller than the normal hindlimb. In 1 tadpole of *M. ornata* a new normal looking tail regenerated beyond the EHL. The EHLs were reversed along the AP axis of the body (Fig. 3). This observation shows two distinct pathways operating during regeneration process and supports the model as proposed by Bryant and Gardiner (1992).

The Hox genes are the class of molecules which are known to regulate pattern formation, have capacity for regeneration and carry positional information. As mentioned by Maden (1993) the Hox genes are likely to be components of homeotic transformation of the tail to legs. There is evidence that RA can activate the Hox genes (Mavilio *et al.*, 1988; Tabin, 1991; Charite *et al.*, 1994; Johnson and Tabin, 1997; Lu *et al.*, 1997). Some genes regulating limb development include Hoxd-9 through Hoxd-13 (Lauter *et al.*, 1994) and Hoxa-9 to Hoxa-13 (Yokouchi *et al.*, 1991). The other genes that play important roles in establishment of axes and regulation of limbs are the Hox-4, Hox-3 and Hox-1 clusters (Simon and Tabin, 1993). The Hox-3 cluster is expressed specially in normal and regenerating posterior appendages, like the hindlimbs and tail. In homeotic transformation, tail is always converted into hindlimbs; so a gene of Hox-3 cluster may be involved in the development of tail to legs. According to the new nomenclature (Scott, 1992) the vertebrate homeobox genes Hox-4, Hox-3 and Hox-1 can be referred to as Hox-d, Hox-c and Hox-a, respectively. From the above findings it is resonable to speculate that vitamin A directly induces expression of Hox genes which play important role in establishment of axes and regulation of limb development.

According to Maden (1993) thyroid hormone (TH) and specific thyroid hormone receptors (TRS) may be involved in homeotic transformation since hindlimb development is induced by rising level of TH. In *P. maculatus* the development of normal hindlimbs were totally as well as partially suppressed and in 3 experimental tadpoles partial suppression of limbs occurred which also developed EHL (Mahapatra and Mohanty-Hejmadi, 1994). Therefore, we have stated that the process of homeotic transformation may not be totally dependent on TH or TRS as proposed by Maden (1993).

The EHLs were always innervated (Das and Mohanty-Hejmadi, 1998).

Another interesting observation was suppression of fore and hindlimb development, ranging from partial to complete suppression in experimental tadpoles (48.6%) of *P. maculatus* (Mahapatra and Mohanty-Hejmadi, 1994). However, in the tadpoles of remaining 3 species only partial suppression was observed. The incidence decreased (6.6%) in *U. systoma* and *B. melanostictus*. In *M. ornata* only 1.6% tadpoles developed suppressed limbs. To explain limb suppression Bryant and Gardiner (1992) have proposed that global application of RA in developing vertebrate limb bud causes all cells to be reprogrammed towards uniform positional value. Lack of positional diversity in the progress zone leads to the failure of growth and pattern formation and to the formation of reduced or truncated limbs.

The duplication of hindlimbs in *U. systoma* and both fore and hindlimbs in *B. melanostictus* indicate that vitamin A treatment somehow or other splits the limb bud into two distinct ones, each capable of differentiating into a limb. Only hindlimb duplication has been reported in *B. melanostictus* (Das and Dutta, 1996; Das and Mohanty-Hejmadi, 2000). Bruschelli and Rosi (1971) have also reported duplication of hindlimbs in *B. vulgaris* under the influence of vitamin A with continuous exposure from early tail bud till the development of opercular folds. In chick, similar limb duplication has been observed following RA bead implantation in the developing limb bud. The exogenous RA changes anterior cells into ZPA (Zone of polarising activity) cells which in turn provide the actual pattern duplicating stimulus (Wanek *et al.*, 1991). The ectopic ZPA cells express sonic hedgehog gene which leads to mirror image duplication initiating the transcription of the cluster of Hox genes responsible for limb duplication. In axolotl, pattern duplication can be induced by RA bead implantation into the developing limb bud (Bryant and Gardiner, 1992). However, in the present study global application of vitamin A causes limb duplication. It is possible that vitamin A is absorbed by the limb bud cells and duplication results from a similar mechanism.

Vitamin A suppresses limb development, duplicates limb and induces homeotic transformation of tail to limb. We asume that all these actions are mediated through respective genes. The details can be revealed by molecular and immunohistochemical probing. Recent findings (Tickle, 1998; Kanegae *et al.*, 1998; Bushdid *et al.*, 1998) indicate that certain transcription factors like NF-kB play a role in vertebrate limb development. As more and more factors are reported to influence limb development it

is appropriate to end with a statement of Tickle (1998) who has aptly said "although genes bring different worlds together, the challenge remains to understand how gene expression is translated into anatomy".

Table 1: **Mortality in the tadpoles of *P. maculatus, M. ornata, U. systoma* and *B. melanostictus* after exposure to vitamin A.**

Species	Sets (hour of vitamin A 10IU/ml treatment)						
	Control (0)	I (24)	II (48)	III (72)	IV (96)	V (120)	VI (144)
P. maculatus	0[1]	0	0	0	10	30	30
M.ornata	0	60	80	80	70	70	100
U.systoma	0	40	70	50	30	50	60
⋆*B.melanostictus*	0	10	30	60	-	-	-

1. Percentage of the tadpoles
⋆ Tadpoles did not survive beyond 72 h treatment

Table 2: **Tail abnormality in the tadpoles of *P. maculatus, M. ornata, U. systoma,* and *B. melanostictus* after exposure to vitamin A.**

Species	Sets (hour of vitamin A 10IU/ml treatment)						
	Control (0)	I (24)	II (48)	III (72)	IV (96)	V (120)	VI (144)
P. maculatus	0[1]	60	100 (10)[2]	90 (20)	100 (20)	100 (20)	100
M.ornata	0	0	60	80 (30)	100	100	100
U.systoma	0	90 (10)	100	100 (20)	100 (20)	100 (30)	100 (50)
⋆*B.melanostictus*	0	100	100	100 (20)	-	-	-

1. Percentage of tadpoles
2. Percentage of tadpoles with ectopic limbs
⋆ Tadpoles did not survive beyond 72 h treatement

Table 3: **Abnormalities in the regenerated tails of the experimental tadpoles of *P. maculatus, M. ornata, U. systoma* and *B. melanostictus*.**

Tail abnormalities	*P. maculatus* (N=60(%)[1]	*M. ornata* N=60(%)	*U.systoma* N=60(%)	*B. melanostictus* N*=30(%)
Normal looking tail	5(8.33)	16(26.6)	1(1.6)	0(0)
Blunt tip[2]	0	10(16.6)	12(19.2)	1(3.3)
Pouch[3]	0	15(25)	19(31.6)	1(3.3)
Bulbular mass with a straight axial tissue	13(21.6)	2(3.3)	4(6.6)	5(16.6)
Bulbular mass with upwardly curved axial tissue	5(8.33)	1(1.6)	1(1.6)	2(2.6)
Bulbular mass with downwardly curved axial tissue	12(20)	1(1.6)	1(1.6)	2(2.6)
Upwardly curved axial tissue	1(1.6)	6(10)	6(10)	6(20)
Downwardly curved axial tissue	8(13.3)	3(5)	3(5)	5(16.6)
Laterally curved axial tissue	2(3.3)	0	0	0
Suppression of ventral tail fin	0	1(1.6)	0	6(20)
Total suppression of tail fin	7(11.6)	1(1.6)	0	0
EHL development at the cut end of tail	7(11.6)	3(5)	13(21.6)	2(6.6)
Normal tail beyond ectopic limbs	0	1(1.6)	0	0

N. number of tadpole;
treatment period ranged from 24-72 h while in other 3 species it ranged from 24-144 hr;
1. Percentage of tadpoles;
2. These died very early (within 2 d);
3. Pouch persisted at the tail tip which died within 3 d postamputation. All the tadpoles are combined in each species.

Table 4: Onset of metamorphosis in the tadpoles of *P. maculatus, M.ornata, U. systoma,* and *B. melanostictus* after exposure to vitamin A.

Species	Sets (hour of vitamin A 10IU/ml treatment)						
	Control (0)	I (24)	II (48)	III (72)	IV (96)	V (120)	VI (144)
P. maculatus	21-30[1] (27.4)[2]	31-43 (35.6)	30-44 (39.8)	45-59 (50.4)	34-61 (45.4)	32-61 (46.4)	36-56 (46.3)
M.ornata	31-37 (33.4)	36-60 (40.8)	36-66 (42.7)	34-65 (40.5)	37-71 (50.4)	37-71 (51.8)	#
U.systoma	11-18 (13.6)	15-29 (18.4)	10-18 (15.6)	12-17 (15.4)	15-36 (24.8)	15-43 (27.6)	17-36 (24.8)
⋆*B.melanostictus*	10-15 (13.4)	15-24 (20.5)	21-24 (23.7)	21-27 (23.7)	-	-	-

1. Days taken for the onset of metamorphosis
2. Mean days

\# In *M. ornata* the tadpoles did not survive till the emergence of forelimbs from set VI (144 h)

⋆ Tadpoles did not survive beyond 72 h treatment

Table 5: Snout to tail tip (S-T) length at the emergence of forelimbs in the tadpoles of *P. maculatus, M. ornata, U. systoma* and *B.melanostictus* after exposure to vitamin A.

Species	Sets (hour of Vitamin A 10IU/ml treatment)						
	Control (0)	I (24)	II (48)	III (72)	IV (96)	V (120)	VI (144)
P. maculatus	38.6	34.8	29.8	28.7	26.9	25.7	25.1
M.ornata	22	20.37	21.5	19.1	17.25	17.83	#
U.systoma	31.4	23.5	22.8	20.5	18.2	18.0	17.8
⋆*B. melanostictus*	18.7	17.4	15.4	14.3	-	-	-

1. Standard deviation

\# In *M. ornata* the tadpoles did not survive till the emergence of forelimbs from set VI (144 h)

⋆ Tadpoles did not survive beyond 72 h treatment

Table 6 **Limb supression in the tadpoles of *P. maculatus, M. ornata, U. systoma* and *B. melanostictus* after exposure to vitamin A.**

Species	Sets (hour of Vitamin A 10IU/ml treatment)						
	Control (0)	I (24)	II (48)	III (72)	IV (96)	V (120)	Vi (144)
P. maculatus	0	40[1] + (10)[2]	10 + (40)	40 + (40)	30 + (20)	30	20 + (10)
M.ornata	0	0	0	10	0	0	0
U.systoma	0	0	10	20	0	10	0
⋆*B.melanostictus*	0	10	0	10	-	-	-

1. Percentage of tadpoles with partial limb suppression
2. Percentage of tadpoles with total limb suppression

⋆ Tadpoles did not survive beyond 72 h treatment

Table 7: **Limb duplication in the tadpoles of *U. systoma* and *B. melanostictus* after exposure to vitamin A**

Species	Sets (hour of Vitamin A 10IU/ml treatment)						
	Control (0)	I (24)	II (48)	III (72)	IV (96)	V (120)	VI (144)
U.systoma	0[1]	0	0	0	0	0	10
⋆*B.melanostictus*	0	50	50	20	-	-	-

⋆ Tadpoles did not survive beyond 72 hr treatment.

1. Percentage of tadpoles

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SEASONAL DISTRIBUTION OF ANOPHELINE MOSQUITOES AND EPIDEMIOLOGY OF MALARIA IN ROHTAK DISTRICT, HARYANA, INDIA

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ABSTRACT

A survey of the anopheline fauna of Rohtak district of Haryana, India was carried out for two consecutive years so as to explore the taxonomic composition and seasonal fluctuations in the occurrence of different species. A total of six anopheline species were collected during the present study. These species, in the descending order of their abundance were - *Anopheles subpictus* > *A. stephensi* > *A. annularis/ A. culicifacies* > *A. fluviatilis* > *A. hyrcanus* 'group'. The population densities and seasonal pattern of abundance have been correlated with the various abiotic factors and the epidemiology of malaria, prevalent at that particular time. *Anopheles* mosquitoes were found during the whole year except in March. The occurrence of malaria was noted to be a vector density-dependent phenomenon. Under the present circumstances, the studies on the vectorial capacity of the common anopheline species is highly desirable.

Key words: *Anopheles*, seasonal abundance, malaria

INTRODUCTION

Malaria is a major public health problem in tropical countries of the world. Anophelines are well known vectors of human malaria all over the world where more than 2 billion people are exposed to infection. It is estimated that approximately 500 million malaria cases occur each year resulting in more than 3 million deaths, mostly in children (WHO, 1996). The malaria situation in India has, however remained static since 1983, with the incidence of about 2 million cases annually (NMEP, Annual report, 1995). The widespread emergence of drug resistance strains of parasite, development of resistant anopheles mosquito to commonly used insecticides, inadequate infrastructure for

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delivery of control measures, population growth and movement of non-immune population to malarious regions have all contributed to the persistence and in many cases, worsening of the malaria problem. The growth of industrialisation, development of commerce and trait, difficulties in water management, lacunae in the implementation of antilarval operation and increasing mosquitogenic condition, has further aberrated the urban ecosystem tremendously. This has stimulated interest in alternative methods for malaria control without causing ecological imbalance. Growing international awareness of the impact of malaria, and in particular, the prospect of an imminent catastrophe in Africa, has generated new efforts in controls measures.

Anopheline mosquitoes are found in all parts of India upto an altitude of 3530 meters (Rao, 1981). Out of 420 recognized anopheline species throughout the world, 70 are considered as important vectors of malaria. In India, 58 species are reported (Nagpal and Sharma, 1994) of which 10 have been implicated in malaria transmission (Rao, 1981). Among these, 6 species *viz., stephensi, culicifacies, fluviatilis, minimus, dirus* and *aundaicus* are the major, while 4 species *viz., annularis, philippinensis, jeyprieonsis* and *varuna* are minor or secondary vectors in transmission of malaria.

In India, malaria is endemic except for some mountainous areas above 1880 meters from sea level and well drained coastal areas of western and eastern ghats. Malaria has for years been described as a rural problem in India which is not correct. The principal vector of urban malaria in India is *A. stephensi* and the secondary vector is *A. culifacies* (Hati, 1997). Studies from different areas indicate that seasonal prevalence of *A. stephensi* varies widely (Gakhar and Vandana, 1996; Hati, 1997). Due to the numerous constraints in the eradication or control of the disease such as the changed epidemiological situation, insecticidal pressure and the numerous developmental activities leading to enormous changes in the environment, attention is once again focussed towards the intensification of entomological studies. Vectorial efficiency of the malaria vectors need to be re-evaluated since lack of this information adversely affects malaria eradication activities.

Malaria epidemiological magnitude is the result of interaction between parasites, vectors and abiotic factors such as temperature, rainfall, humidity, vast breeding places and the prolonged transmission season. These factors, however, create extremely suitable conditions for the realization of vector borne diseases among which malaria is a leading one. There is an intricate ecological association between mosquito vector species and ecosystem of breeding grounds. Therefore, the emerging concept of

malaria eradication to a great extent embodies the indepth knowledge of population ecology of the vector.

The factors governing the changes in the population densities of the mosquitoes are frequently complex, an understanding of which is essential for the effective implementation of control measures. Barring few studies regarding the taxonomic composition (Gakhar and Vandana, 1994), seasonal prevalence and biology of different anopheline species (Ansari *et al.*, 1982; Sharma *et al.*, 1983; Subbarao *et al.*, 1984; Sharma, 1991), no such studies have been carried out in Haryana, particularly in Rohtak district. However, the reports on such aspects are available from other parts of India like Delhi, Nagaland, Orissa, Madhya Pradesh and Gujarat (Bhatia *et al.*, 1958; Sarkar *et al.*, 1980; Das *et al.*, 1990; Kulkarni, 1990; Bhatt *et al.*, 1991).

The objective of the present research was to know the detailed seasonal occurrence and variations in the population densities of the larval and adult anopheline species, population structure and malaria prevalence. The knowledge gained might help in having a better understanding on the ecology of vector species and which in turn might help in planning better control strategies.

MATERIALS AND METHODS

The anopheline survey was carried out for two successive years, *i.e.* from October 1995 to September 1997. The meteorological data of Rohtak was obtained from Indian Meteorological Department, New Delhi (Gakhar and Vandana, 1994). The mosquito collections were carried out once in a month during morning hours from 10 different localities of Rohtak. These sites were natural open drains, cattle sheds and human dwellings for immature mosquitoes and mixed dwellings for adults. The collection sites and the methods of collection of immature and adult mosquitoes were as described earlier (Gakhar and Vandana, 1994). The data on human *falciparum* and *vivax* malaria in Rohtak per month basis was collected from District Malaria Office, Rohtak. The urban malaria cases were reported on passive surveillance basis. The data has been extrapolated to correlate malaria epidemiology in the following ways:

i. **Annual Parasite Index (API):**

$$\frac{\text{Number (No.) of slides positive for malaria}}{\text{Population under surveillance}} \times 100$$

ii. **Annual Falciparum Index (AFI):**

$$\text{AFI} = \frac{\text{No. of slides positive for } \textit{falciparum} \text{ malaria}}{\text{Population under surveillance}} \times 100$$

iii. **Slide Positivity Rate (SPR):**

$$\text{SPR} = \frac{\text{No. of slides positive for malaria}}{\text{No. of slides collected}} \times 100$$

iv. **Slide Falciparum Rate (SFR):**

$$\text{SFR} = \frac{\text{No. of slides positive for } \textit{falciparum} \text{ malaria}}{\text{No. of slides collected}} \times 100$$

RESULTS AND DISCUSSION

A total of 6 anopheline species, in the following descending order of their abundance-*A. subpictus* > *A. stephensi* > *A. annularis* / *A. culicifacies* > *A. fluviatilis* > *A. hyrcanus* 'group', were collected during the present study. The *Anopheles* mosquitoes were found during the whole year except in the month of March (Fig.1). The temporal profiles of seasonal pattern of abundance for two years were classified on the basis of magnitude of fluctuations in relation to different seasons for different species, i) Unimodal monsoon - *A. subpictus*, ii) Bimodal spring and monsoon - *A. stephensi*, *A. annularis*, and iii) Bimodal winter and monsoon - *A. culicifacies*.

Quantitatively, the population density of total anopheline was high from July to November and very low during December to June. The highest density was present in the month of November (2723.2/m^2 for immatures and 35.3 pmh (per man hour) for adults). High rainfall and moderate temperature are the important factors in determining the population as rain provides freshwater conducive for *Anopheles* breeding. The water in the breeding sites remained polluted during rest of the months. This pattern is in contrast to that of the *Culex* fauna where the lowest density was recorded during rainy season, *i.e.* from August to October (Gakhar and Vandana, 1996). The density pattern of different species is also discussed below:

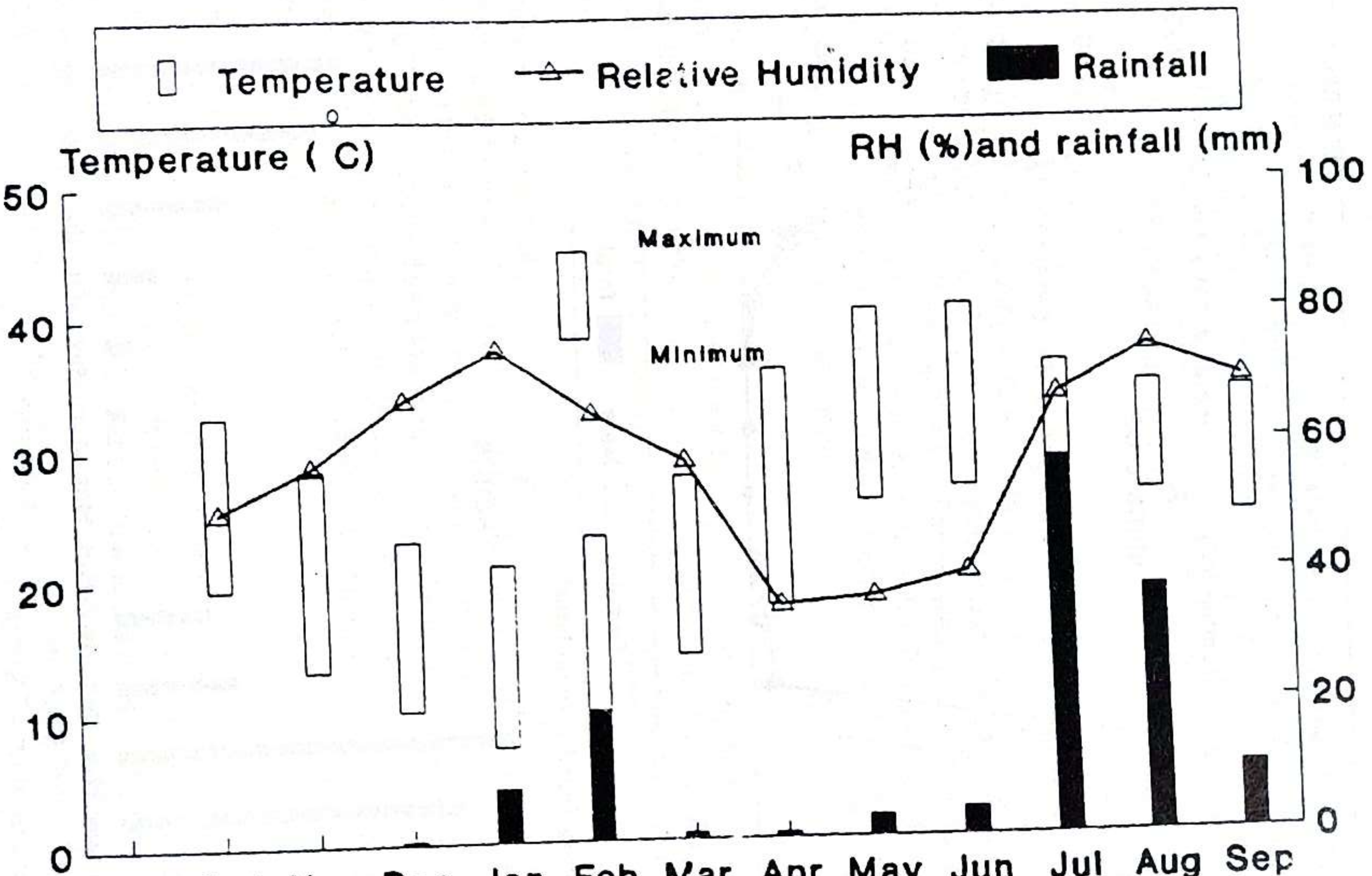
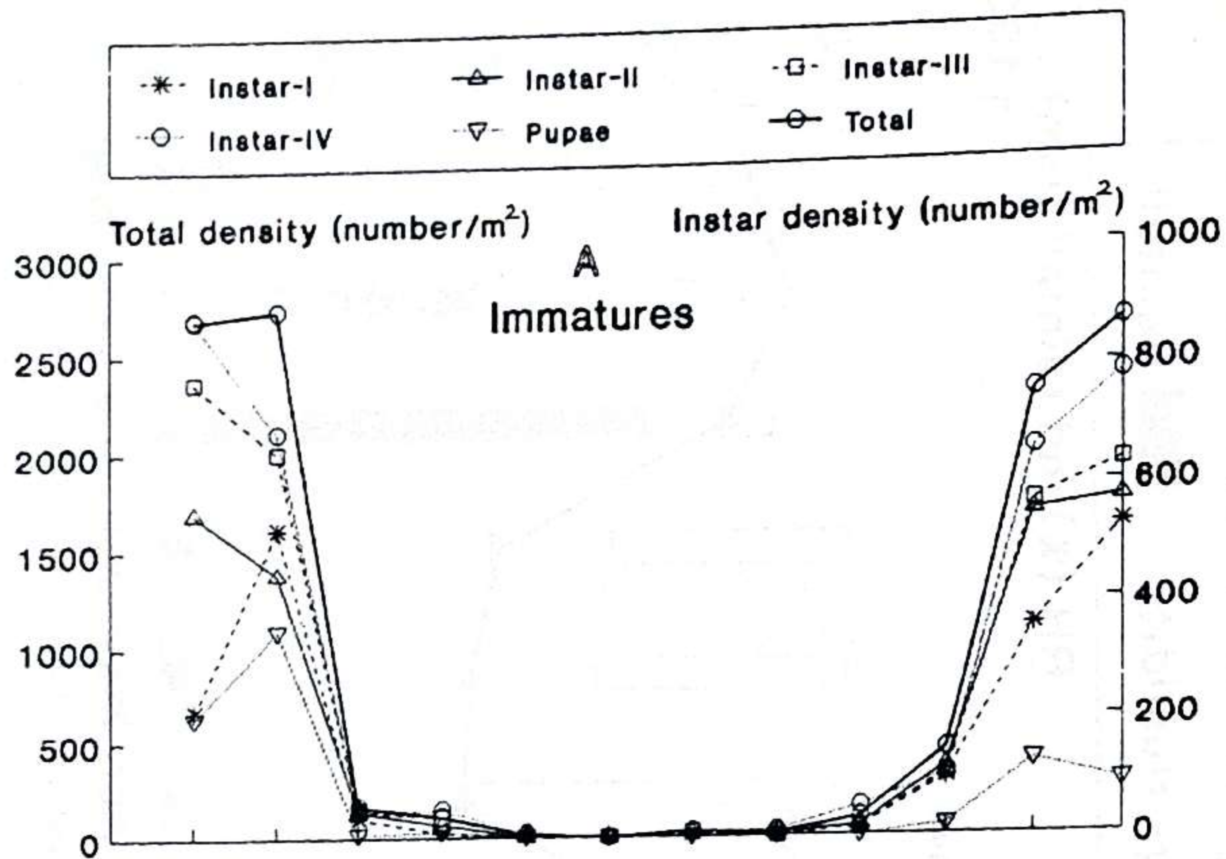


Fig. 1: Monthly variations in the atmospheric temperature, relative humidity and rainfall in Rohtak.

Fig. 1.



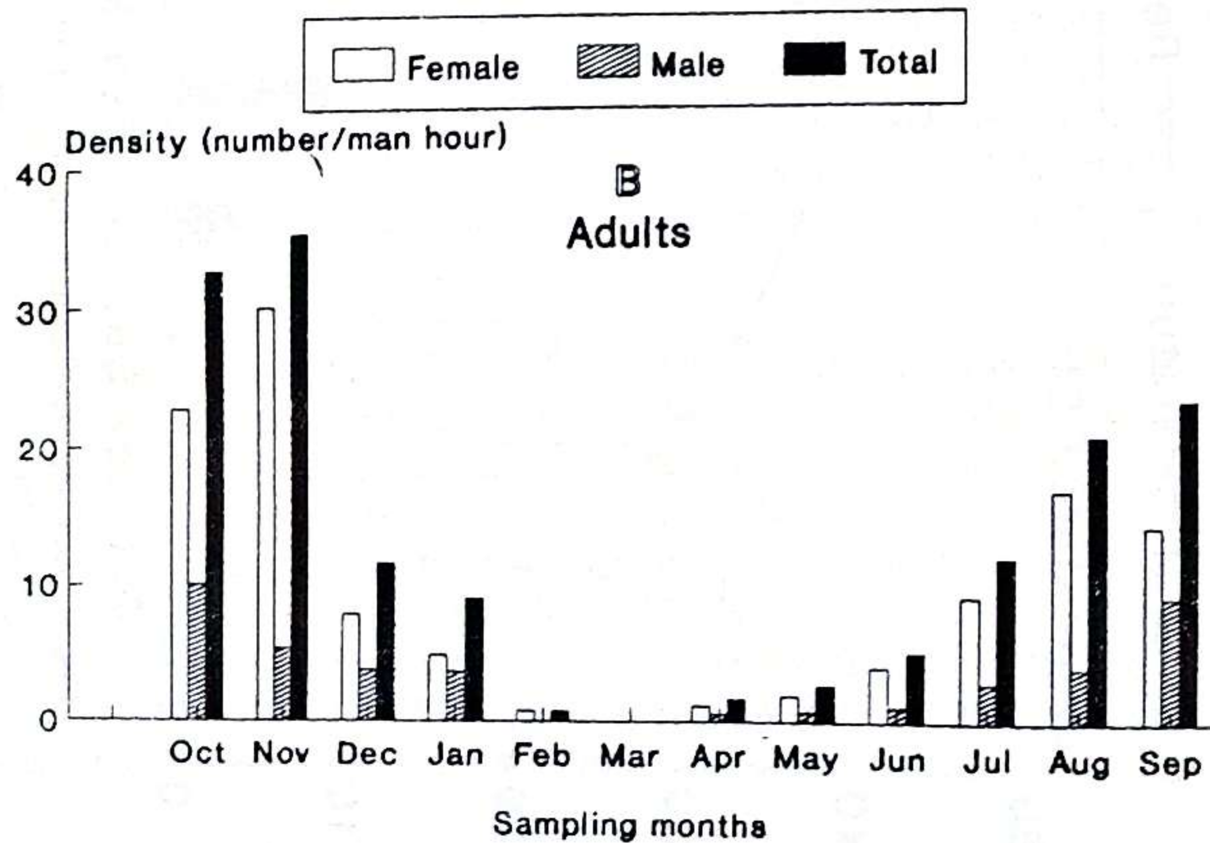


Fig. 2

Figs. 2A&B: Monthly density pattern of *Anopheles* from October 1988 to September 1990 in Rohtak.

Anopheles annularis

This species was found in very low densities both during larval and adult collections. The adults were present only from August (0.91 pmh) to November (1.83 pmh) (Fig.2A) and contributed 4.39% of total anopheline fauna. This species seems to be characterized by high levels in monsoon and post-monsoon months as reported earlier (Reisen and Milby, 1986; Bhatt *et al.*, 1991).

Anopheles culicifacies

This species was encountered only during adult collections and was third dominant among anophelinae contributing 4.39% of total collection. The adults were altogether absent from February to June (Fig. 2B). The highest density was noticed in October (2.9 pmh). The density pattern of this species in Rohtak was quite similar to that found in other parts of country (Das *et al.*, 1990; Kulkarni 1990; Bhatt *et al.*, 1991). The most important controlling factor seems to be the formation of fresh water ponds devoid of amorphous matter during monsoon months.

Anopheles fluviatilis

Only a few adult female mosquitoes could be trapped during July and August 1989 from a human dwelling. The period coincided with heavy rains. Reisen and Milby (1986) also reported the reduced abundance during the hot desiccating pre-monsoon and cold dry winter.

Anopheles hyrcanus `group'

Only a few adult females of *A. hyrcanus* `group' found in November 1988 are indicative of the fact that this species is not indigenous to this area. Bhatia *et al.*, (1958) and Batra *et al.*, (1979) also recorded very low numbers from Delhi and Tamilnadu. However, this is the most dominant anopheline species in Nagaland and Bastar, Madhya Pradesh (Sarkar *et al.*, 1980; Kulkarni, 1990). It reaches peak in these areas in October/November.

Anopheles stephensi

A. stephensi was second dominant species amongst the anophelines in both immature and adult collections of Rohtak contributing 19.7% and 10.8% respectively of the total collection. The adults were mainly found in the human dwellings and were altogether missing the cattle sheds. This species was almost absent from December to March (Fig. 3A). The immature density was highest in November (1389.3/m^2). Two peaks were revealed in the adult density over the year, first in August (2.74 pmh) and second in May (2.57 pmh). Similar pattern of population densities was found in the nearby district of

Gurgaon, Haryana (Sharma, 1991). The low temperature could be unfavourable for this species. The high temperature and heavy rainfall seem to be favourable factors for its abundance. The monsoon rain water perhaps washes the natural breeding places to restrict the density. Kaur and Reuben (1981) also noted that heavy rainfall caused heavy mortality of immature stages.

Anopheles subpictus
This was the most dominant anopheline species influencing the total *Anopheles* population. It contributed 80.2% of the immature and 79.4% of the adult anopheline collection. In contrast to *A. stephensi,* the adults of this species showed preference for cattle sheds and mixed dwellings over human dwelling. The immatures were abundant in temporary muddy water collections. The species was almost absent from February to May (Fig. 3B). The density was quite high during August to November and low during January to June. The highest level was noticed in September for immatures (2494.8/m^2) and November for adults (31.26 pmh). Sharma (1991) from Gurgaon and Ansari *et al.*, (1982) from Basantpur village of Harayana have recorded the highest density in August. This species shows a definite seasonal trend and seems to be a typical monsoon type. A similar trend was also found by Kulkarni (1990). A low density has been found during winter months from December to February in Tamilnadu and Gujarat (Batra *et al.*, 1979; Bhatt *et al.*, 1991).

Temperature seems to have some influence on the occurrence of this species because in January (13°C) and February (17°C) of 1989, no immature or adult mosquitoes were encountered though there was sufficient rainfall during that period. *A. subpictus* breeds particularly in temporary water pools and such type of pools abound during monsoon. This explains the seasonal trend depicted by the species in this region.

Epidemiology of malaria
Annual *falciparum* index (AFI) and Annual parasite index (API) for the years 1988,1989 and 1990 in Rohtak have been presented in Table 1. Incidence of malaria was highest in 1990. The epidemiological data on Slide Positivity Rate (SPR) and Slide *Falciparum* Rate (SFR) have been shown in Fig. 4. The *falciparum* malaria was prevalent only in the months of October and November with a few cases reported in August. Its contribution was only 9.55%. Slide *falciparum* rate was high during July to October. The highest level of 0.983% was noted in August. The period of highest SPR in July to November coincided with the peak densities of the anophelines. The occurrence of malaria thus seemed to be a vector density-dependent phenomenon as suggested by

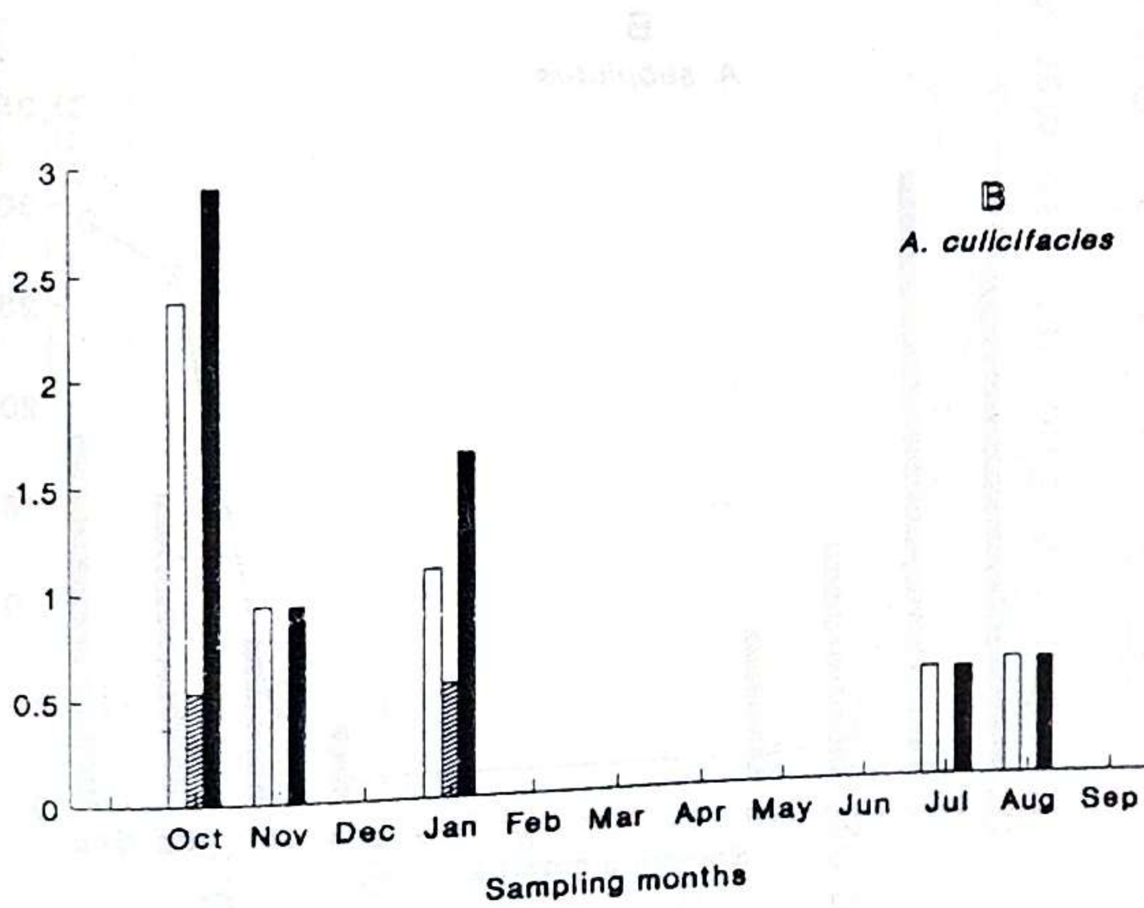
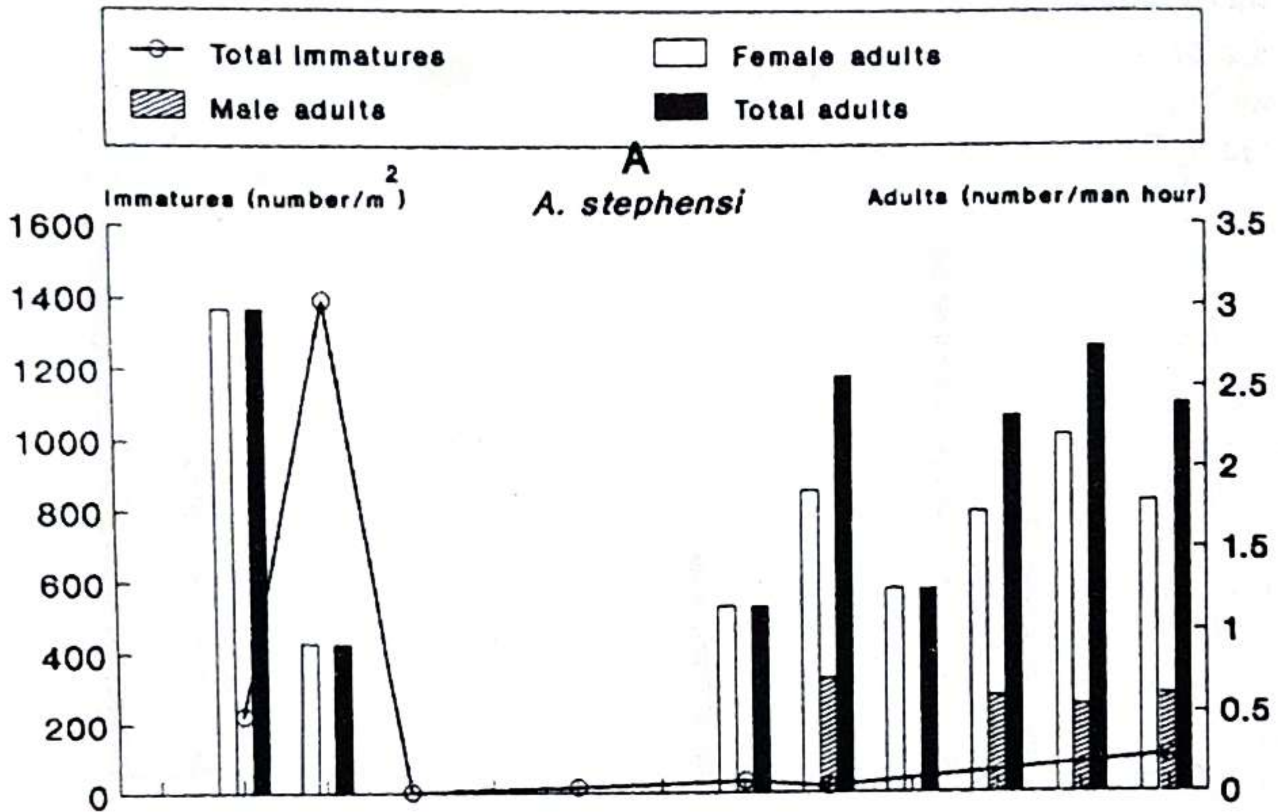


Fig. 3.

Figs. 3A&B: Monthly density pattern of *A. annularis* and *A. culicifacies* from Oct. 1988 to September 1990 in Rohtak.



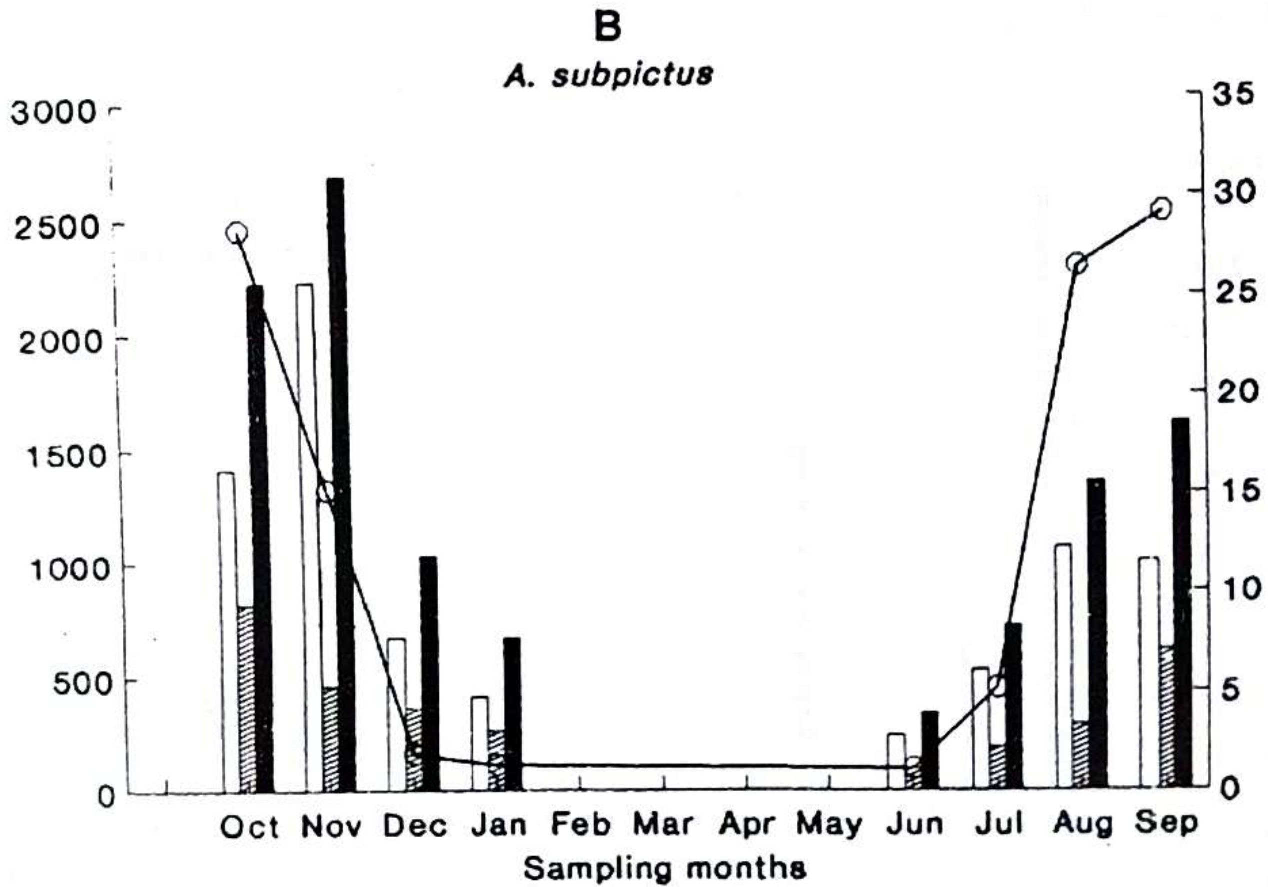


Fig. 4.

Figs. 4A&B: Monthly density pattern of *A. stephensi* and *A. subpictus* from October 1988 to September 1990 in Rohtak.

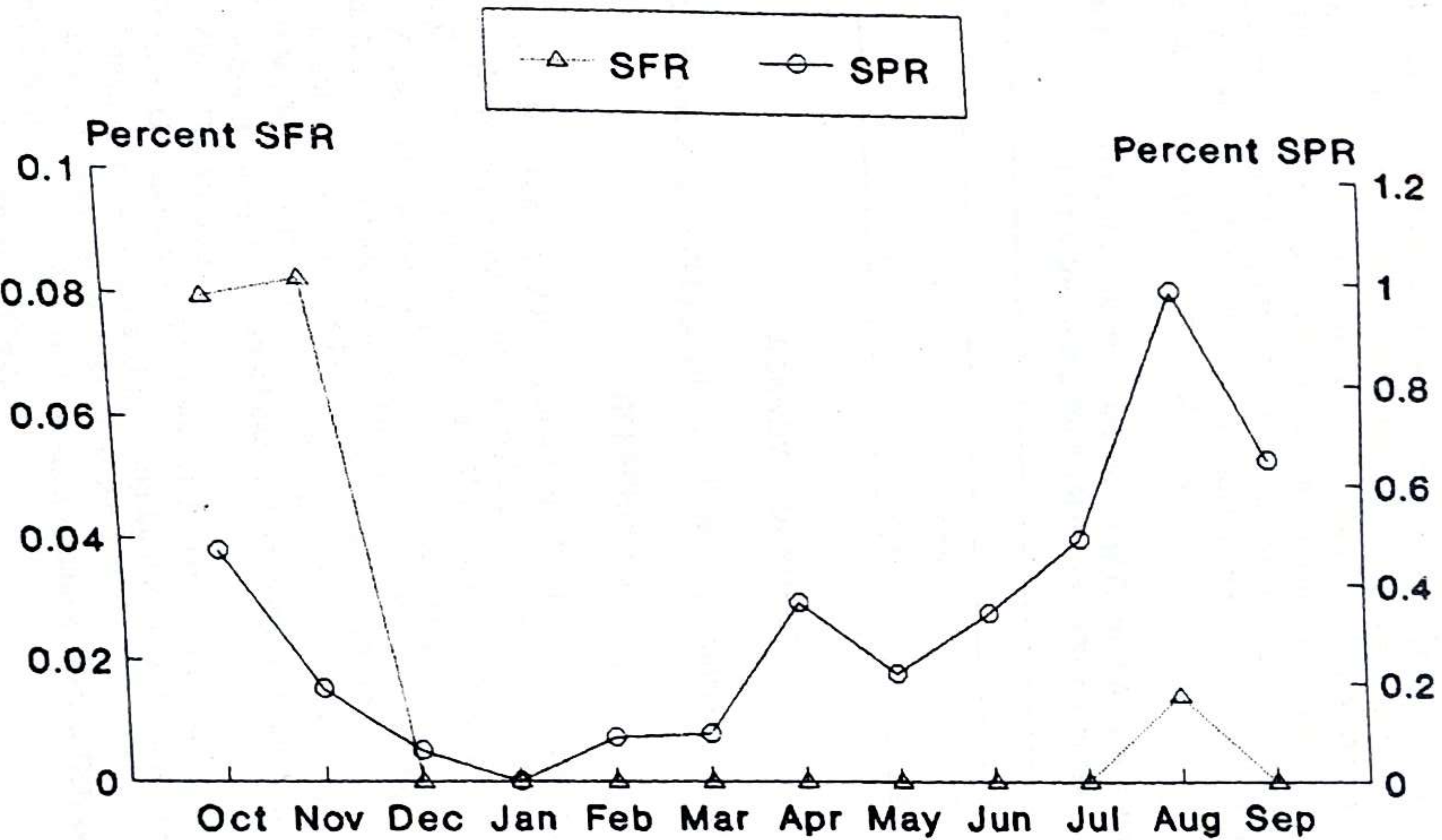


Fig. 5: Monthly data of Slide *Falciparum* Rate (SFR) and Slide Positivity Rate (SPR) for malaria from October 1988 to September 1990.

other workers (Batra *et al.*, 1979 in Tamilnadu; Das *et al.*, 1990 in Orissa; Kulkarni, 1990 in Madhya Pradesh). Another low peak of SPR was noted in April (0.358%). This resurgence of malaria coincided with the built up of *A. stephensi* mosquitoes at that time. However, the recognized vector species *A. culicifacies* was very scarce in indoor hand catches during the present collections. Under these circumstances, the studies on the vectorial capacity of this species and also of other common anopheline species like *A. stephensi* in this region are under way.

Table 1: Annual *falciparum* incidence (AFI) and Annual parasite incidence (API) for the years 1988,1989 and 1990 in Rohtak.

	1988	1989	1990
AFI	0.031	0.025	0.106
API	0.317	0.56	0.838

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LENS DIFFERENTIATION IN THE FISH TILAPIA, *OREOCHROMIS MOSSAMBICUS* - AN OVERVIEW

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ABSTRACT

During the development of the eye lens, a large number of tissues from different sources contribute towards its formation. The endoderm (inductor tissue) and the mesoderm induce the epidermis to form the lens placode and the lens vesicle. The lens varies considerably in shape and in consistency in different vertebrates. There also occurs different biochemical changes in the lens with increase in age. The age related changes of some biochemical components in the lens of the fish, *Oreochromis mossambicus* is discussed.

Key words : *Tilapia*, lens differentiation, lens protein.

INTRODUCTION

Of all the organs formed in the body of vertebrate, the eye has a more complex organization. A large number of tissues from different sources contribute to form such a unique organ. In this respect, the lens situated in between the pupillary portion of iris and the vitreous body, plays a major role in transmitting image-bearing light beam towards the retina as long as it remains transparent. Various workers consider the lens as a fascinating subject of research. As a result, manyfold aspects from different points of view have been worked upon animals from different orders and suborders of amphibians, birds and mammals. Reptiles to some extent can be included in the list.

It is known that the lens develops through a series of developmental steps and/or tissue interactions. The inductor tissue *i.e.* the endoderm and the mesoderm induce the epidermis to form the lens placode and lens vesicle, which in turn is induced by the neural retina thus establishing a solid lens (Nace, 1970; Van Doorenmalen, 1981). After the lens-inducing process is completed, differentiation, growth and maintenance of the lens continues life long. In the next phase of differentiation, epithelial cells at the equator of the lens transform into lens fiber cells. The early fiber cells arrange concentrically, layer after layer, to form the lens nucleus, whereas, the cortex is formed

by the newly laid-down fiber cells (Papaconstantinou, 1967). At the end of this terminal differentiation, the lens fiber cells lose their nuclei, particularly those at the centre and become filled with specific proteins-crystallins, to perform a highly specific function. Since the fibers lack nuclei, it can be concluded that because crystalline synthesis continues, the messengers involved are stabilized (Bloemendal, 1977).

Although, eyes of all vertebrates perform similar function, the lens, however, varies considerably in shape and in consistency. At the same time, there occur considerable changes in the protein profile and other components between different species. Hence, a lens of a particular species is expected to posses biochemical components of its own, subject to alteration with age. In this paper, an overview of age related changes of some biochemical components in the lens of the fish *Oreochromis mossambicus* has been given. Further, histological investigations have also been designed to obtain an insight of the changes and their functional correlation in maintaining structural integrity of the lens.

The fish lens, when studied in the age range covering 30 to 750 d, consisting of 6 age groups, maintained a water content of about 54.95% to 40.55% which is maximum in earlier age groups (Sahu and Kundu, 1989). This concomitant decrease of the percentage of water in different age groups of the fish lens has relationship with the quantity of proteins studied. This indicates a gradual and appreciable increase of quantity and aggregated forms of proteins in older age groups.

A chemical analysis shows that the lens is rich in protein. Besides, it contains inorganic materials namely Na^+, K^+, Mg^+ and Ca^{++}. The concentration of these electrolytes, varies in different age groups. Judging from the results of chemical analysis of electrolytes, at different ages, it can be stated that the *Tilapia* lens seems to have an intricate system to produce high level of potassium and calcium ions. Could this be attributed to the nature of the lens protein?.

In most studies, the lens tissue is homogenised in distilled water and by centrifugation one can obtain a water soluble (WS) and water insoluble (WI) part. The quantity of total protein, estimated for *Tilapia* lens agrees with the data recorded that lens contains 35% structural protein (Kuck, 1970; Clayton, 1974; Bloemendal, 1981). The interesting aspect is that the quantity of total proteins in different age groups of the *Tilapia* does not remain constant. Since, the quantity of water soluble crystallins in terms of the percentage of total protein decreases, and that of the water insoluble crystalline increases, the ratio of WS/WI decreases.

The water soluble proteins can be separated into three main classes of proteins as alpha, beta and gamma crystallins on the basis of their size and/or charge (Harding and Dilley, 1976). Separation into classes and sub-classes is done by gel chromatography or by ion-exchange chromatography. Analysis of fractions obtained is mostly performed by electrophoresis in the presence of sodium doecylsulphate (0.1%).

Crystalline elution pattern during development

The present study of gel filtration with Sephadex G-200 material for convenient separation of WS crystalline is worth mentioning. Proteins separated by Sephadex indicate that the fish lens contains as many as four components and not less than three components of WS proteins in the older and earlier age groups of the lens (Fig.1). This suggests that higher age groups have newly emerged protein fractions, whereas in the earlier groups there are no proteins of Low Molecular Weight (LMW=10,000 Da.) indicating a change in protein composition in aging fish lens.

Identification of the fraction with molecular weight

There are indications that the first fraction F_1 of WS proteins of the fish lens eluted on Sephadex gel filtration is alpha crystallin, as determined from its molecular weight (640,000-687,000 Da) and V_e/V_o value (0.97 to 1.80) and compared with other vertebrates. The second fraction F_2 shows a molecular weight range between 154,000 to 190,000 Da, except in the age group of VI. It may well be beta high crystalline. The third major fraction (F_3) of all age groups has a molecular weight between 64,000 Da to 64,400 Da. The last fraction (F_4) possess a molecular weight of 37,000 Da and 40,600 Da with V_e/V_o ratio of 2.34 to 2.27.

The patterns of lens crystalline in *Tilapia* are very interesting because (i) it consists of alpha and beta crystalline in all the age groups studied. (ii) The proportionate distribution of different classes of proteins determined by column chromatography is widely variable. (iii) The "LMW" protein fraction is present in significant proportions in the last three age groups in comparison to the F_3 and F_4 fractions of a particular age group (iv) These crystallins comprise of a heterogeneous group and beta crystallin is the most heterogeneous population of the WS lens proteins. (v) LMW protein is absent in the three early age groups.

Size and molecular weight of crystallin polypeptides

During lens differentiation these crystallins undergo gradual changes both quantitatively and qualitatively in the polypeptide composition (Clayton, 1970, 1974). SDS gel electrophoresis resolves polypeptides according to size and number of bands. The change with age could be correlated.

The first fraction is of high molecular weight protein (33,000 Da) in all age groups studied and comprises of subunits with minimum two (in the age group VI) and maximum five (in the age group III) bands of different molecular weights, suggesting variability among age groups. The 2nd, 3rd and 4th fraction consist subunits of either two of three bands (Fig. 2). SDS-PAGE studies for subunits of different crystallins show an increase of 14,000 to 18,000 Da polypeptides particularly in the age group of VI.

A relative abundance of identical and closed molecular weight polypeptides for alpha crystallins is seen in different age groups. Similarly, for the beta crystallin, 7000, 15,000 and 16,000 Da polypeptide are present in initial and final age groups exhibiting an age dependent relationship. This variation among the polypeptide composition of crystallin in the lens of aging fish is the resúlt of post-synthetic (Hoenders and Bloemendal, 1983) or conformational modification (Kundu and Sahu, 1992). In the higher age group, lenses demonstrate marked differences of LMW proteins in the second and third subunits with molecular weights 2000 and 6000 Da, respectively.

Histological picture

The lens of *Tilapia* is transparent, more or less round and is composed of (outer to inner) the lens cupules, lens epithelium and lens fibers. (Fig. 3a). Towards the equator, lens epithelial cells gradually become flattened with changes in the shape of nuclei (Fig. 3b). These epithelial cells transform into fiber cells. With the increase of age, the fiber cell nucleus becomes elliptical and appears rod-like (Fig. 3c) indicating an active DNA synthesis. Gradual disappearance of the nuclei with the transformation and accumulation of fibers are the prominent features as aging proceeds. Lenses of higher age groups show more differentiation of lens protein fibers than the earlier ones. Lens fibers are mostly pronounced in the posterior surface of retina suggesting retinal influence in fiber formation.

CONCLUSION

Differentiation of the lens is associated with a series of biochemical events and thus a lens procures all its functional properties. The alterations so far observed are mainly associated with respect to the structural proteins, which constitute a major percentage

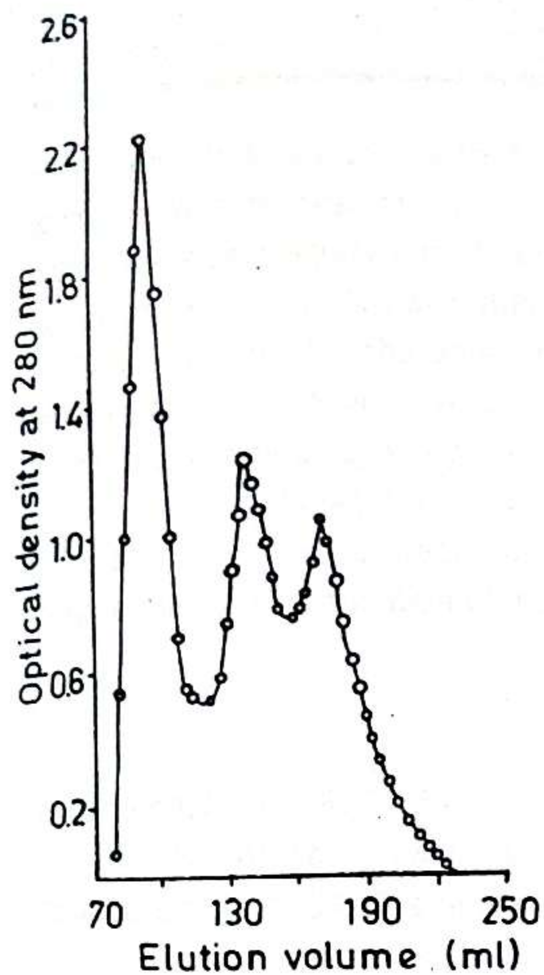

Fig. 1

Fig. 3a

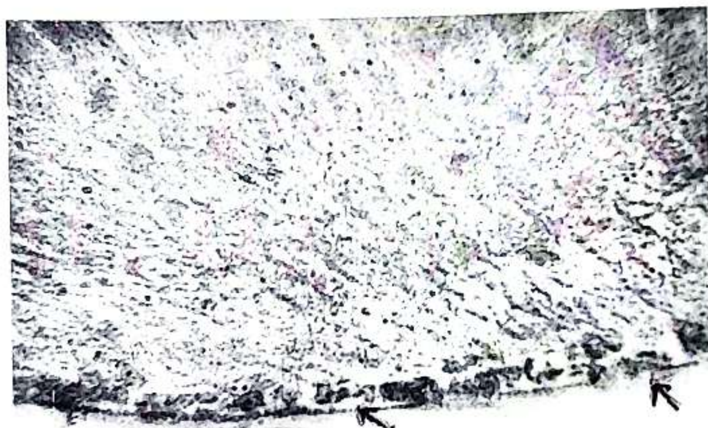

Fig. 3b

Fig. 3c

Fig. 2

Fig.1. Elution pattern of water soluble lens extract of fish *Tilapia* (age groups I & VI) on sephadex G-200 column 50 ; **Fig.2.** SDS-PAGE of sephadex gel filtrated water soluble lens protein fractions of age group (V). F1-F4 indicates the first to fourth fraction ; **Fig.3a.** Vertical meridional section through normal eye of *O. mossambicus* showing the position of the lens; **Fig.3b.** Section of the lens at equatorial region showing disposition of the lens fibers and epithelial cell nuclei (arrow); **Fig.3c.** Epithelial cells transforming into lens fibers in the region posterior to the equator. Note differentiating fiber cells with rod like nuclei (arrow).

of the lens. It is reasonable that alterations in protein synthesis due to aging can be correlated with the change in ion concentrations. An existing species specificity of lens crystallin is demonstrated. Probably, deficiency of gamma and delta crystallins, during aging are noted, which are specific for the species concerned. Presence of LMW protein is a specific character in the lens of *Tilapia* during aging. As far as the polypeptides of crystallins are concerned, LMW protein exhibits heterogeneity in charge with the increase of age suggesting a major change with age. The influence of either protein or nucleic acid in the lens fiber formation is characteristic of lens cell differentiation. The appearance of lens-specific proteins with increase in age, characterises differentiation of the lens.

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CHRONOLOGICAL DEVELOPMENT OF ORGANS AND ORGAN SYSTEMS AT DIFFERENT DEVELOPMENTAL STAGES OF OLIVE RIDLEY SEA TURTLE, *LEPIDOCHELYS OLIVACEA*

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ABSTRACT

The development of various organs and organ systems of olive ridley (*Lepidochelys olivacea*) sea turtles have been worked out under laboratory conditions. It has been found that all the organ systems, including the differentiation of gonads develop between stage 3 and stage 18 of Yntema. The development of head and head processes, brain, eye, nose, ear, gut and its derivatives, circulatory system, kidney, gonads, limbs, carapace, etc. are reported here.

Key words : *Lepidochelys olivacea*, development, organ system

INTRODUCTION

The olive ridley sea turtle, *Lepidochelys olivacea* is one of the endangered marine turtles included in Schedule-1 of Indian Wildlife (protection) Act of 1972. As regards to its development, early studies on the embryology of turtles made important contributions to the understanding of reptilian development (Rathke, 1848; Agassiz, 1857; Parker, 1880; Mitsukuri, 1894; Allen, 1906; Jordan, 1917a,b; Yntema, 1968; Crastz, 1982; Behera and Mohanty-Hejmadi, 1985; Mohanty-Hejmadi *et al.*, 1985; Moracio *et al.*, 1989; Janzen and Paukstis, 1991). A complete developmental series was not described for any species of marine turtles until 1982 when Miller (1982) described the embryological series of *Chelonia mydas, Caretta caretta, Eretomchelys imbricata, Dermochelys coriacea*, which was the basis for his review paper on the embryology of marine turtles (1985). However, in the present work a chronological development of various organs and organ systems of *L. olivacea* have been done according to Yntema

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(1968) stages of *Chelydra serpentina* which is the standard for all turtle species. The embryos at different days of incubation at various temperatures (33.5°C, 30°C and 27.5°C) have been well compared by Behera (1989) with the developmental stages of Yntema (1968) for *C. serpentina* (Crastz, 1982), for *L. olivacea* and *D. coriacea* (Miller, 1982). The unique representation of the current work facilitates a more detailed and comparative study on the development of various organs at different stages.

MATERIALS AND METHODS

The freshly laid eggs were incubated in Remi B.O.D. incubators at constant temperatures of hot (33.5°C) and cold (27.5°C). Before incubation the eggs were washed with tap water without changing the orientation. They were then drained, measured, weighed and marked. Batches of eggs were wrapped with two layers of moist cotton and put in different enamel pans. Two hundred eggs were incubated in hot temperature and 200 eggs were incubated in cold temperature. From the beginning of the incubation upto hatching, 2 eggs from hot and 2 eggs from cold were taken out of the incubators at regular intervals of 24 h for morphological and anatomical studies. The early stages of embryos were separated from the egg shells by Kleineberg's fixative, then washed in 80% ethanol and finally fixed in 70% ethanol for further study. The embryos of late stages were anaesthetized in MS 222 before fixing in Bouin's fluid. All the developmental stages were compared with the 27 stages of Yntema (1968) for *C. serpentina*. The early stages of embryos were serially sectioned to study the growth of internal organs of the species. The slides of the serial sections of the embryos were prepared and studied under microscope. The main characters were tabulated to show the developmental chronology.

RESULTS AND DISCUSSION

The rate of growth of *L. olivacea* embryos was dramatically slower for embryos incubated at cold temperature compared with those incubated in hot temperature. The incubation period of hot batches and cold batches were 46 d and 52 d, respectively.

The first distinguished feature of the embryos was the development of head processes which was found at stage 3. The anterior neuropore closed at stage 5 and the brain vesicle and epiphyses were apparent at stage 8 and stage 11, respectively. The development of eye occurred between stage 7 and stage 17+, during which period, the development of optic vesicle (stage 7), optic cup (stage 8) and lens vesicle (stage 9+) took place. The lens pit closed (stage 10) and the iris appeared (stage 17). The

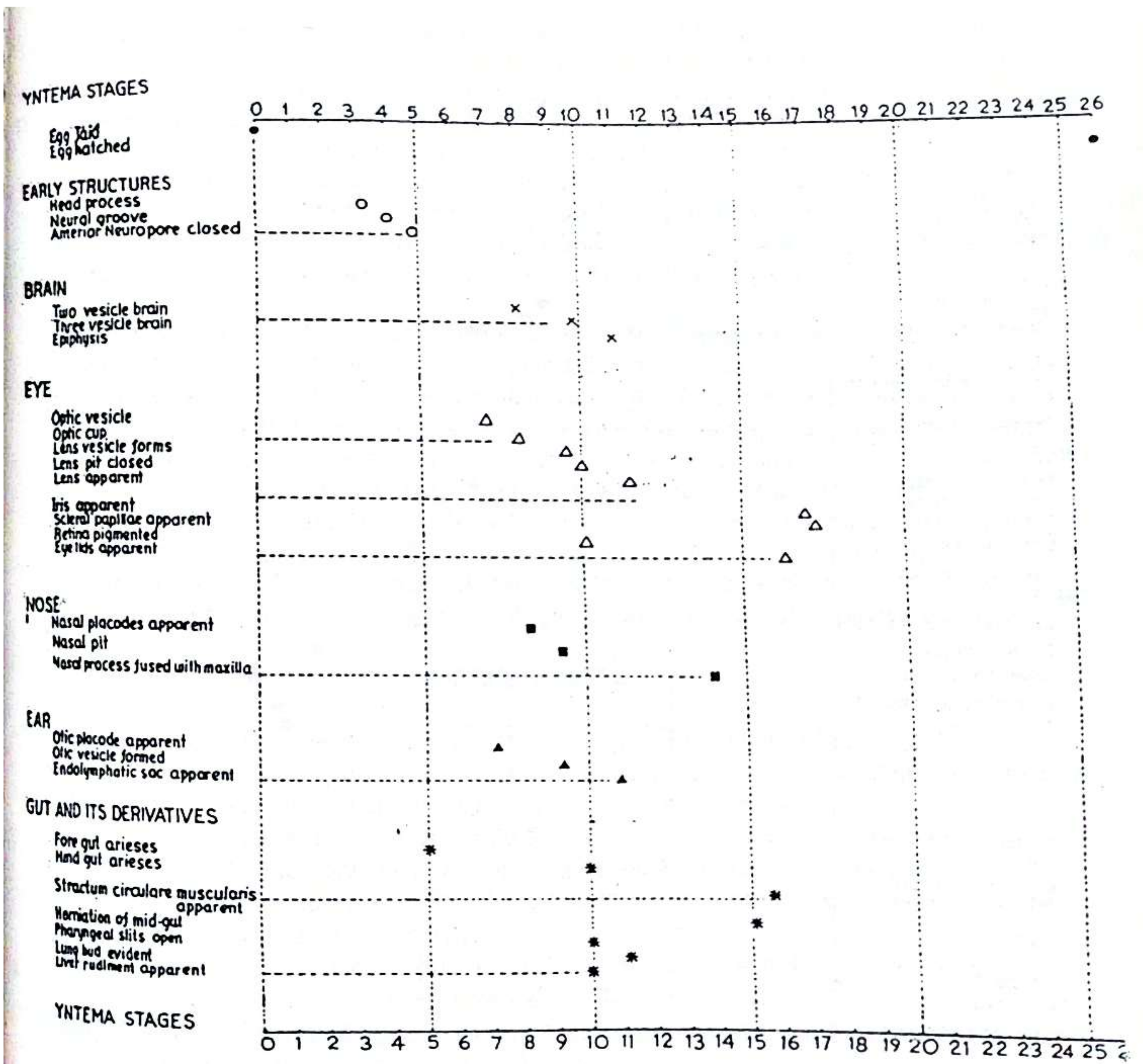

Fig. 1 : Chronological summary of ontogeny of organs at different stages of Yntema

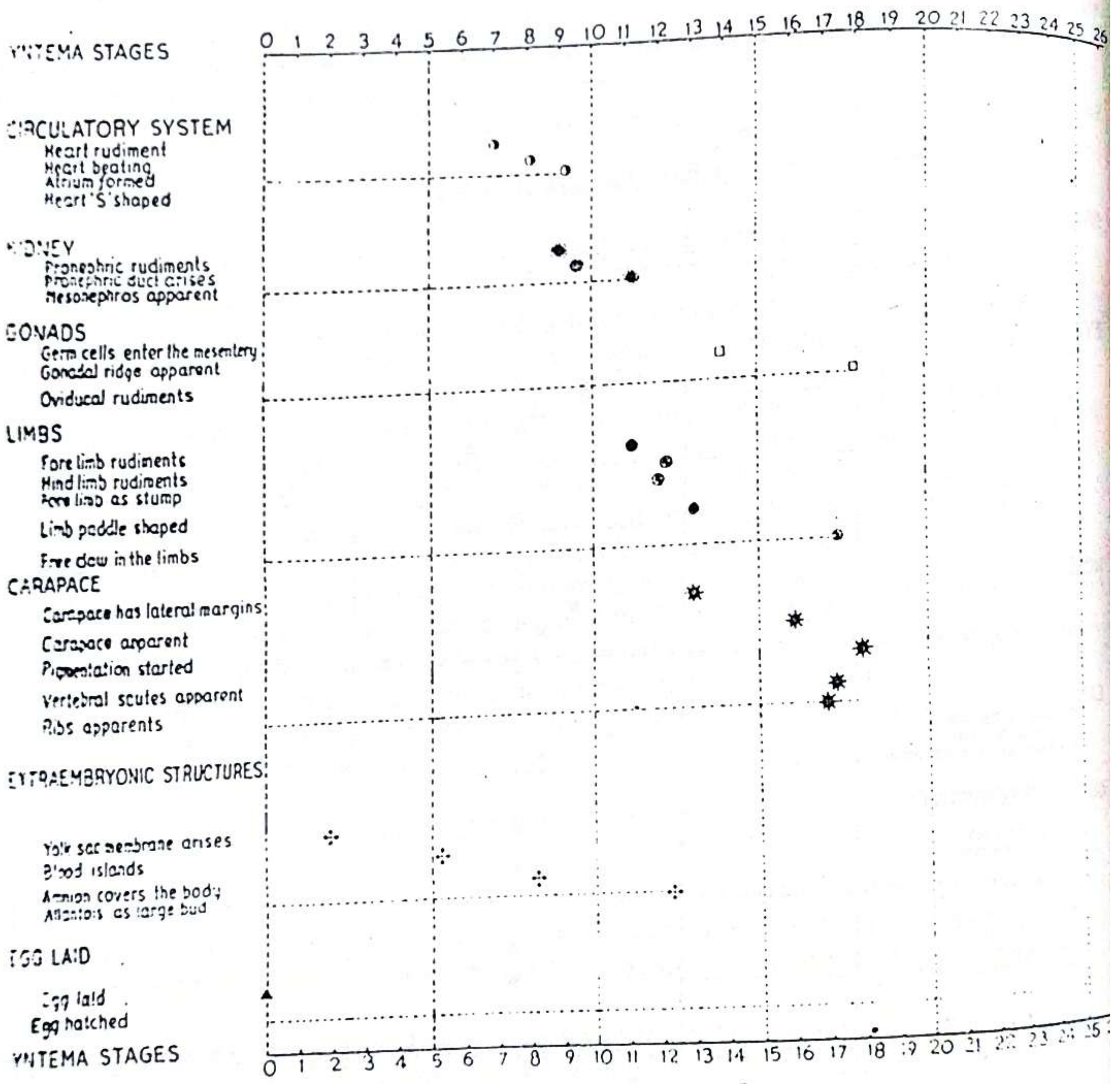

Fig. 2 : Chronological summary of ontogeny of organs at different stages of Yntema

development of nasal processes took place between stage 8 and stage 14 during which the otic placode (stage 6+), otic vesicle (stage 9) and endolymphatic sac (stage 10+) became apparent. The foregut became apparent at stage 5 whereas the hindgut developed at stage 10. The pharyngeal gill slits and liver rudiments were noticed at stage 10. The lungbud developed at stage 11+ and the heart rudiments were found at stage 7. The pronephric rudiment developed at stage 8+ which was followed by the formation of mesonephros at stage 11+. The differentiation of gonads took place between stages 13+ and 17+. The forelimb rudiments were visible at stage 11+ and that of the hind limb at stage 12+. Finally, the eggs hatched at stage 27.

The morphological criteria proposed by Crastz (1982) were unsuitable for advance stages of development. Therefore, we used the morphological criteria used for *C. serpentina* by Yntema (1968) for embryological stages. Due to slow rate of growth the embryos incubated at cold temperature were more suitable for observation and comparison with the Yntema stages to tabulate the development of various organ systems. Although several workers (Crastz, 1982; McCoy *et al.*, 1983; Behera and Mohanty-Hejmadi, 1985; Horacio *et al.*, 1989; Janzen and Paukstis, 1991) have described the development of olive ridley, no reports are available in detail on the growth of early embryonic organ systems of this species. Figs. 1 and 2 shows a graphical representation of the various stages of development of *L. olivacea*.

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HISTOCHEMISTRY OF OOGENESIS IN A FRESHWATER TELEOST, *TILAPIA MOSSAMBICA* (PETERS)

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ABSTRACT

The seasonal histological changes of the ovary has been studied. During the process of oogenesis, six different stages of oocytes differing in structure and chemical composition have been observed. The Gonosomatic Index (GSI) and ova diameter were recorded every month over one year to determine the monthly change in the state of the gonad and annual ovarian activity. Moreover, the histochemical study of the ovary has been made to ascertain the presence of various proteins, lipid and carbohydrates. Histologically, the ovary is found to have six different stages of oocytes. The yolk content from one oocyte to the other is variable. Histochemistry of vitellogenesis reveals the presence of histidine, tryptophan and amino acids containing -SS and -SH groups, mucopolysaccharides, glycolipids, lecithin, cephalin and long chain unsaturated fatty acids.

Key words: *Tilapia*, histochemistry, oogenesis, yolk, ovary

INTRODUCTION

In order to meet the protein demand of the vast population many researchers have diverted their attention towards reproductive biology of fishes with the intention of increasing fish production by application of scientific technique. Many earlier workers like Andrew and Pinto (1957), Gopal Dutt and Govindan (1969), Bisht and Joshi, (1975) have made their effort to trace the origin of fresh crop of oocytes. Guraya *et al.*, (1975), Bose and Bose (1964), Rastogi (1968), Bisht and Joshi (1975) have contributed a lot on the possible role played by nuclear extrusions in vitellogenesis. Many workers have made indepth study of the origin, possible contribution of the organelles in vitellogenesis and the chemical composition of the yolk. The present paper deals with the origin of oocytes, origin of yolk in the vitellogenic oocytes, role played by nucleolus, secondary nucleoli and yolk nucleus and histochemical composition of the yolk. Constituents of proteins, lipids and carbohydrates and their distribution in various parts of the oocytes of *Tilapia mossambica* have been worked out.

MATERIALS AND METHODS

The alive fishes were sacrificed and the ovaries were dissected out. The weight of the ovary and the weight of the fishes were taken to determine the Gonosomatic Index (GSI) of the fish every month. The ovaries were fixed in Bouin's fluid, Carnoy's fluid, 10% neutral formalin and formol calcium. Paraffin sections were used for histological and histochemical preparations for the study of proteins, carbohydrates while formol calcium fixed frozen sections were used for the study of lipids. To study the chemical composition of yolk, the vitellogenic oocytes were subjected to various histochemical tests as described by Pearse (1975). The GSI of a fish is calculated by the formula

$$\frac{\text{Mean ovarian weight}}{\text{Mean body weight}} \times 100$$

RESULTS

The ovaries are paired and attached to the dorsal wall of the body cavity by a thick mesovarium. The ovarian wall consists of outer peritoneal layer, middle tunica albuginea and inner ovarian epithelium from which ovigerous lamellae project into the ovocoel. A number of oocytes in different stages of development are arranged along the ovigerous lamellae (Fig.1). The fresh oocytes originate from germinal epithelium. The developing oocyte passes through a series of maturation stages before it is transformed into a yolky ripe egg. Based on the nuclear and the cytoplasmic components of the developing oocytes, the following stages may be distinguished.

Stage I

The oocyte of this stage measures an average about 80 µm in diameter. It has a large vesicular nucleus with a homogenous cytoplasm. The oocyte nucleus has a centrally concentrated chromatin mass, in which lampbrush chromosome may be located (Fig. 2). Some of the oocytes appear to posses almost a peripheral yolk nucleus in their cortical ooplasm. The yolk nucleus is seen to have an outer lighter zone and darkly stained inner zone (Fig. 3). Histochemical tests reveal the presence of protein and RNA in the yolk nucleus. The follicular cells appear to form a membranous squamous epithelium around the oocytes. Thecal cells are found close to the follicular epithelium.

Stage II

Oocytes of this stage measure an average about 150 µm in diameter. Lambrush chromosomes with large number of perinuclear bodies may be distinctly observed. The

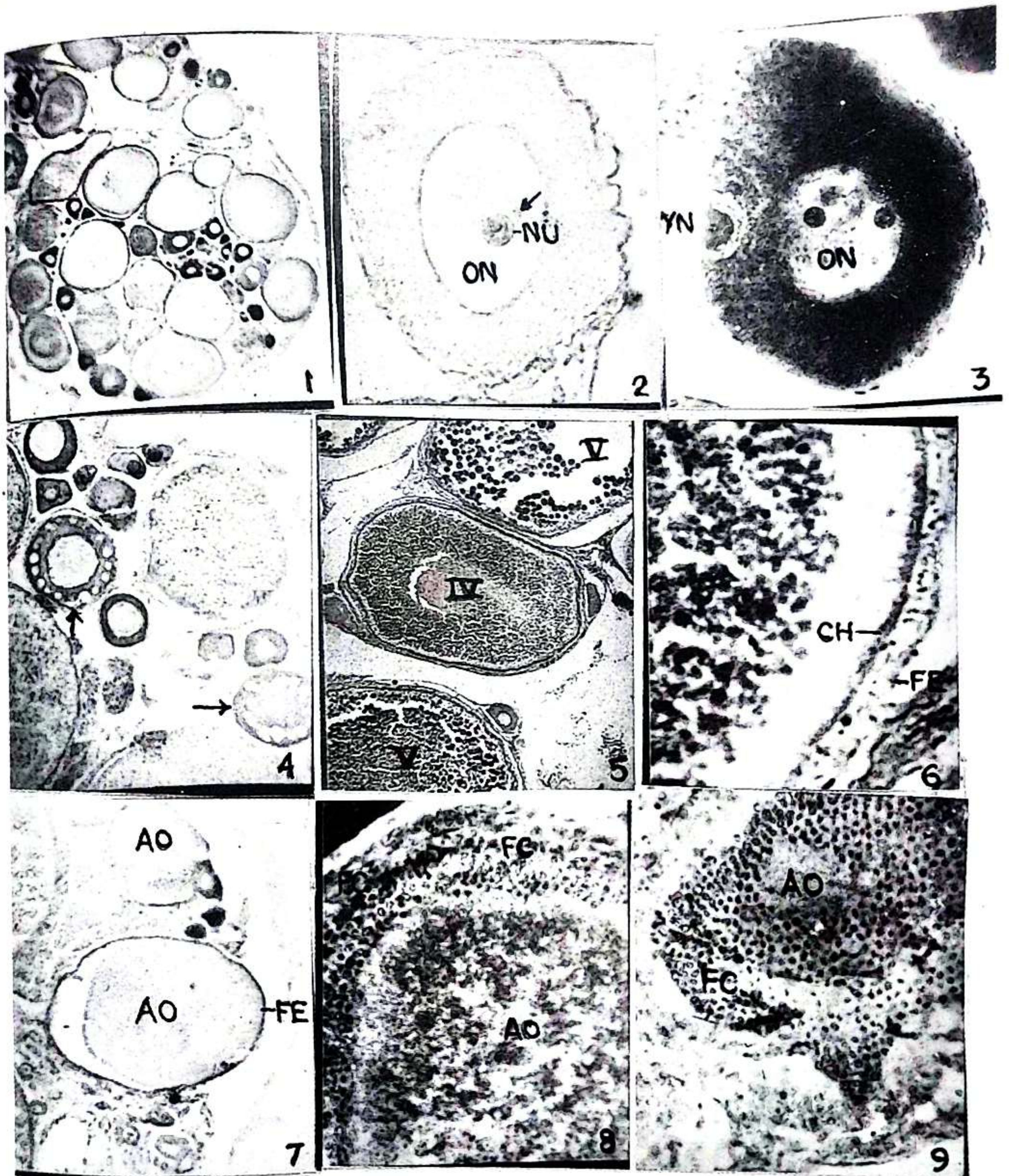

Fig. 1 : Ovigerous lamellae with oocytes of different growing stages. Haematoxylin/Eosin X 200 ; **Fig. 2 :** Stage 1 oocyte with perinuclear body (arrow) and nucleolus (NU) in the chromatin mass of the oocyte nucleus (ON). MG/PY X 1200 ; **Fig. 3 :** Stage I oocyte with yolk nucleus (YN) in the peripheral ooplasm. TB X 900 ; **Fig. 4 :** Oocytes in different growing stages. Stage II oocytes (arrow) with a number of vacuoles in the peripheral region. T.B. X 400 ; **Fig. 5 :** Oocytes of stage IV and V. Note the accumulation of yolk granules in the ooplasm of stage IV oocytes. Haematoxylin/Eosin X 900; **Fig. 6 :** Stage VI oocyte filled with yolk. Note the formation of Chorion (CH) beneath the follicular epithelium (FE). Hg BPB X 1200; **Fig. 7 :** Atretic oocyte (AO). Ninhydrin - Schiff X 900; **Fig. 8:** Atretic oocyte (AO) with hyperactive follicular cells (FC). Hg BPB X 900 ; **Fig. 9 :** Atretic oocytes (AO) invaded almost entirely by the follicular cells. Haematoxylin/Eosin X2000.

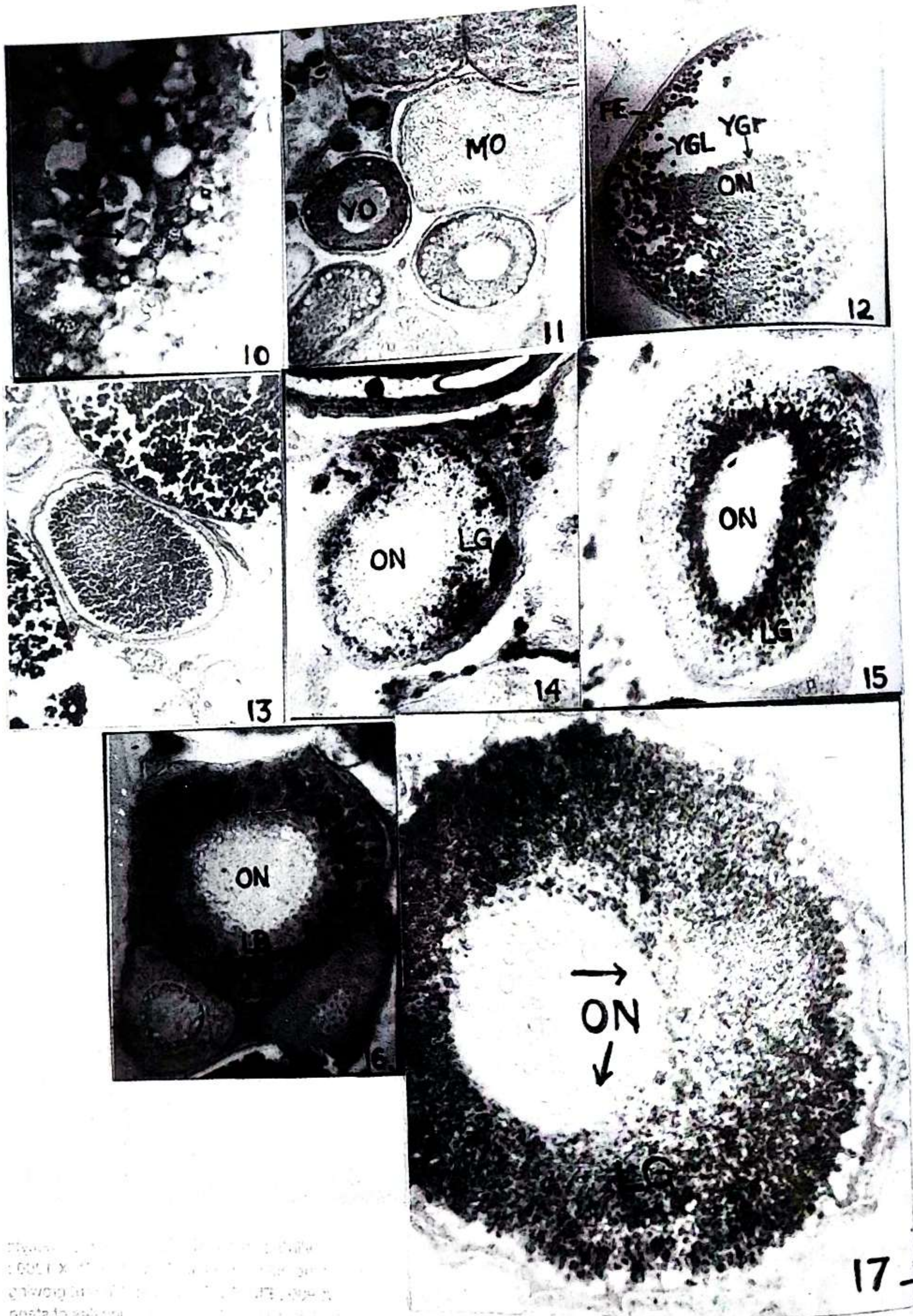

Fig. 10 : Atretic postovulatory oocyte with dissolving yolk globules (arrow). Hg BPB X 2000 ; **Fig. 11 :** Oocytes of different growing stages. Young oocytes (YO) stained positively and matured oocytes (MO) stained negatively with TB. TB X 400; **Fig. 12 :** Stage V oocytes packed with yolk granules (YGr) and yolk globules (YGL). Hg BPB X 900 ; **Fig. 13 :** Mature oocyte filled with yolk. CTZ X 400 ; **Fig. 14 :** Oocytes of stage II with randomly scattered lipid granules (LG). SBB X 900; **Fig. 15:** Showing accumulating lipid granules (LG) in the perinuclear zone of the stage III oocytes. SBB X 900 ; **Fig. 16 :** Stage IV oocyte with dispersed lipid bodies in it's ooplasm. SBB X 900 ; **Fig. 17 :** age VI oocyte with lipid granules (LG). Note the negative reaction in the nucleoli (arrow) SBB X 900.

nucleoli have increased in size and number and tend to migrate towards the nuclear membrane. In the ooplasm there appear a number of vacuoles in the peripheral zone (Fig. 4). Some oocytes of this stage are seen possessing the yolk nucleus. The follicular epithelium appears to consists of cuboidal cells. The thecal cells cannot be distinctly marked from the follicular epithelium.

Stage III

Oocytes of this stage measure about 250 μm in diameter. Almost all the nucleoli are arranged along the nuclear membrane. The vacuoles in the ooplasm which appear to become reduced in size, tend to remain in the cortical zone. No oocyte of this stage possesses a yolk nucleus. The follicular epithelium consists of cuboidal cells. The fibrous thecal cells are distinctly observed near the follicular epithelium.

Stage IV

During this stage, the oocytes measure about 310 μm in diameter. The nuclear membrane appears irregular and some oocytes posses nuclei having wavy outline with outpushing pockets containing nucleoli. The vacuoles in the ooplasm are confined to the cortical zone. The peripheral ooplasm contains large number of granules which tend to move towards the peripheral ooplasm (Fig. 5). The follicular cells become columnar in shape leaving narrow intercellular spaces, through which possibly the infiltration of yolk precursors into the oocyte takes place. The thecal cells become hyperactive and distinctly form few layers around the follicular epithelium.

Stage V

The diameter of the oocytes at this stage measure about 375 μm. The nuclear membrane remains irregular with reduced number of nucleoli. A layer of cortical alveoli is distinctly visible in the cortical zone of the ooplasm. Small yolk granules appear in the ooplasm. A protective layer of *zona pellucida* (chorion) is seen around some oocytes. The follicular cells with intercellular space become hyperactive and so also the thecal cells.

Stage VI

Oocytes of this stage measure about 430 μm in diameter. The oocyte nucleus is pushed towards the peripheral ooplasm due to deposition of yolk granules. The ooplasm becomes packed with yolk granules and yolk globules. The nucleoli of the oocyte nucleus in most cases become indistinct. The cuboidal follicular cells become flat and fit closely to the follicular epithelium leaving no intercellular space.

Follicular atresia

All oocytes, however, do not undergo maturation process. Some of them become atretic. Atresia has been observed in all the stages of the oocytes. Stage I and II oocytes possibly initiate atresia by vacuolisation. In most cases atresia is apparently caused by hyperactive follicular cells either directly as phagocytes or indirectly through their secretions of lytic substances which cause dissolution of the ooplasmic components (Figs. 7-10).

Annual ovarian cycle

Based on morphological details of the ovary studied fortnightly throughout the year, the following six phases of the ovary could be distinguished.

1. **Resting phase (First week of January, May and September) :** The ovaries are small and transparent. Most of the oocytes are in stage I. The mean diameter of the oocytes is 80±7.90, 185±7.90 and 160±9.35μm in the first week of January, May and September, respectively.

2. **Maturing phase (Third week of January, May and September and First week of February, June and October) :** The ovaries increase in size and appear light yellow in colour. They visibly occupy half of the length of the body cavity. The oocytes are found to grow to stage III, IV, V and VI in the first week of February, June and October. The mean ova diameter and GSI show increasing trend (Table 1).

3. **Mature and pre-spawning phase (Third week of February, June and October):** The ovaries are yellow and occupy more than half of the body cavity. About 50% of the oocytes develop to stage VI during this phase.

4. **Spawning phase (First week of March, July and November) :** The ovaries are bright yellow in colour and occupy whole of the body cavity. Most of the oocytes (90%) are in the final stage (Stage VI) of maturation. The mean ova diameter and GSI have further increased in comparison to prespawning phase (Table 1).

5. **Post-spawning phase (Third week of March, July and November) :** The ovaries shrink in size to a great extent and appear almost transparent. Most of the mature oocytes are in atretic stage and a number of post ovulatory follicles exist. Most of the oocytes (75%) are in Stage I. The mean ova diameter and GSI show a decreasing trend.

6. **Spent-phase (First week of April, August and December)** : The ovaries appear shrunken and reduced. Most of the oocytes are in stage I (92%) and few are in stage II (8%). The mean ova diameter and GSI have minimum value in the month of December (Table 1).

GSI and Ova diameter

The GSI of the fish samples were calculated from January to December of a calender year. When GSI and ova diameter were plotted against their corresponding months, a characteristic graph was obtained, which indicated the relation between GSI and ova diameter and annual ovarian activity. It can be inferred that the ovary of *Tilapia mossambica* is at the peak of its activity in the month of March, July and November (Fig. 18). Curiously enough *Tilapia mossambica* spawns three times in a calender year, which is in agreement with three peaks as shown in Fig. 18.

Histochemistry of vitellogenesis

Histochemical tests reveal that two types of yolk, namely, the yolk granules and yolk globules are synthesised in the developing oocytes of *Tilapia*. The precursors of the yolk granules infiltrate through the intercellular spaces in the follicular epithelium and accumulate in the peripheral ooplasm where they coalesce to form yolk granules. The second category of yolk, the yolk globules develop *de novo* in the central part of the ooplasm. Histochemically, yolk globules are found to be made up of proteins and yolk granules are constituted of proteins, carbohydrates and lipids.

Carbohydrate yolk : The yolk granules and the precursors of yolk migrating into the oocyte show strong PAS-positivity. Yolk globules do not respond to PAS test. Treatment with Schiff's reagent and PAS after acetylation and deacetylation reveals the presence of polysaccharides containing vic-glycols. Incubation with salivary amylase and malt diastase confirmed the presence of glycogen in yolk granules. Treatment with Alcian Blue (AB) indicated the presence of non-sulphated acid muco-polysaccarides in stage VI oocytes, cortical alveoli and chorion. Test with Azure A (pH 3.0 and above) emphatically ascertains their sialomucin contents. Occurrence of neutral mucopolysaccharides in the ovarian tissue was negated after testing with phenyl hydrazine.

Proteid yolk : On treatment with mercuric bromophenol blue (HgBPB) all the components of the ovary were stained blue showing the presence of proteins. Development of yolk granules from extraneous sources appears to be certain as their protein histochemistry is similar to that of the follicular epithelium. Test with Ninhydrin-Schiff followed by deamination, CTZ test followed by extraction with performic acid, DNFB and benzoylation confirm the presence of tryptophan, tyrosine and histidine

(Fig.13). The ferric ferricyanide and performic AB tests reveal the presence of sulphydryl and disulphide group-containing proteins in the yolk. The protein histochemistry of both the yolk granules and yolk globules appear to be similar.

Fatty yolk : Oocytes of stage 1 and stage 2 show Sudan Black B (SBB) positive granules randomly scattered in the ooplasm (Fig.14). The infiltrating yolk granules in the stage IV-VI oocytes and the yolk precursors found in the inter-cellular spaces of the follicular epithelium show positive response to lipid test (Figs. 15-17). The yolk globules are sudanophobic and contain no lipid. Treatment of yolk granules with ethanolic SBB and propylene glycolic SBB, extraction with a hot mixture of chloroform and methanol confirmed their lipid content. The extra oocytic precursor of yolk including yolk granules on testing with various histochemical reagents like hot ether, cold acetone, Sudan III+IV, Oil red O, Pyridine, Fischler's and PFAS and Schultz tests revealed the presence of glycolipids, lecithin and cephalin-containing phospholipid, unsaturated fatty acid chain and non fatty lipid elements. However, the yolk granules and their precursors contain no cholesterol.

DISCUSSION

Literature on teleost fishes reveal conflicting views on the source of origin of oocytes. In *Tilapia mossambica* the pre-existing oogonia (residual oogonia) are the source of origin of oocytes. The same observation has been reported by Bisht and Joshi (1975) in *Schizothorax richardsonii.* Based on histological and histochemical tests the oocytes of *T. mossambica* shows six different stages. The basis of staging is in agreement with that of Guraya *et al.*, (1975). The secondary nucleoli originate from the granular elements attached to the lampbrush chromosomes as has been reported by Gopal Dutta and Govindan (1975). Nucleoli are the site of synthesis of ribosomal RNA. As revealed by biochemical studies nucleolus plays an important role in transmitting information via RNA from the nucleus to the cytoplasm. The role of lambrush chromosome in the oocyte of *T. mossambica* is probably to form protein yolk. The yolk nucleus has been observed in the periphery of the ooplasm and it disappears before the yolk bodies develop. It is deduced that the yolk nucleus plays no role in vitellogenesis. The nuclear extrusions containing extruded nucleoli apparently play no role in the formation of yolk, as has been reported by earlier workers (Verma *et al.*, 1981). Histochemical details of the fish under study reveal the fact that the yolk precursors and yolk granules have infiltrated through the follicular cells and/or the intercellular spaces of the follicular epithelium to the peripheral ooplasm. However, the yolk globules develop *de novo*, *i.e.* under the influence of certain ooplasmic organelles. Many of the histochemical studies made in *Tilapia* are in agreement with the recent histochemical findings of Pisca and Prasadam (1991) in *Salmostoma bacaila*, Schwalme and Mackay (1992) in *Esox lucius* and Grau *et al.* (1996) in *Sericola dumerili.*

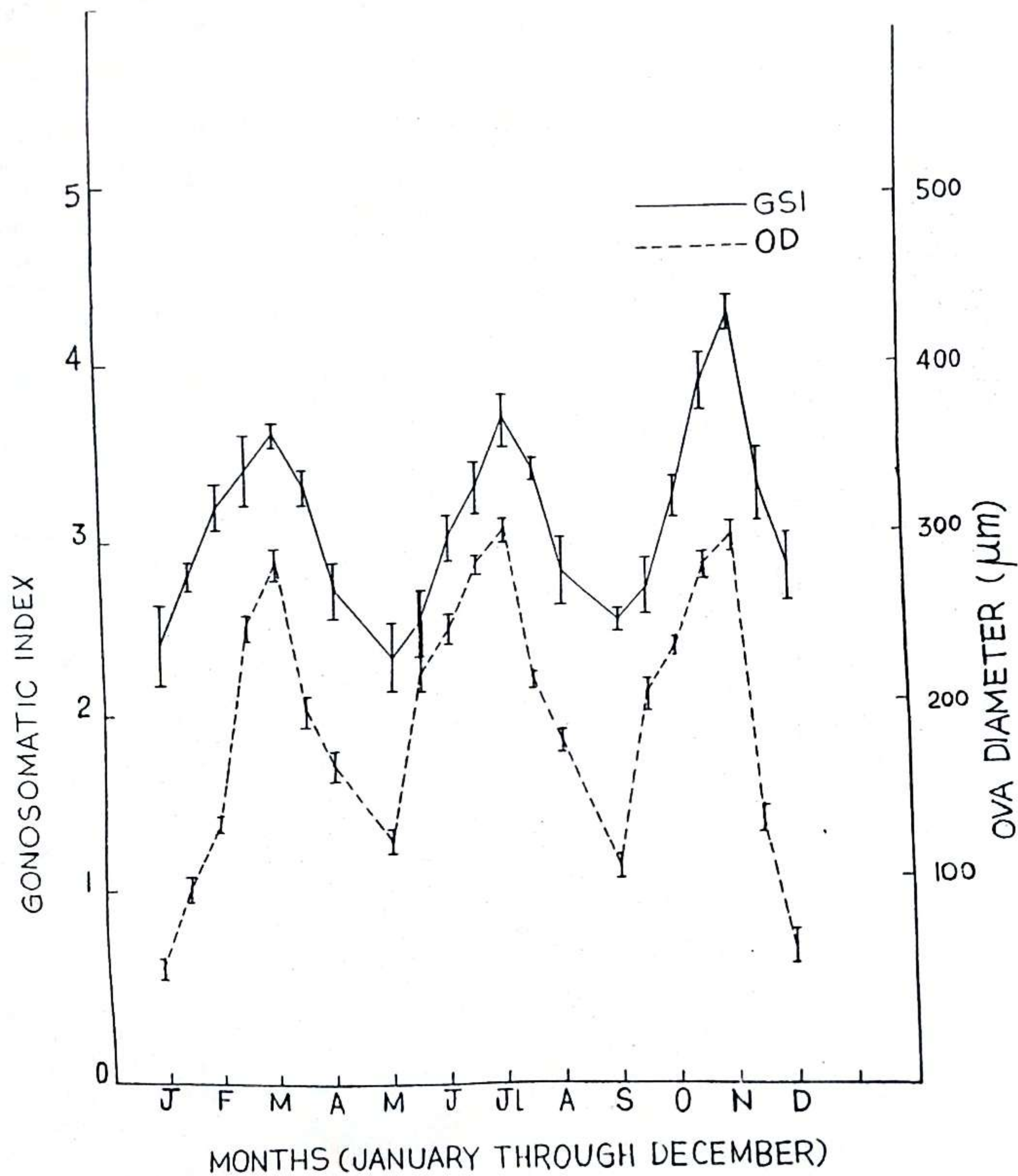

Fig. 18 : Seasonal variations in the mean ova diameter and gonosomatic index in *Tilapia mossambica*.

Table 1: Fortnightly variation in mean GSI and ova diameter of *Tilapia mossambica*

Month	Average weight of the fish in gms	Average weight of the ovary in gms	Mean G.S.I	Average ova diameter in µm
January				
1st week	45.408	1.086	2.4±0.25*	80.±7.90
3rd week	47.393	1.327	2.8±0.09	150±9.35
February				
1st week	55.993	1.793	3.2±0.14	205±6.12
3rd week	46.529	1.582	3.4±0.19	375.10.61
March				
1st week	43.695	1.573	3.6±0.07	430±12.75
3rd week	40.151	1.325	3.3±1.10	300±12.75
April				
1st week	51.628	1.393	2.7±0.16	250±12.51
May				
1st week	64.825	1.507	2.3±0.19	185.±7.90
3rd week	58.360	1.459	2.5±0.19	330±14.58
June				
1st week	59.516	1.824	3.0±0.14	370±18.71
3rd week	41.750	1.378	3.3±0.14	430±14.14
July				
1st week	40.700	1.505	3.7±0.16	460±13.59
3rd week	36.294	1.234	3.4±0.07	325±6.12
August				
1st week	54.186	1.517	2.8±0.19	270±10.61
September				
1st week	63.160	1.566	2.5±0.07	160±9.35
3rd week	45.111	1.218	2.7±0.17	310±12.75
October				
1st week	53.165	1.543	2.9±0.12	350±3.81
3rd week	43.974	1.715	3.9±0.16	425±9.35
November				
1st week	43.895	1.887	4.3±0.10	450±12.51
3rd week	40.757	1.345	3.3±0.22	200±10.61
December				
1st week	67.565	1.891	2.8±0.19	90±14.14

* Means±SD

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RETARDED EMBRYONIC DEVELOPMENT AFTER IMPLANTATION IN *HIPPOSIDEROS LANKADIVA LANKADIVA* (KELAART)

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ABSTRACT

In *Hipposideros lankadiva lankadiva* preimplantation stages of embryo are observed until second week of September after which the blastocyst implants at the cranial end of the uterus in a preformed implantation chamber, which anatomically appears as a swollen bulb. By mid-October to mid-March the bats become semitorpid for about 5 months. The bulb size does not increase from September to December, but thereafter there is a gradual but slight increase. By mid-March there is significant increase in the arousal period from semitorpor to reaching maximum, at term. The implanted bilaminar blastocyst remains at the embryonic-disc stage throughout semitorpor exhibiting retarded embryonic development. The diffuse trophoblastic placenta occupies half the thickness of the endometrium and consists of a syncytiotrophoblastic shell amidst which lie maternal capillaries lined by distinct endothelium. The undifferentiated cytotrophoblast marks its foetal boundary. The endometrium beyond the synciotrophoblastic shell is compact with cut ends of distal segments of the uterine glands. The epiblast/ embryonic disc during October-December is in contact with the basal cytotrophoblast. During January-February, formation of trophoepiblastic cavity is noticed which enlarges by March. In January the cytotrophoblast becomes active - its cells divide and form hillocks and by March they penetrate the syncytiotrophoblastic shell. After mid-March development accelerates and by mid-April advanced stages of pregnancy are noticed. The primitive streak develops about 7 months, postcoitally. Deliveries take place during late May or early June.

Key Words: Retarded embryonic development, bat.

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INTRODUCTION

One of the reproductive strategies employed by bats is the phenomenon of embryonic delays during development. Two types of embryonic delays occur in bats:

1. Developmental diapause or delayed implantation

The fertilized ovum after it develops in a normal manner upto the blastocyst stage gets into a period of developmental quiescence and remains in an unimplanted condition for a variable length of time. This is a common type of delay occurring in mammals. Apart form order Chiroptera it occurs in 6 distantly related orders *viz.* Marsupilia, Insectivora, Edentata, Carnivora, Rodentia and Artiodactyla (Enders, 1963; Daniel, 1970; Wimsatt, 1975; Renfree and Calaby 1981; Heideman, 1989; Mead, 1993; Khan, 1996). Among bats, developmental diapause or delayed implantation occurs in *Corynorhinus rafinesquei* (Pearson *at al.*, 1952), *Eidolon helvum* (Mutere, 1967; Fayenuwo and Halstead, 1974), *Rhinolophus landeri* (Menzies, 1973), *Rhinolophus rouxi* (Ramakrishna and Rao, 1977), *Miniopterus schreibersii* from France and Africa (Peyre and Herlant, 1963, 1967; Anciaux de Faveaux, 1977; Bernard, 1980, 1994; Bernard *et al.*, 1996; van der Merwe, 1979, 1986), *Miniopterus minor* from Kenya (McWilliams, 1988), *Miniopterus schreibersii natalensis* from South Africa (van der Merwe, 1980; Bernard, 1980) and *Miniopterus frateculus* from South Africa (Bernard, 1980).

2. Retarded embryonic development

The blastocyst implants on schedule but after implantation the embryonic development slows for a period of time. Post implantation embryonic delay or retarded embryonic development after implantation is uncommon and is reported to occur only in bats from both suborders- Mega and Microchiroptera (Heideman, 1989; Khan, 1996). Retarded embryonic development after implantation has been observed in *Haplonycteris fischeri* (Heideman, 1989), *Cynopterus sphinx* (Krishna and Dominic, 1983), *Hipposideros caffer caffer* (Bernard and Meester, 1982), *Macrotus californicus* (Bradshaw, 1962; Burns *et al.*, 1972; Bleier, 1975a; Burns and Easley, 1977), *Artibeus jamaicensis* (Fleming,1971), *Carollia perspicillata* (Rasweiler and Badwaik, 1997) and *Natalus stramineus* (Mitchell, 1965)

A literature survey on retarded embryonic development in bats did not reveal any histological details occurring during the delay for any species studied so far. The present paper therefore, aims to study the details of retarded embryonic development after implantation in *Hipposideros lankadiva lankadiva* (Kelaart).

MATERIALS AND METHODS

The present report is based on the study of 214 females of *Hipposideros l. lankadiva* collected from Chandrapur in Maharashtra and Mandu in Madhya Pradesh, India. The bats were collected with the help of a butterfly net. The collections were made during 1985-1995 in such a way that every calender month was represented by one or more collections. The animals were killed with chloroform and their body weight and other morphological details were recorded. The genitalia was removed and fixed in alcoholic Bouin's fluid or formol alcohol or buffered formalin for 24h. The tissues were dehydrated by passing through graded ethanol, cleared in xylene and embedded in paraffin (m.p. 58°C-60°C). The sections were cut at 6-8 μm and stained with haematoxylin-eosin.

RESULTS

Breeding Biology and organization of female Reproductive Tract

Hipposideros l. lankadiva a monotocous bat breeds once in a year, breeding commencing at the peak of rainy season during late August-early September and parturition takes place by the end of May, the gestation period being 260-270 d. Morphologically the female genitalia is bicornuate and bilaterally symmetrical but physiologically the left ovary and uterine cornu is dominant over the right (62.1%). Ovulation invariably occurs from the left ovary with pregnancy occurring in the uterine cornu, ipsilateral to the new corpus luteum (CL). The embryo descends into the uterus as a morula and develops into a bilaminar blastocyst prior to implantation. In females the preimplantation changes are localized to a small cranial segment which is seen anatomically as a swollen bulb carrying early pregnancy.The anti-mesometrial part of the uterine lumen forms a pocket/implantation chamber in which the morula/blastocyst is lodged while the rest of the uterine lumen towards the mesometrial side remains as a wide slit.

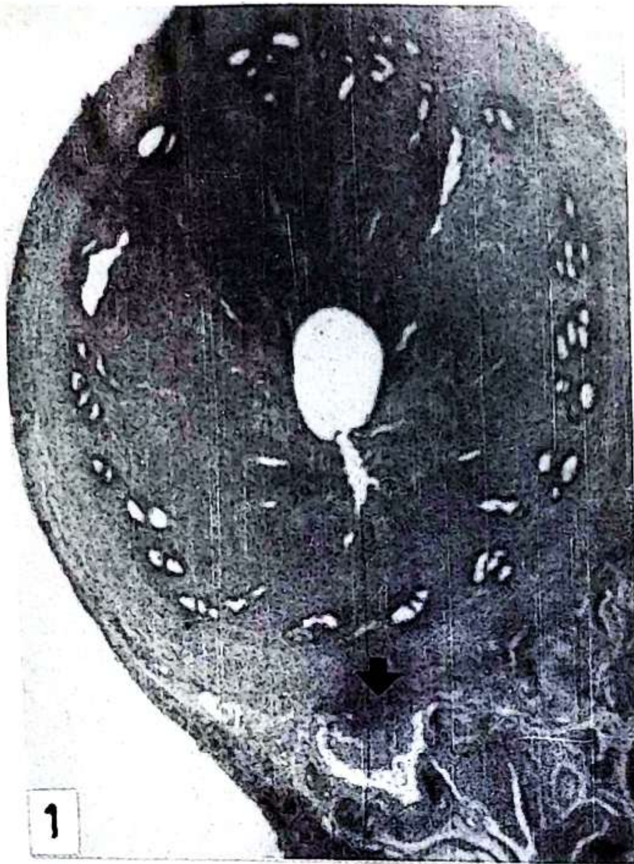
Implantation

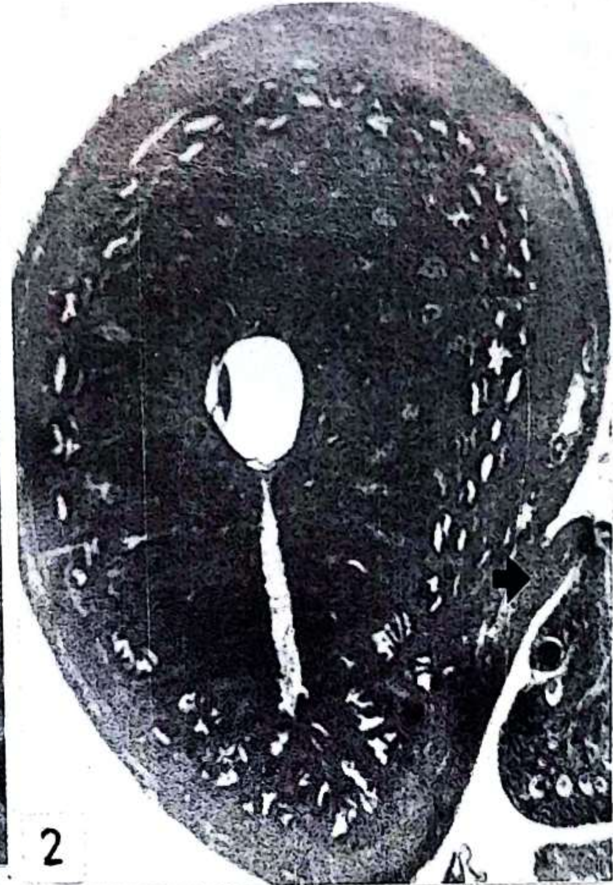
The earliest implanting blastocyst was collected on 14.09.88. The blastocyst expands (Figs. 1-3) and its trophoblast establishes contact with the reduced and discontinuous uterine epithelium of the preformed implantation chamber, implantation being superficial and circumferential, while the rest of the uterine lumen on the mesometrial side remains slit-like and is lined by a distinct columnar epithelium (Fig. 4). The embryonic disc is biconvex and shows variable orientation -oriented towards the anti-mesometrial side of the uterus (Fig. 2) towards the lateral side of the uterus (Figs. 1,3) and in between the lateral and antimesometrial side of the uterus.

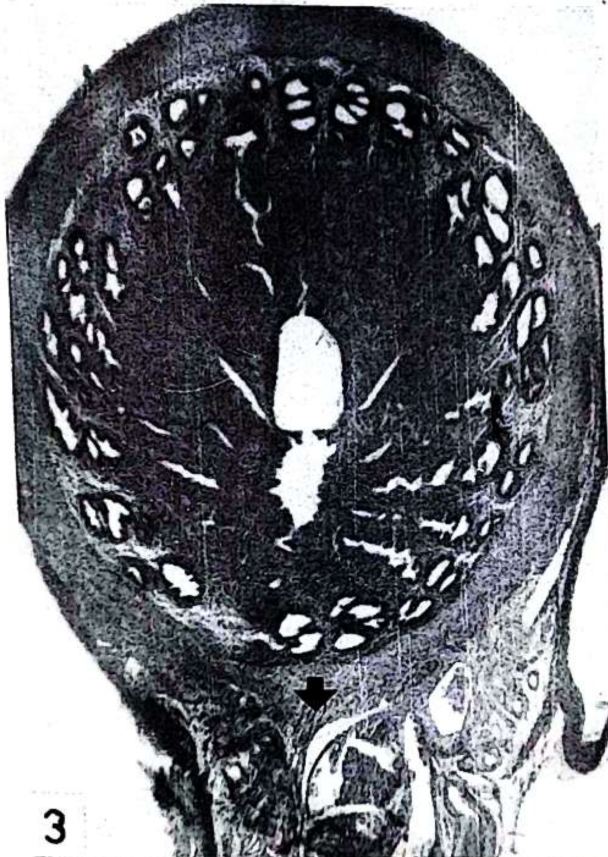
In the embryonic region the trophoblast invades the uterine endometrium forming small hillocks (Figs. 5-7). The reduced uterine epithelium appears disrupted (Figs. 6,7) in the region where the trophoblast has penetrated. The embryonic disc is biconvex and is made up of small polygonal cells. The endoderm consists of fusiform cells which underline the embryonic disc (Figs. 5-7) and also forms a complete lining below the trophoblast (Fig. 8). The endometrium surrounding the implantation chamber and the slit-like uterine lumen can be differentiated into two distinct regions (Figs. 1-4): an inner two-thirds of the endometrium just below the invading trophoblast appears compact. In this region the proximal parts of most of the uterine glands observed at the free uterine blastocyst stage have broken down and merged with the endometrial stroma (Figs. 1-4). However, cut ends of a few proximal parts of the uterine glands in the process of merging are still visible. A close examination of this region (Fig. 8) shows the penetrating trophoblast, the decidual endometrial stromal cells, the cells of the proximal parts of the uterine glands which have merged with the decidual cells and numerous maternal capillaries lined by distinct endothelial lining. The myometrium is thick. The endometrium below the myometrium is lightly stained as the endometrial cells are sparsely arranged. This region is characterized by the presence of distinct coiled cut ends of the distal parts of the uterine glands (Figs. 1-4).

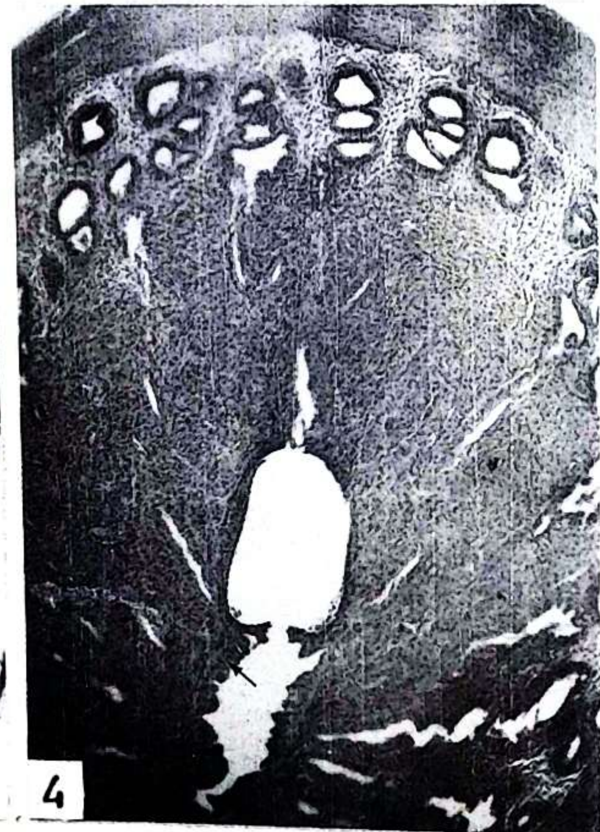
Changes occurring during retarded embryonic development

From mid-October to mid-March (5 months) the bilaminar blastocyst shows retarded embryonic development which coincides with semi-torpor. The bulb size (Table 1) does not increase from September to December. From December onwards there is a gradual but slight increase in bulb size and by mid-March (the period of arousal of bats from semi-torpor) the bulb size shows significant increase which reaches maximum at term *i.e.* pregnancy. Thus, from September to mid- March implanted bilaminar blastocyst with

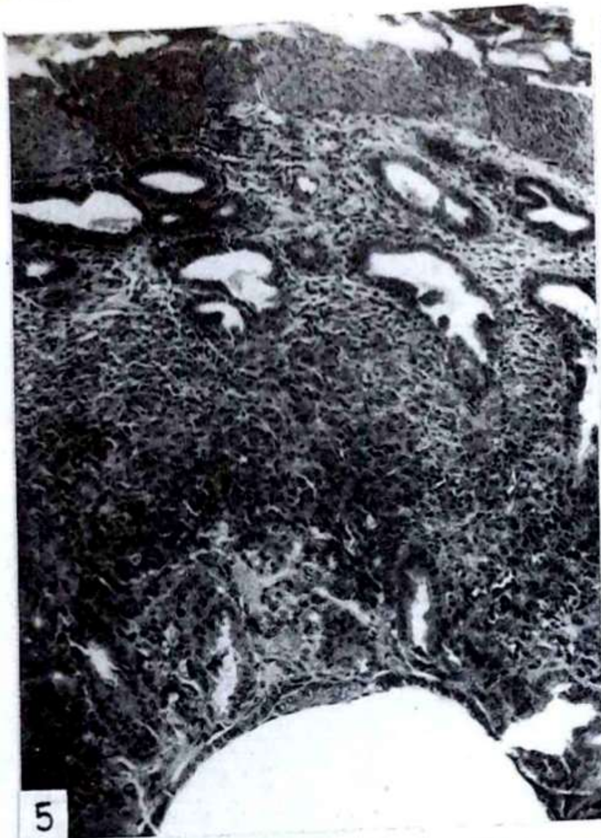


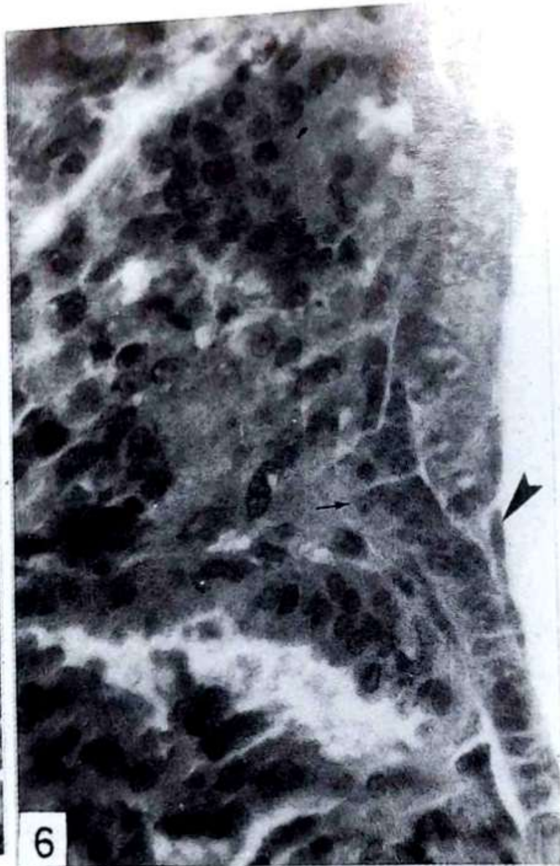


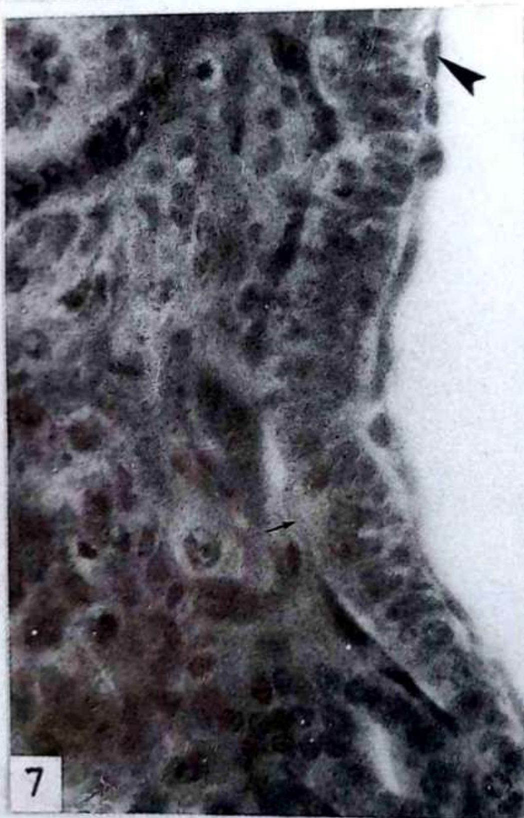




Figs. 1-3 : Transverse section (T.S.) of left uterus showing an implanting bilaminar blastocyst in a preformed implantation chamber formed on the antimesometrial side of the uterus while the rest of the uterine lumen on the mesometrial side remains slit-like. Proximal segments of most of the uterine glands have broken down and merged with the endometrial stroma. Distinct coiled cut ends of the distal parts of the uterine glands are seen below the myometrium. The embryonic disc is oriented towards the lateral side of the uterus in the blastocyst in Fig. 1 and Fig. 3 while it is oriented towards the antimesometrial side of the uterus in the blastocyst in Fig. 2. Arrow points towards the mesometrium. Fig. 1: X44; Fig. 2: X42; Fig. 3: X56 ; **Fig. 4:** Part of Fig. 3 magnified to show the implanting bilaminar blastocyst in a preformed implantation chamber. Note that rest of the slit-like uterine lumen on the mesometrial side is lined by distinct columnar uterine epithelium (arrow). X128







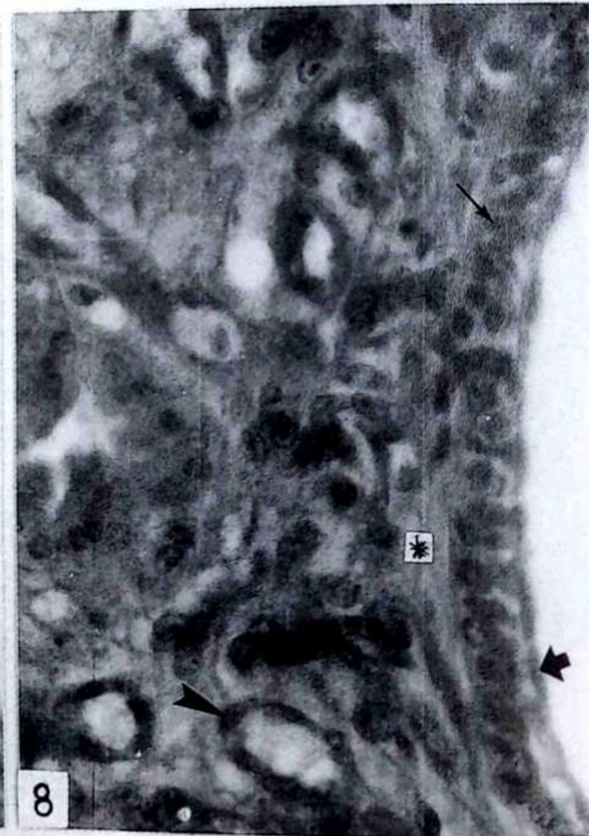


PLATE-II

Fig. 5: Part of the embryonic area of an early implanting bilaminar blastocyst. X180 ; **Fig. 6:** Part of Fig. 5 magnified to show that the embryonic disc is made up of polygonal cells and the trophoblast invading the endometrium. The uterine epithelium appears disrupted (arrow). The endoderm (arrowhead) underlines the embryonic disc and the trophoblast. X560 ; **Fig. 7:** Part of the embryonic area of an early implanting bilaminar blastocyst showing thick trophoblast in contact with the disrupted uterine epithelium (arrow). The fusiform endodermal cells (arrowhead) form a lining to the trophoblast. X560 ; **Fig. 8:** Part of the endometrium surrounding the implantation chamber at early implanting bilaminar blastocyst showing the invading trophoblast, the cells of the broken uterine glands and decidual endometrial cells. Numerous maternal capillaries (arrowhead) lined with distinct endothelium lie in this region. Thin arrow points towards the trophoblast and thick arrow points towards the endothelium. Asterisk marks the area of disrupted uterine epithelium. X712.

embryonic disc stage is noticed (Figs. 1-3,9-17). The noticeable changes occurring during this period are:

i) The blastocyst expands and the uterine lumen is completely obliterated at the level of implantation.

ii) The embryonic disc enlarges in size and undergoes lateral expansion (Figs. 9-11, 13-17). The embryonic disc shows variable orientation; oriented anti-mesometrially, oriented between lateral and antimesometrial side and in a few cases oriented towards the lateral side of the uterus.

iii) The embryonic disc/epiblast during October-December is in contact with the basal layer of cytotrophoblast (Figs. 9-11,18,19). During January-February the formation of tropho-epiblastic cavity is noticed (Figs. 14,20).

iv) By March the tropho-epiblastic cavity- the cavity between the epiblast embryonic disc and the basal layer of cytotrophoblast enlarges (Figs. 15-17,21).

v) The endometrium during October-March can be distinguished into 2 distinct regions: the region below the basal layer of cytotrophoblast - the syncytiotrophoblastic shell (Figs. 10,14,17,19,20,22) occupying about half the thickness of the endometrium and amidst which lie maternal capillaries lined by distinct endothelium (Fig. 23). At the maternal border of the syncytiotrophoblastic shell necrotic zones of the trophoblast (Figs.13, 20) are observed that mark the boundary of the syncytiotrophoblastic shell. Beyond this the endometrial stroma is compact. Below the thick myometrium lie cut ends of the distal segments of the uterine glands.

vi. In January the cytotrophoblast at few places becomes active, its cells divide and form hillocks (Fig. 24) and by March they penetrate the syncytiotrophoblastic shell (Fig. 25).

vii. The CL has a long life and persists approximately for 8 months till ovulation through retarded embryonic development (semi-torpor) until the limb-bud stage. A regressed CL was observed at the late limb-bud stage of development of the embryo.

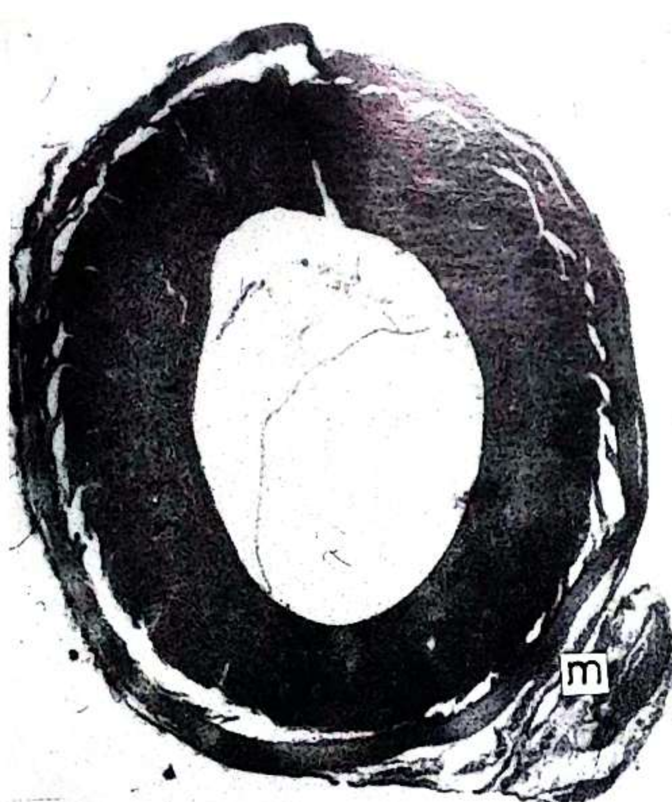
viii. After 12th March, the arousal period of bats from semi-torpor development accelerates (both embryonic and placental) and by 2nd week of April progressively advanced stages of development are obtained *viz.* trilaminar blastocyst, early and late primitive streak stages, allantoic diverticulum stage,

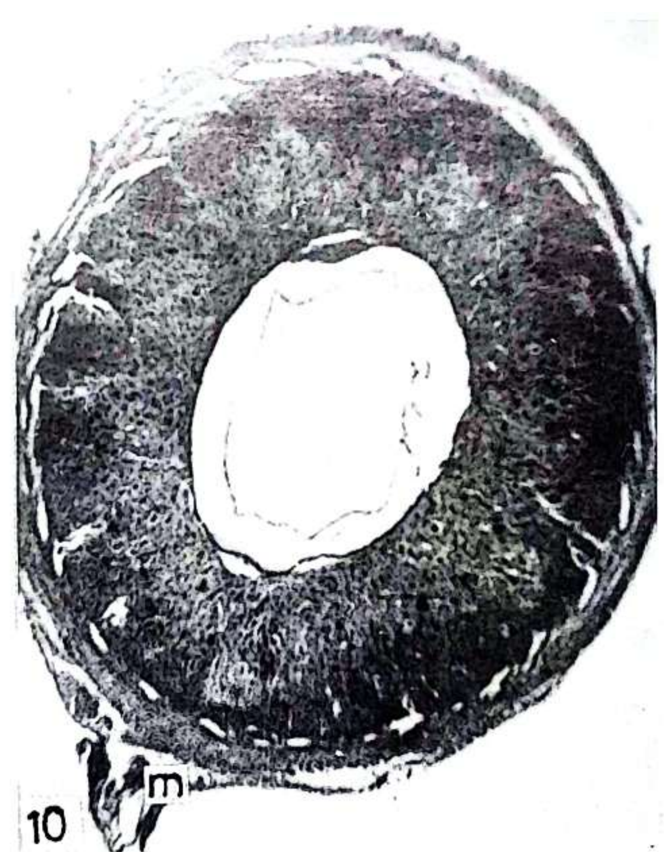
early and late limb bud stages (Figs. 26-34, 36-41) and a well development placenta is observed at the late primitive streak stage (Fig. 35)

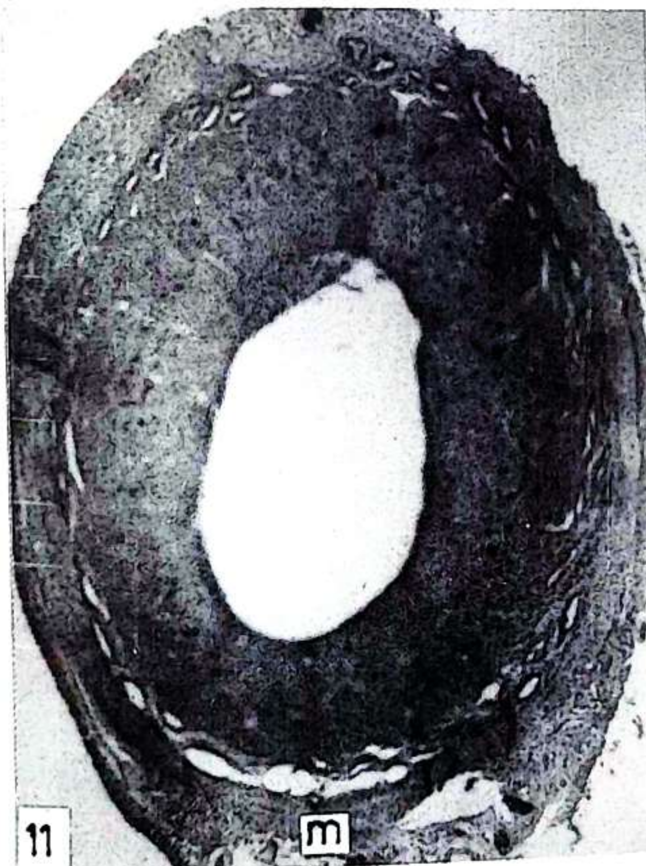
ix. The primitive streak (Figs. 29-31) develops approximately 7 months after post-coitum (06.04.88). Table 1 shows the details of bulb size correlated with various stages of development.

DISCUSSION

Recent research work on delays in embryonic development in bats is directed to establishing the role of hormones of the thyroid, CL (progesterone) and those of the anterior pituitary. Mead (1993) summed up information on *Macrotus californicus* which is as follows: maternal blood levels of thyroxine are depressed during retarded embryonic development in *Macrotus californicus,* but increase when embryonic development is renewed. Maternal triiodothyronine are only slightly elevated during winter months and then appear to decline when embryonic development resumes (Burns, 1981). Repeated injection of thyroxine for 20 d failed to increase the rate of embryonic development. Gonadal steroids are secreted in a biphasic pattern during pregnancy. An early rise in estrogen and progesterone secretion occurs at the time of implantation and placental development (October-December). However, the precise stage of development was not confirmed in these animals. This is followed by a period of marked reduction in both oestrogen and progesterone which coincides with mid-delay period (January-March). Secretion of both hormones again increased significantly during the more rapid and final stages of embryonic development (April-May). Thereafter, progesterone levels rapidly declined prior to parturition (Burns and Wallace, 1975; Burns and Easley, 1977). Unilateral removal of the right functional ovary bearing the CL at any stage of pregnancy, consistently resulted in abortion whereas sham ovariectomy was without any effect (Burns, 1981). Changes in ultrastructure of luteal cells of this bat are correlated with changes in plasma levels of progesterone (Bleier, 1975b; Crichton *et al.*, 1990). These observations suggest that reduced luteal function is somehow linked to the retardation of embryonic development. Richardson (1979, 1981) reported an increase in the number of prolactin (PRL) cells prior to renewed embryonic development. This led him to hypothesize that PRL was somehow implicated in the process of retarded embryonic development. Prolactin might be essential to stimulate increased luteal function. Alternatively, PRL might somehow influence uterine function or growth of the embryo.







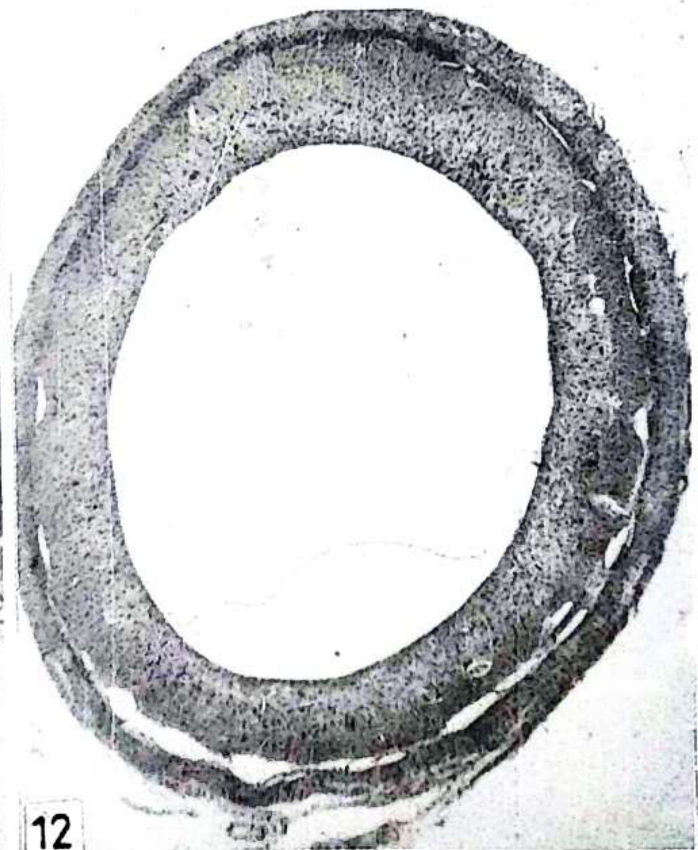
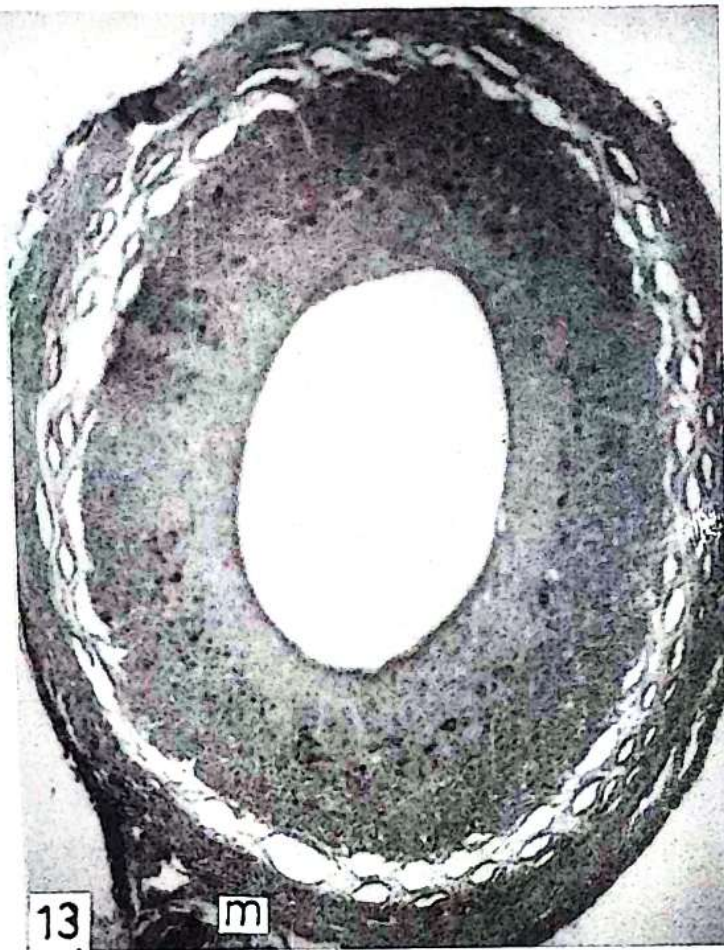


Fig. 9: T.S. of uterus showing an implanted bilaminar blastocyst at the embryonic disc stage during semi-torpor. The blastocyst has expanded and the uterine lumen is completely obliterated. The embryonic disc has expanded laterally and is in contact with the basal layer of cytotrophoblast and is oriented in between the antimesometrial and lateral side of the uterus; m - mesometrium. X54 ; **Fig.10:** T.S. of uterus showing an implanted bilaminar blastocyst at the embryonic disc stage. The embryonic disc has peeled away from its contact with the basal layer of cytotrophoblast during fixation. The embryonic disc is oriented towards the antimesometrial side of the uterus. Note the diffuse syncytiotrophoblastic shell around the implantation chamber. m - mesometrum. X60 ; **Fig. 11:** T.S. of uterus showing an implanted bilaminar blastocyst at the embryonic disc stage. The embryonic disc is oriented towards the antimesometrial side of the uterus and is in contact with the basal layer of cytotrophoblast; m - mesometrium. X62 ; **Fig.12:** T.S. of uterus containing an implanted bilaminar blastocyst at the embryonic disc stage. The embryonic disc is not seen in the photomicrograph. X64.



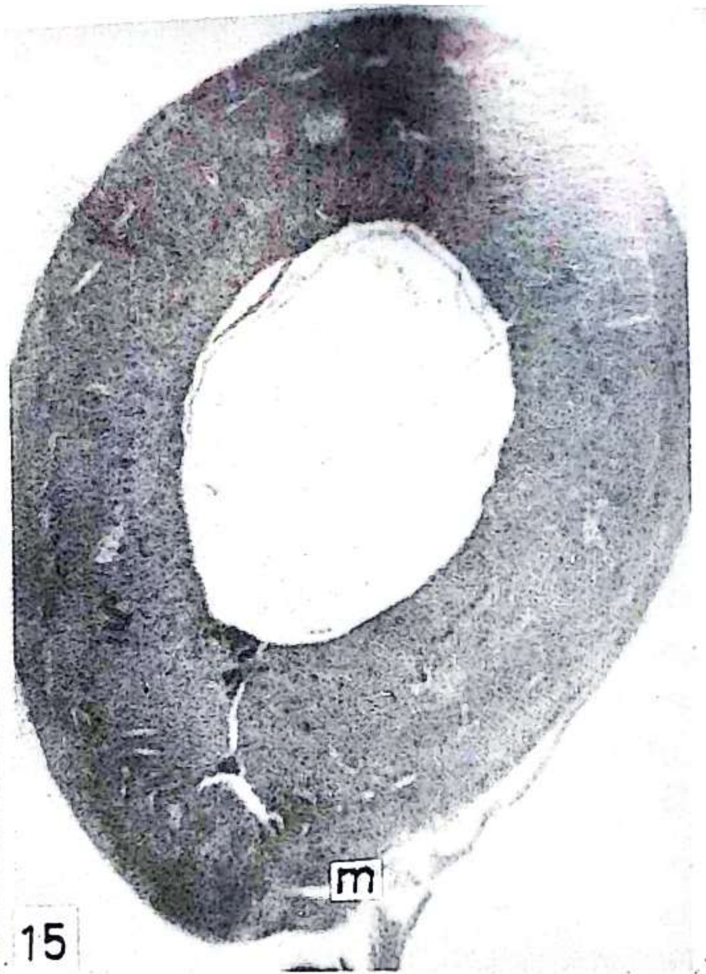
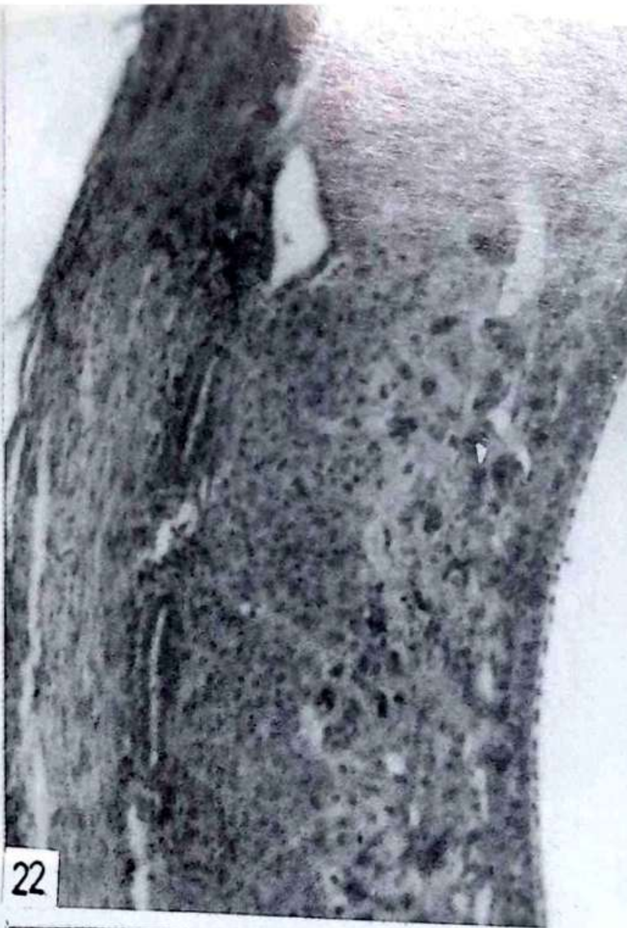
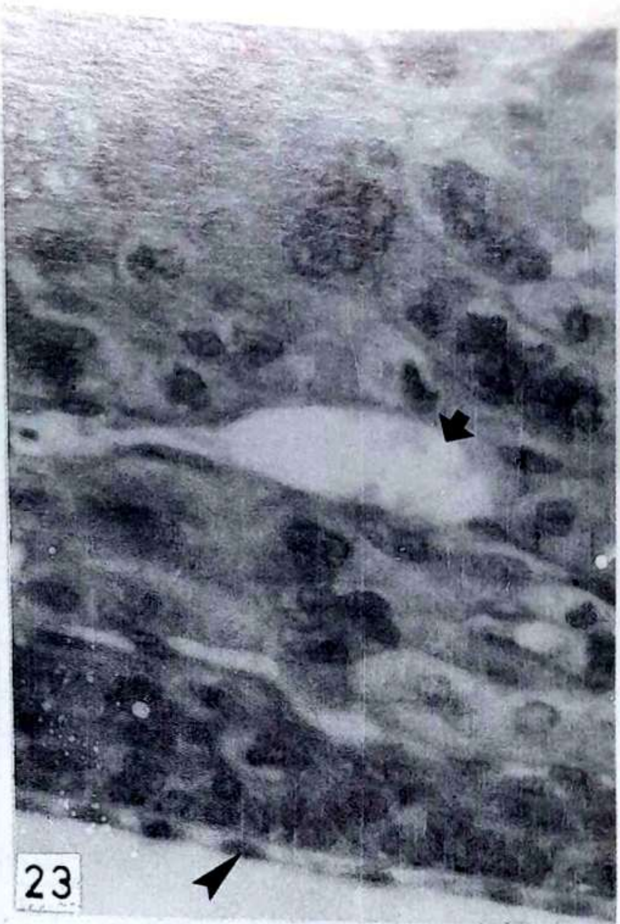


Fig. 13: T.S. of uterus showing an implanted bilaminar blastocyst at the embryonic disc stage. Small trophoepiblastic cavities appear in between the epiblast and the basal layer of cytotrophoblast. The embryonic disc is oriented towards the antimesometrial side of the uterus; m - mesometrium. X66 ; **Fig. 15:** T.S. of the uterus showing an implanted bilaminar blastocyst with a large trophoepiblastic cavity in between the epiblast and the basal layer of cytotrophoblast. The embryonic disc/epiblast is oriented towards the antimesometrial side of the uterus; m - mesometrium. X35 ; **Fig. 16:** T.S. of the uterus containing an implanted bilaminar blastocyst with an extensive epiblast and a large trophoepiblastic cavity; the amniotic cavity lies in between the epiblast and the basal layer of cytotrophoblst. The epiblast is oriented towards the lateral side of the uterus; m - mesometrium. X35.





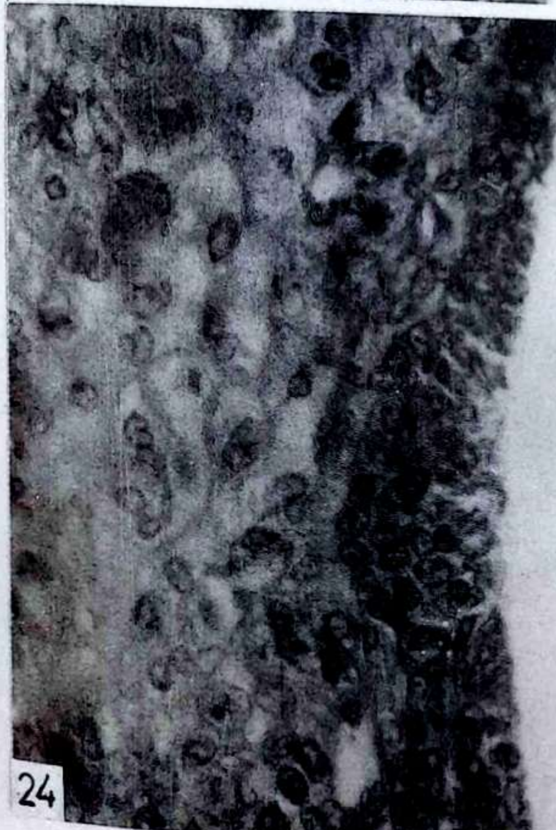
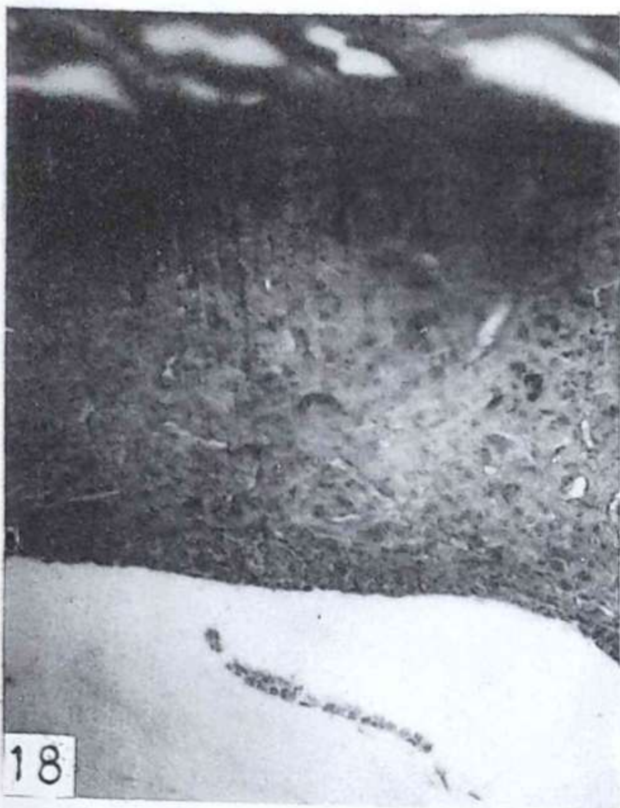
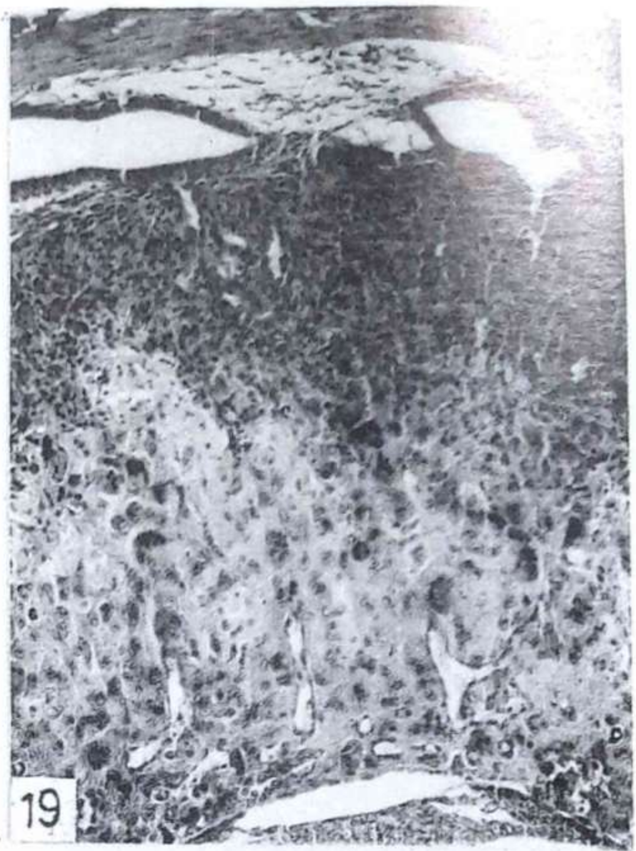
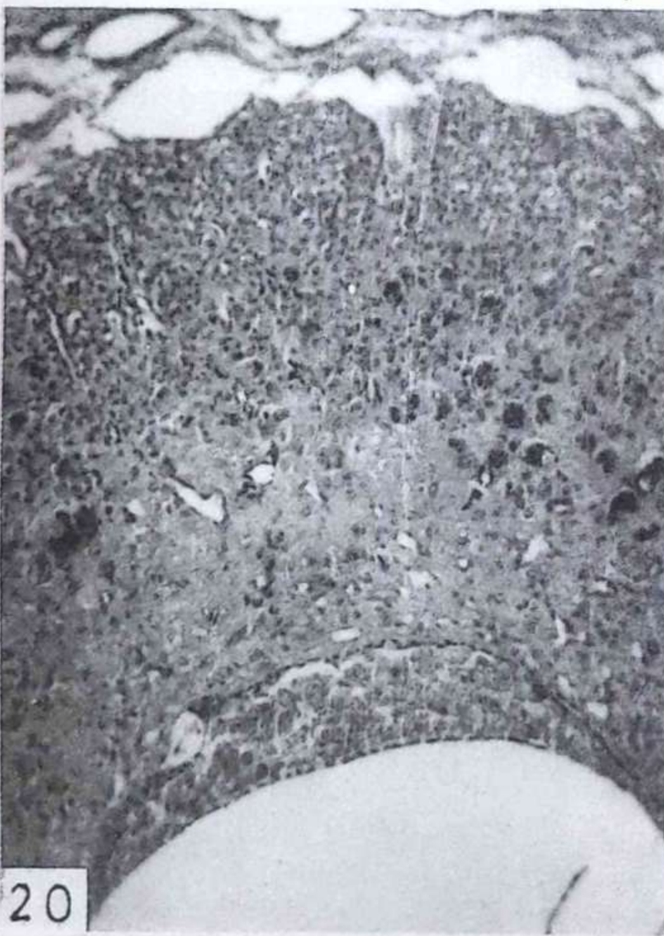


Fig. 22: Part of the lateral region of an implanted bilaminar blastocyst during January to show the syncytiotrophoblastic shell which occupies half the thickness of the endometrium. X180 ; **Fig. 23:** Part of syncytiotrophoblastic shell at the implanted bilaminar blastocyst stage magnified to show the symplasmic mass formed by the syncytiotrophoblast in which lie maternal capillaries (arrow) lined by distinct endothelium. The cytotrophoblast marks the foetal border of the syncytiotrophoblastic shell. Arrowhead points towards the endoderm. X712 ; **Fig. 24:** Part of the adembryonic area of an implanted bilaminar blastocyst during January showing the piling up of the cytotrophoblast cells. X560 ; **Fig. 25:** Part of the adembryonic are of an implanted bilaminar blastocyst during March showing the invasion of the basal cytotrophoblast layer (arrow) into the syncytiotrophoblastic shell. X140







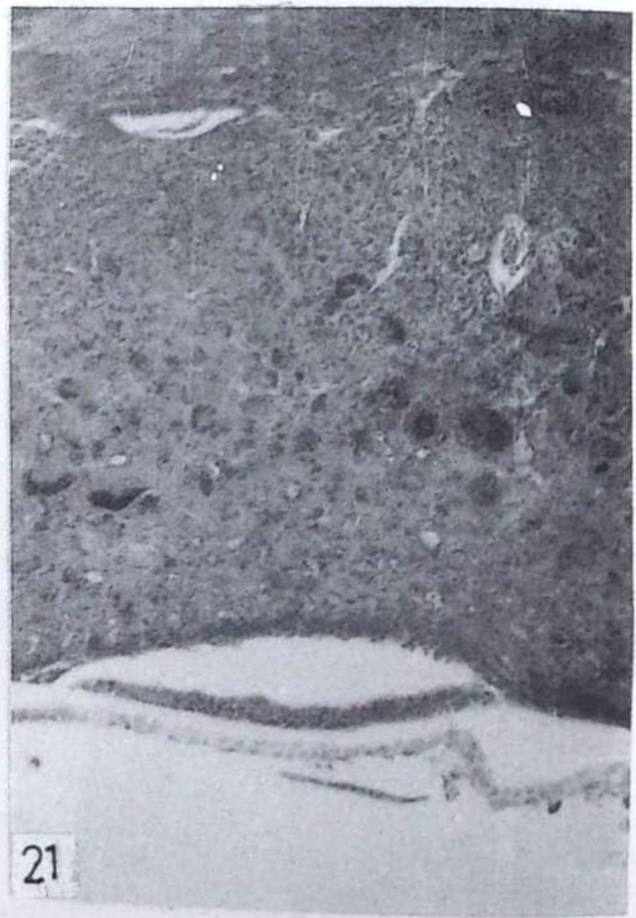
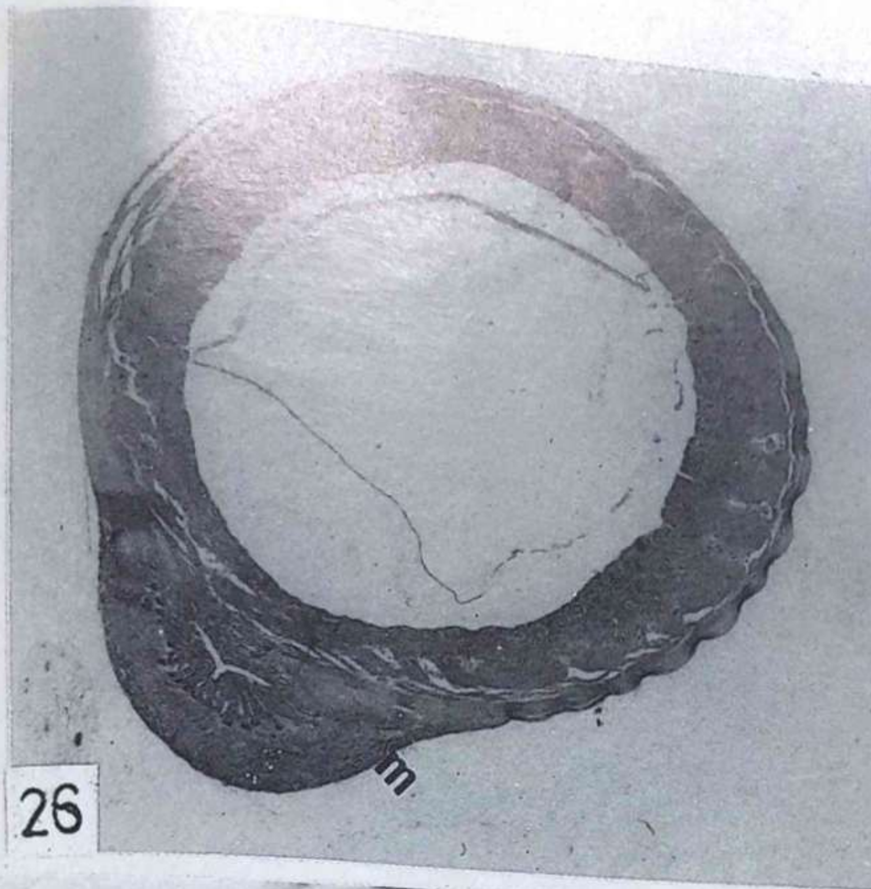


Fig. 18: Part of the embryonic area in Fig. 9 magnified to show the laterally expanded epiblast in contact with the basal layer of cytotrophoblast. X160 ; **Fig. 19:** Part of the embryonic area in Fig. 10 magnified to show the embryonic disc in contact with the basal layer of cytotrophoblast. The basal layer of cytotropholast and the embryonic disc have peeled off from its contact during fixation X180 ; **Fig. 20:** Part of the embryonic area of an implanted bilaminar blastocyst during February showing an extensive epiblast with the appearance of trophoepiblastic cavities. Note the necrotic zones marking the boundary of the syncytiotrophoblastic shell. X180 ; **Fig. 21:** Part of the embryonic area of Fig. 15 magnified to show a large trophoepiblastic cavity, the amniotic cavity in between the epiblast and the basal layer of cytotrophoblst. X128.



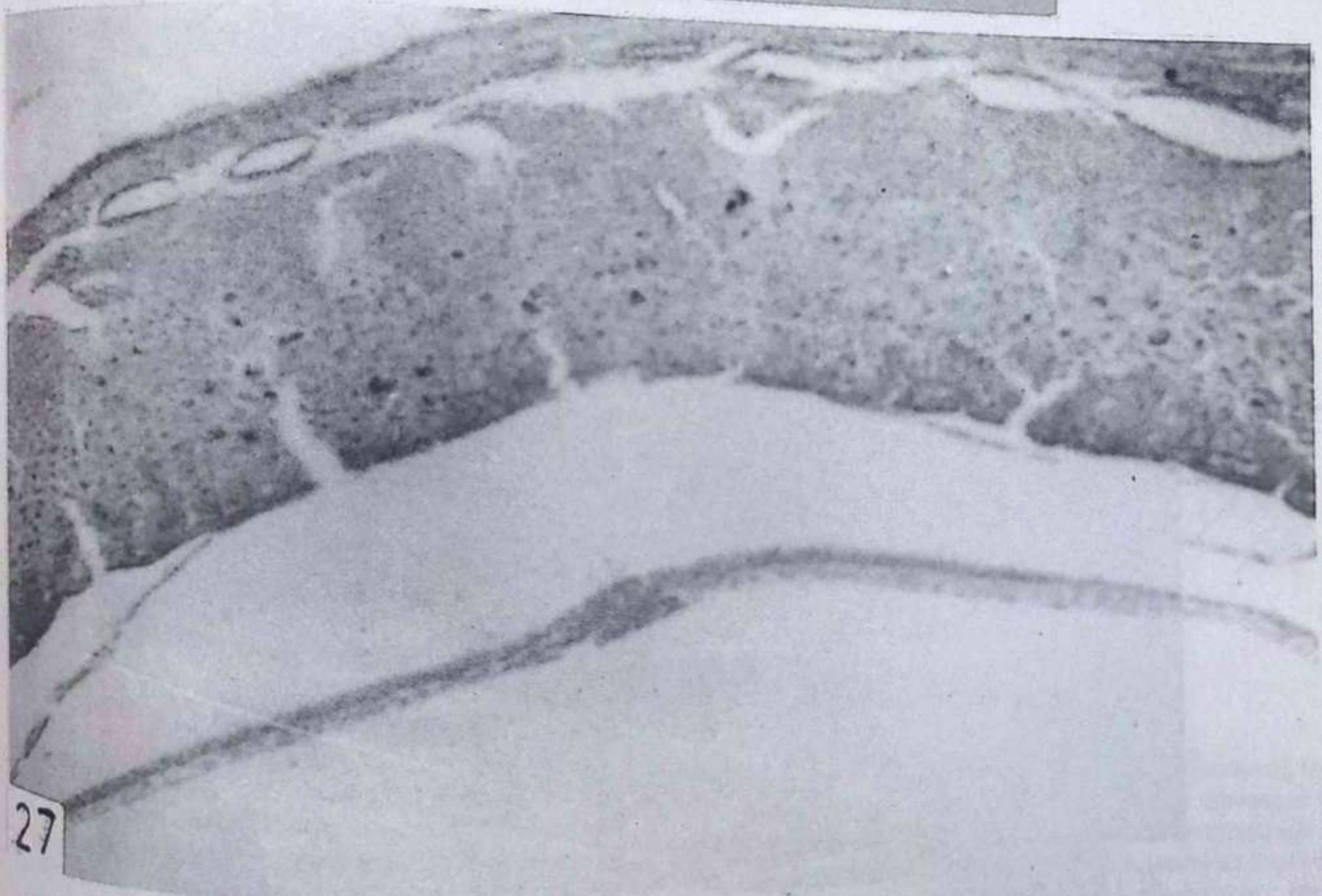
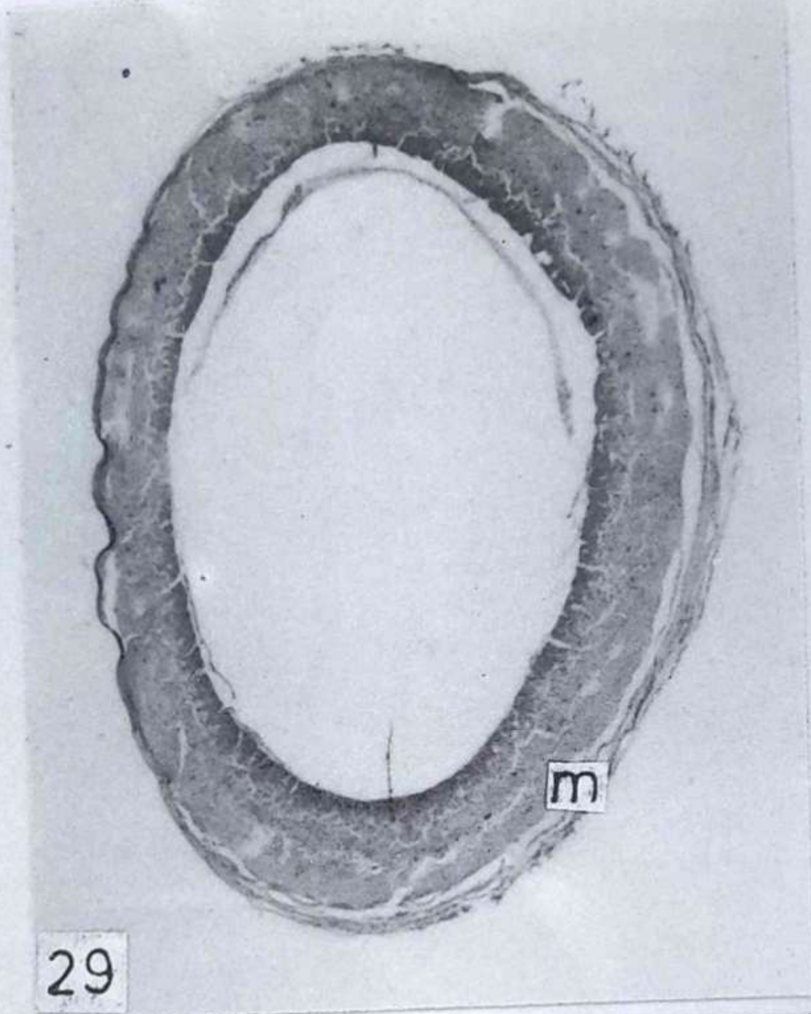


Fig. 26: T.S. of uterus containing a trilaminar blastocyst. The embryonic disc/ epiblast is oriented in between the antimesometrial and lateral side of the uterus. Please see Fig. 28 (Schematic drawing). m - mesometrium. X50 ; **Fig. 27:** Part of the embryonic area of Fig. 26 magnified to show the epiblast composed of compactly arranged cells. Above the epiblast lies the amniotic cavity roofed over by the bilaminar amnion which is in apposition with the trophoblastic placenta. X96.



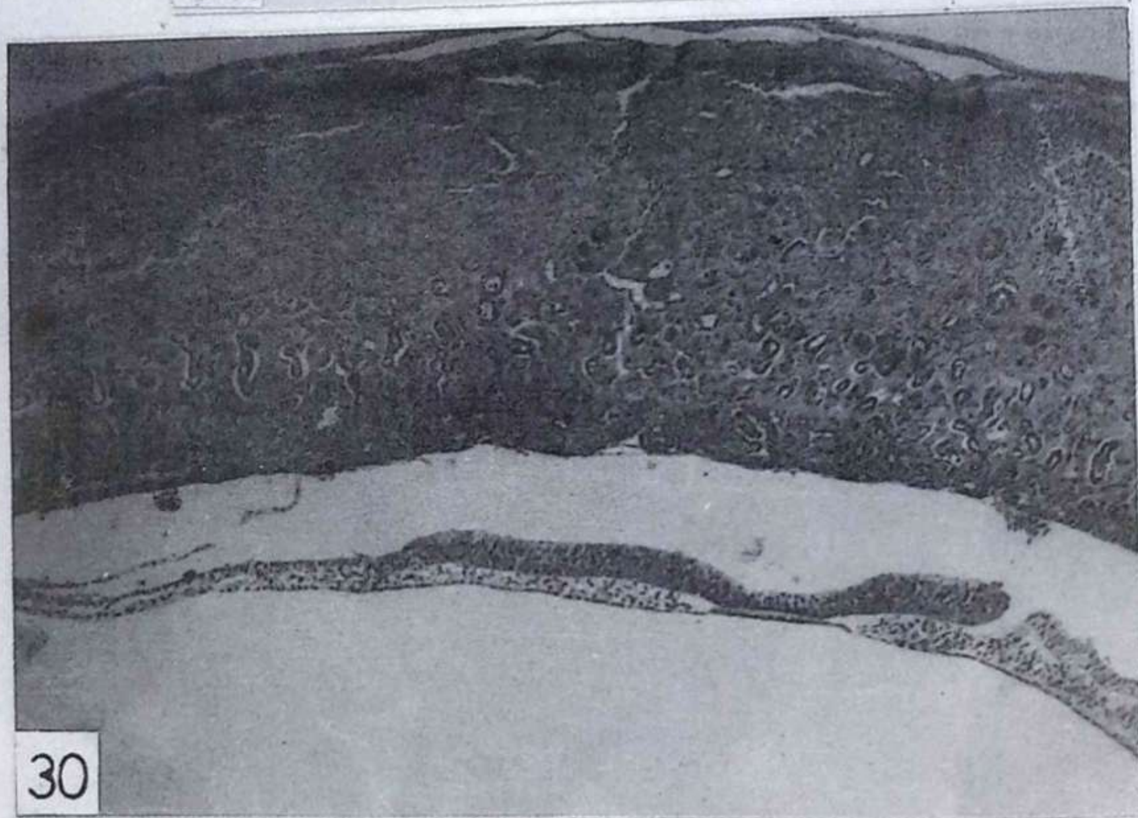


Fig.29: T.S. of the uterus containing an embryo at early primitive streak stage. The epiblast is oriented slightly eccentric to the antimesometrial side of the uterus. Please see Fig. 31 (Schematic drawing), m - mesometrium. X22 ; **Fig. 30:** Part of the embryonic area of Fig. 29 magnified to show the epiblast with a shallow neural groove. Dorsal to the epiblast is the amniotic cavity roofed over by the amnion which is in contact with the trophoblastic placenta. The mesoderm has differentiated in between the epiblast and the endoderm. X112.

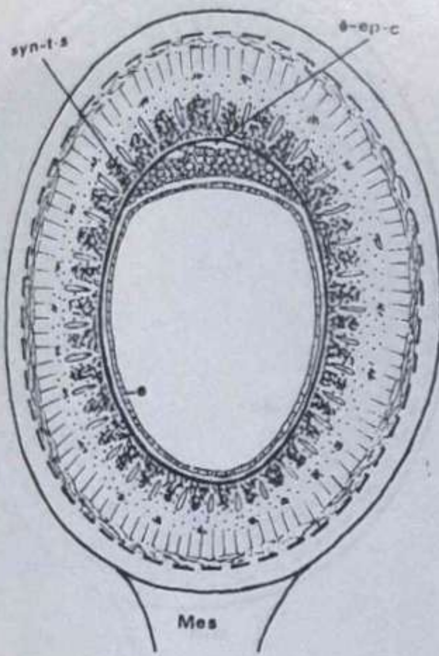


Fig. 14

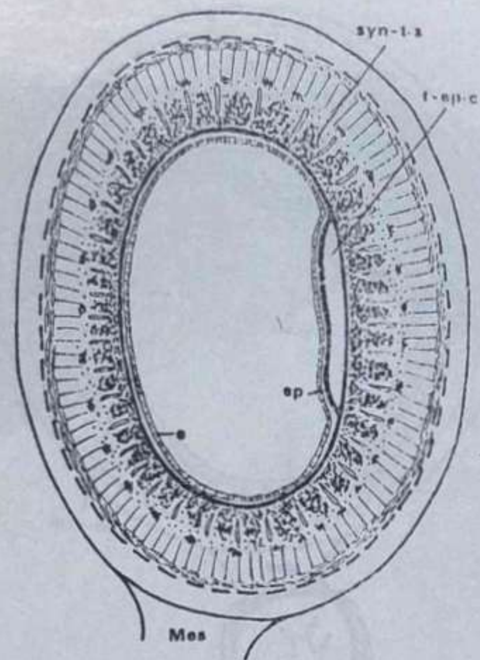


Fig. 17

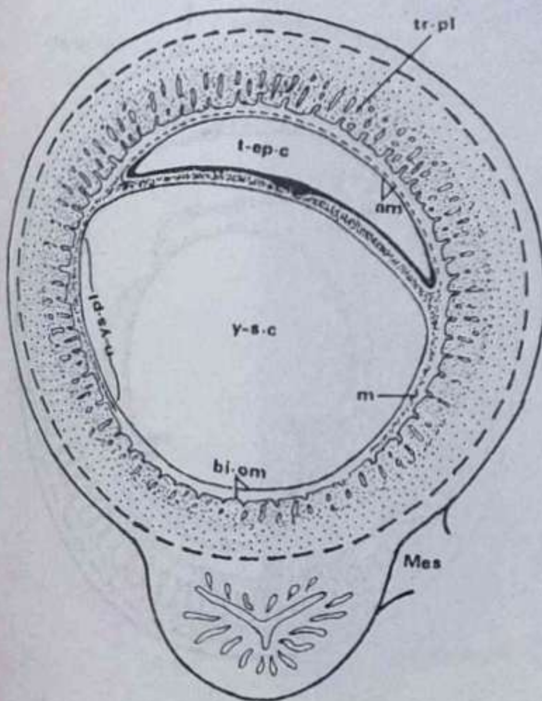


Fig. 28

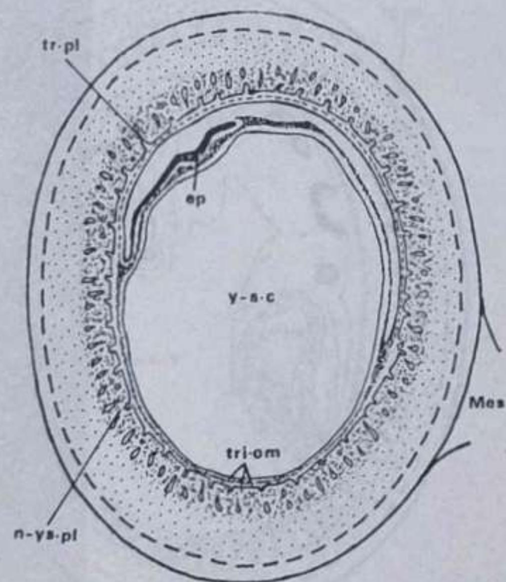


Fig. 31

Fig.14 : Schematic drawing of the implanted bilaminar blastocyst (Hi 118-20 collected on 7-2-88). e: endoderm ; Mes: mesometrium; syn.t.s syncytio trophoblastic shell ; t-ep.c: tropho-epiblastic cavity ; **Fig.17 :** Schematic drawing of the implanted bilaminar blastocyst (Hi 150-9 collected on 12-3-88). e: endoderm ; ep : epiblast : Mes: mesometrium; syn.t.s: syncytio trophoblastic shell ; t-ep.c: tropho-epiblastic cavity ; **Fig. 28 :** schematic drawing of the trilaminar implanted blastocyst (Hi 200-12 collected on 2-4-88). am : amnion; bl.om: bilaminar omphalopleure ; m: mesoderm ; Mes : mesometrium ; n-ys.pl: non-vascular yolk-sac placenta ; tr.pl: trophoblastic placenta, t.op.c: tropho-epiblastic cavity ; y.s.c: yolk-sac cavity ; **Fig. 31 :** Schematic drawing illustrating the arrangement of the foetal membranes at early primitive streak stage (Hi 206-19 collected 6-4-88) ep: epiblast ; Mes : mesometrium ; n-ys.pl: non-vascular yolk-sac placenta ; tr. pl; trophoblastic placenta ; y.s.c: yolk-sac cavity, tri om : trilaminar omphalopleure.

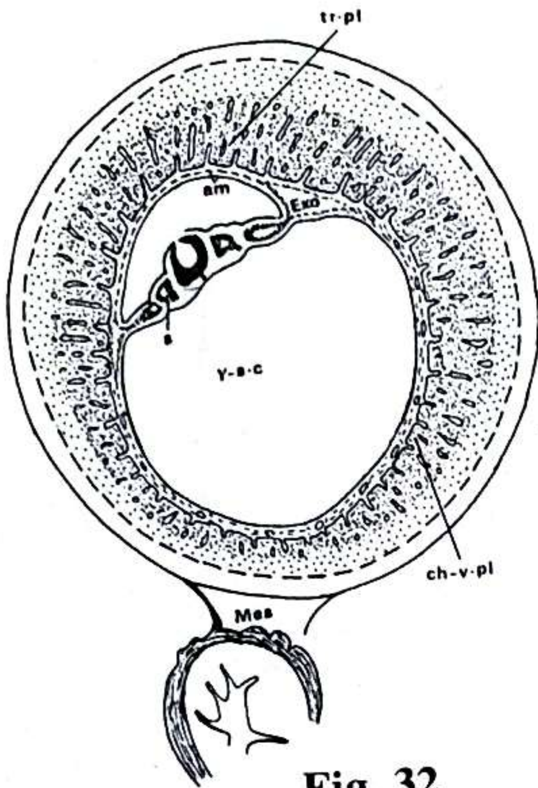


Fig. 32

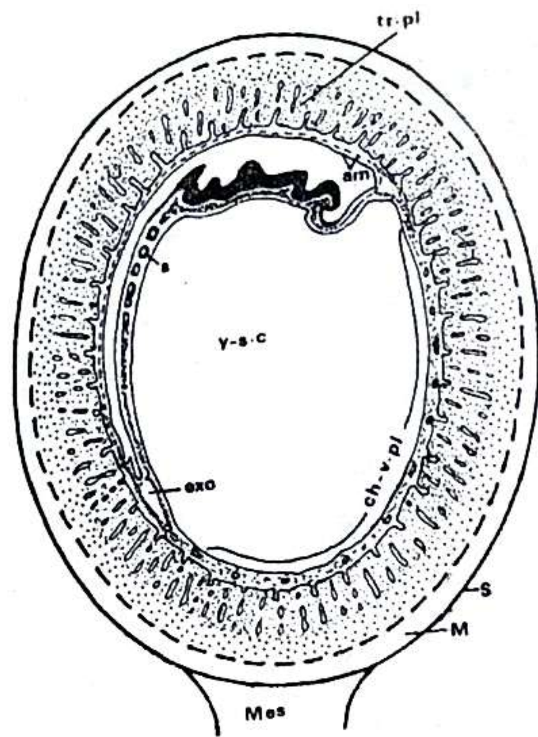


Fig. 36

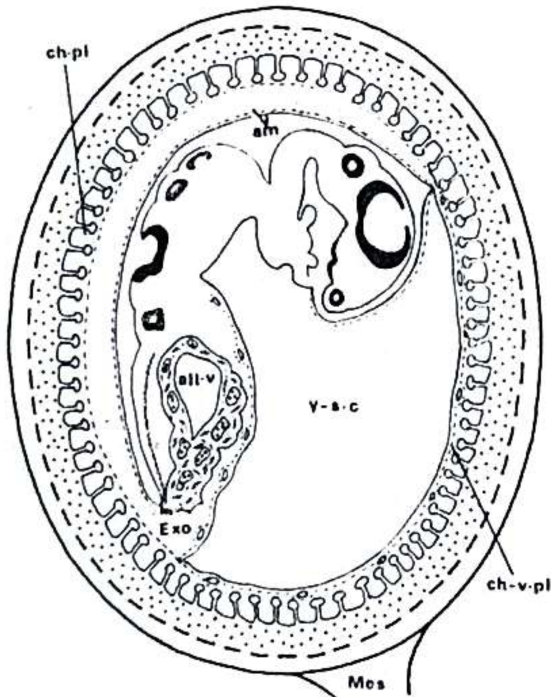


Fig. 38

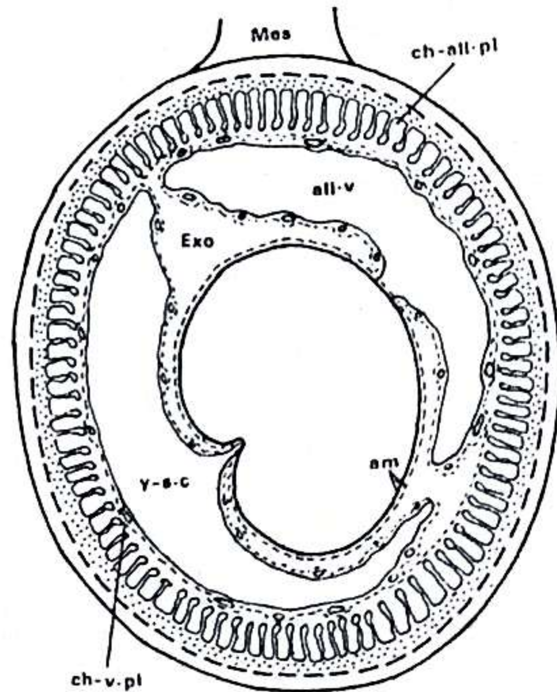
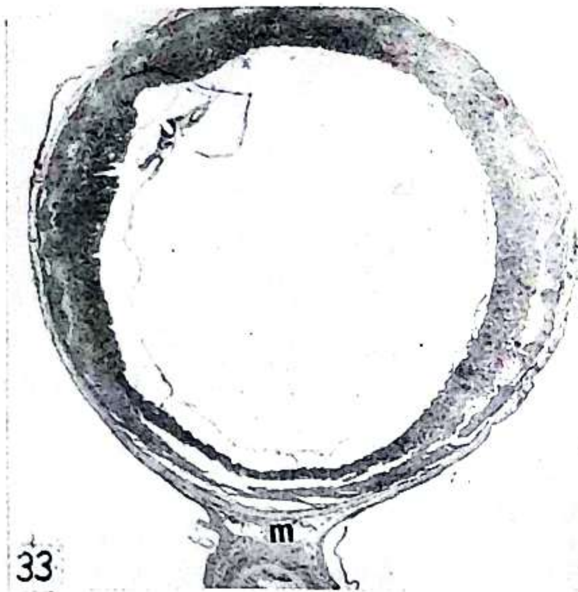


Fig. 40

Fig. 32 : Schematic drawing illustrating the arrangement of the foetal membranes at late primitive streak/somite stage of development of the embryo (Hi 202-24 collected on 6-4-88). am : amnion; ch-v.pl: chorio-vitelline placenta ; exo; exocoelom; M: myometrium ; Mes; mesometrium ; s: somite; tr. pl: trophoblastic placenta; y-s.c: yolk-sac cavity ; **Fig. 36 :** Schematic drawing illustrating the arrangement of the foetal membranes at late primitive streak/somite stage of development of the embryo (Hi 199-23 collected on 6-4-88). am : amnion; ch-v.pl: chorio-vitelline placenta ; exo; exocoelom; M: myometrium ; Mes; mesometrium ; s: serosa; s: somite ; tr. pl: trophoblastic placenta; y-s.c: yolk-sac cavity ; **Fig. 38 :** Schematic drawing to illustrate the arrangement of the foetal membranes at the allantoic diverticulum stage of development (Hi 204-25 collected on 6-4-88) all.v: allantoic vesicle ; am: amnion; ch.pl: chorionic placenta ; ch-v.pl: chorio-vitelline placenta ; exo: exocoelom; Mes : mesometrium. y.s.c: yolk-sac cavity ; **Fig. 40 :** Schematic drawing of the arrangement of the foetal membranes at the early limb-bud stage of development (Hi 203-30 collected on 6-4-88) all.v: allantoic vesicle ; am: amnion; ch-v. pl: chorio-vitelline placenta ; ch-all.pl: chorio-allantoic placenta ; exo: exocoelom ; Mes : mesometrium. y.s.c: yolk-sac cavity.



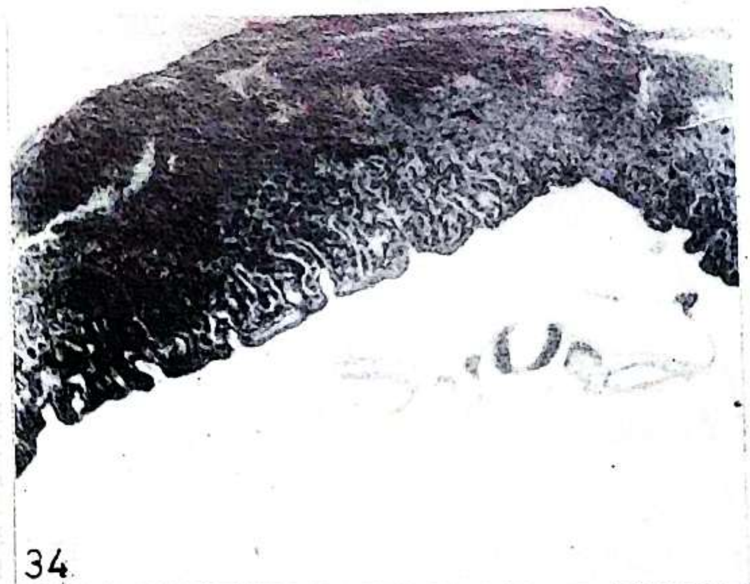
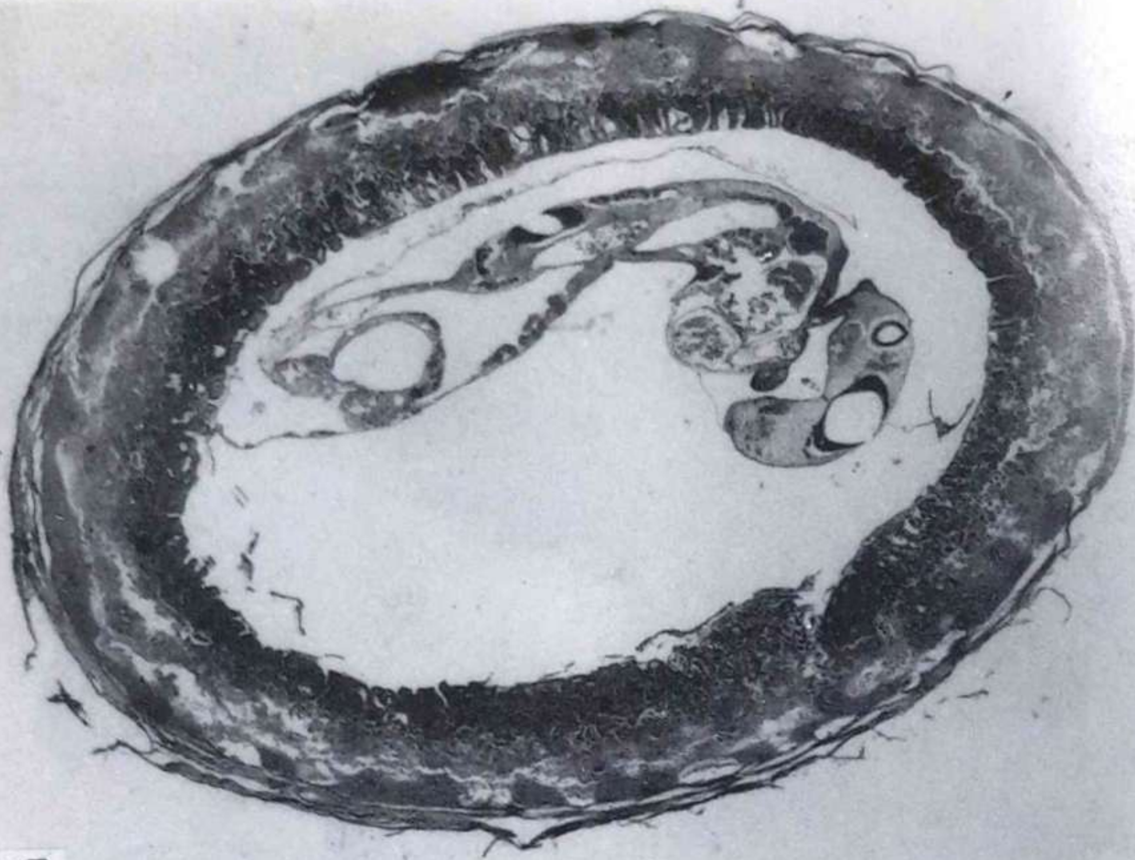


Fig. 33: T.S. of the uterus containing an embryo at late primitive streak stage. The epiblast is oriented in between the antimesometrial and the lateral side of the uterus. Please see Fig. 36 (Schematic drawing). X60 ; **Fig.34:** Part of the embryonic area of Fig. 33 magnified to show a deep neural groove in the epiblast. The amniotic cavity above the epiblast roofed over by the amnion which has peeled away from its contact with the trophoblastic placenta. The mesoderm below the epiblast shows the formation of somites. Also the mesoderm has split to form the exocoelom. X112 ; **Fig. 35:** Part of the well developed trophoblastic placenta at the late primitive streak/somite stage showing the placental tubules hanging from the uterine wall. Note the increase in the number of maternal capillaries in the syncytiotrophoblastic shell. X128



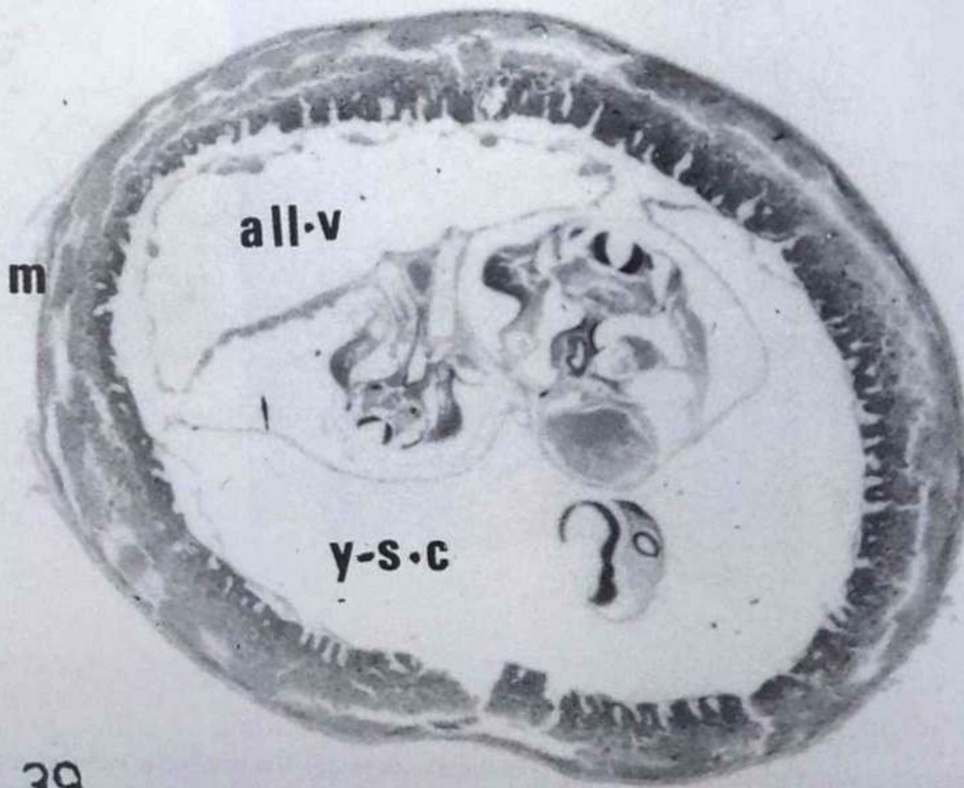


PLATE X

Fig. 37: T.S. of the uterus carrying an embryo at the allantoic diverticulum stage. Please see Fig. 38 (Schematic drawing). X60 ; **Fig. 39:** T.S of the uterus carrying an embryo at the early limb-bud stage of development. Please see Fig. 40 (Schematic drawing). m - mesometrium, all. v - allantoic vesicle, y-s.c - yolk-sac cavity. X50.

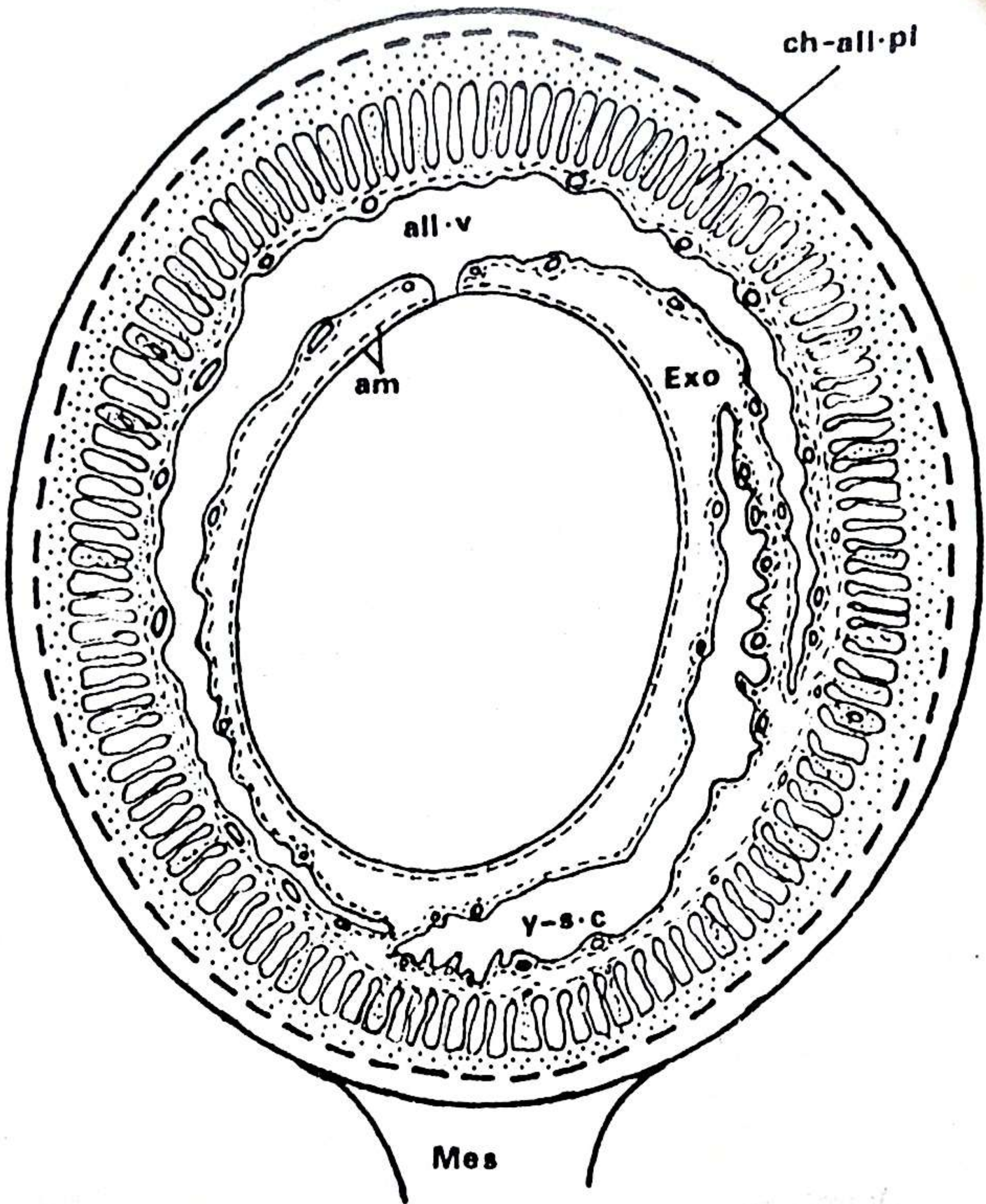


Fig-41

Fig. 41 : Schematic drawing illustrating the arrangement of the foetal membranes at the late limb-bud stage of development of the embryo (Hi 153-25, 35 collected on 2-4-88). all.v: allantoic vesicle ; am: amnion ; ch-all. pl: chorio-allantoic placenta; exo: exocoelom ; Mes: mesometrium : y.s.c. : yolk. sac cavity.

In *Miniopterus schreibersii*, Peyre and Herlant (1967) observed that the CL is poorly developed throughout delayed implantation of 5 months which coincides with hibernation. An arrest in LH secretion after ovulation together with insufficient PRL is responsible for delayed implantation. In the Japanese long-fingered bat, *Miniopterus s. fuliginosus* (Kimura *et al.*, 1987) the plasma progesterone concentration was not significantly elevated during developmental diapause/delayed implantation which occurs before the bats enter hibernation. But the progesterone concentration was significantly lower during retarded embryonic development after the implantation which occurs during hibernation and rose significantly during the rapid embryogenesis that occurs after arousal from hibernation. Changes in CL volume corresponded closely with those of plasma progesterone values. Van der Merwe and van Aarde (1989) have observed that in *Miniopterus s. natalensis* the plasma progesterone concentration increased significantly during the period of delayed implantation. Values peaked at implantation but decreased thereafter. Concentration remained low (60 ng/ml) during initial period of foetal development and attained peak values (85.6-181.3 ng/ml) 216- 222 d after fertilization which coincided with significant increase in placental weight.

Khan (unpublished) has observed that the CL in *Hipposideros l. lankadiva* is well developed during the preimplantation stages of development of the embryo and during implantation of the blastocyst. The plasma progesterone level is high i.e. 34.6 ng/ml in the pre-implantation stage and 40.9 ng/ml after implantation. There is slight reduction in the size of the CL during retarded embryonic development and the plasma progesterone level decreased (12.6 ng/ml). By mid-March, the arousal period of bats from semi-torpor, there is no appreciable increase in the size of the CL but the level of progesterone increases (41.6 ng/ml). After arousal, the embryonic development is accelerated and by second week of April advanced stages of development were observed. The chorioallantoic placenta becomes fully established at the late limb bud stage and the CL regresses. At advanced pregnancy no CL is present but the level of progesterone is high (46.0 ng/ml) which is due to a well developed and functional chorioallantoic placenta. At term, the level of progesterone decreases (12.0 ng/ml).

To sum up, we emphasize that the secretion of ovarian (CL) hormone is controlled by that of the pituitary which in turn is under the influence of the hypothalamus. Thus, the hypothalamus-pituitary-ovarian axis needs further study. In conclusion we agree with Mead (1993) that the phenomenon of embryonic delays in Chiroptera still holds mysteries for scientists to solve.

Table 1: Details of bulb size correlated with various stages of development.

Sl.No.	Animal No.	Date of Collection	Stage of Development	Bulb size (mm)
1	H1170	24.08.88	Uni Bl(+ZP)	1.5 x 1.2
2	H1171	24.08.88	Uni Bl(+ZP)	1.4 x 1.2
3	H1177	09.09.88	Uni Bl(-ZP)	1.8 x 1.2
4	H1179	09.09.88	Uni Bl (-ZP)	1.8 x 1.5
5	H1181	14.09.88	Impl Bi Bl	1.9 x 1.5
6	H123	20.09.88	Impl Bi Bl	2.0 x 1.5
7	H126	20.09.86	Impl Bi Bl	1.9 x 1.5
8	H129	20.09.86	Impl Bi Bl	2.0 x 1.6
9	H161	27.09.87	Impl Bi Bl	2.0 x 1.6
10	H162	27.09.87	Impl Bi Bl	2.1 x 1.8
11	H170	10.10.87	Impl Bi Bl	2.0 x 1.8
12	H171	10.10.87	Impl Bi Bl	2.0 x 1.8
13	H178	27.10.87	Impl Bi Bl	2.1 x 1.7
14	H1	06.11.95	Impl Bi Bl	2.1 x 2.00
15	H185	04.12.87	Impl Bi Bl	2.0 x 1.8
16	H1100	25.12.87	Impl Bi Bl	2.0 x 1.8
17	H1113	17.01.88	Impl Bi Bl	2.5 x 1.8
18	H1185	25.01.89	Impl Bi Bl	2.5 x 1.8
19	H1116	07.02.88	Impl Bi Bl	3.0 x 2.0
20	H1118	07.02.88	Impl Bi Bl	3.0 x 2.0
21	H1396	03.03.92	Impl Bi Bl	5.0 x 4.0
22	H1132	12. 03.88	Impl Bi Bl	6.1 x 5.0
23	H1149	12.03.88	Impl Bi Bl	6.0 x 5.0
24	H1150	12.03.88	Impl Bi Bl	6.1 x 6.0
25	H1153	02.04.88	Late limb bud stage	9.0 x 8.1
26	H1194	02.04.88	Advanced preg.	9.0 x 8.2
27	H1200	06.04.89	Trilaminar Bl	6.0 x 5.0
28	H1202	06.04.89	Late primitive streak stage	7.0 x 6.2
29	H1203	06.04.89	Early limb bud stage	8.0 x 6.2
30	H1204	06.04.89	All diverticulum stage	8.0 x 7.1
31	H1206	06.04.89	Primitive streak stage	7.0 x 5.6
32	H1220	21.04.90	Advance preg.	27.0 x 17.1
33	H1227	21.04.90	Advance preg.	18.2 x 12.4
34	H1238	23.05.90	Term preg.	41.2 x 22.2
35	H1243	23.05.90	Term preg.	42.6 x 24.2

Bi - Bilaminar ; **Bl** - blastocyst; **Impl** - implanted; **preg** - Pregnancy; **Uni** - unilaminar

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COMPENSATORY HOMEOTIC REGENERATION OF EYE IN AMPHIBIAN TADPOLE

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ABSTRACT

Lens regeneration provides a clear example of transdifferentiation of one differentiated cellular type having a distinctive pattern of metabolic activities to another cellular type, which is morphologically distinct from the original one. In the present study, it was found that after removal of both lateral eyes of external gill stage tadpoles, the pineal organ gets transformed into the median eye. The eye ball of the transferred median eye was almost spherical containing cornea, well developed lens and photosensitive cells in the retina.

Key words : Lens regeneration, amphibian tadpoles

INTRODUCTION

Lens regeneration has long been studied as an exceptional example of metaplasia in which previously specialized tissues change into other types. The lens is differentiated from the iris epithelium after lentectomy in urodeles and anuran tadpoles (Stone, 1959; Reyer, 1971; Yamada, 1972; Swami, 1992). Similar observations regarding transdifferentiation or homeotic regeneration have also been reported by Mohanty-Hejmadi *et al.*, (1992) and Mahapatra and Mohanty-Hejmadi (1994) in amphibian tadpoles. They observed not only regeneration of the original structure but also the transformation of the amputated tail of the tadpole to limbs by exposing them to vitamin A. Maden (1993) confirmed these results by using a European frog *Rana temporaria*. Das and Dutta (1996) also gave further evidence of homeotic transformation of supernumerary limbs at the site of tail amputation in anuran tadpoles. These observations motivated the present study with the aim to extend further the knowledge of homeotic regeneration of different organs. In the present study it was found that a new median eye developed from the pineal organ after removal of normal eyes at external gill stage in *Bufo melanostictus*.

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MATERIALS AND METHODS

Bufo melanostictus tadpoles were reared in glass aquaria and were fed on half boiled spinach leaves. Tadpoles of external gill stage were anaesthetized in 1:400 MS 222 and fine oblique cut was made to remove the anteriormost part of the brain including both optic vesicles and keeping the oral armature intact. The operated tadpoles were reared in tap water. Following operation, 70 tadpoles were preserved in Bouin's solution at different time intervals. Experiments were terminated at the end of day 7 after operation.

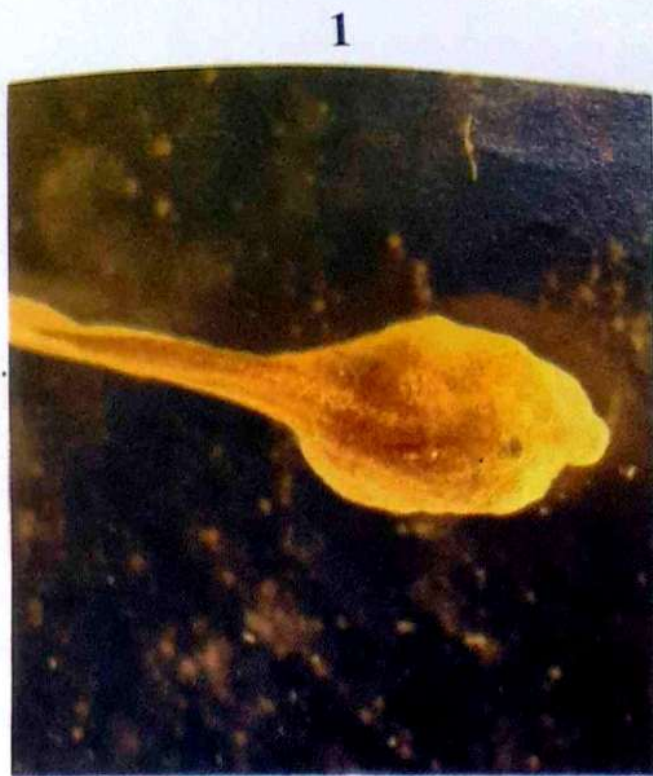
RESULTS

The observations of present study are presented in Table 1. After amputation of both optic vesicles proturbance like structures appeared in the centre of cephalic region. The proturbance later on developed into the median eye in 37 cases out of 70 (Figs. 2 & 3). In 5 out of 70 operated tadpoles fused median eyes (Fig. 4) and in 4 out of 70 operated tadpoles two frontal eyes were observed. In these tadpoles paired eyes developed in normal way but they were arranged closely located in the centre of cephalic region. Histological study revealed that the median eyes were similar to that of normal eyes (Fig. 4). These eyes have normal size with spacious vitreous chamber, well differentiated iris and lens with well-developed crystalline fibres. A spacious aqueous chamber is bound externally by the cornea. The neural retina is also well differentiated. The ganglion cells are arranged in single row on the inner margin. The ganglion layer is separated from inner nuclear layer by inner plexiform layer. The visual cells are also differentiated and thus the transformed organ looks as good as a functional eye. In few cases stalked median eye developed.

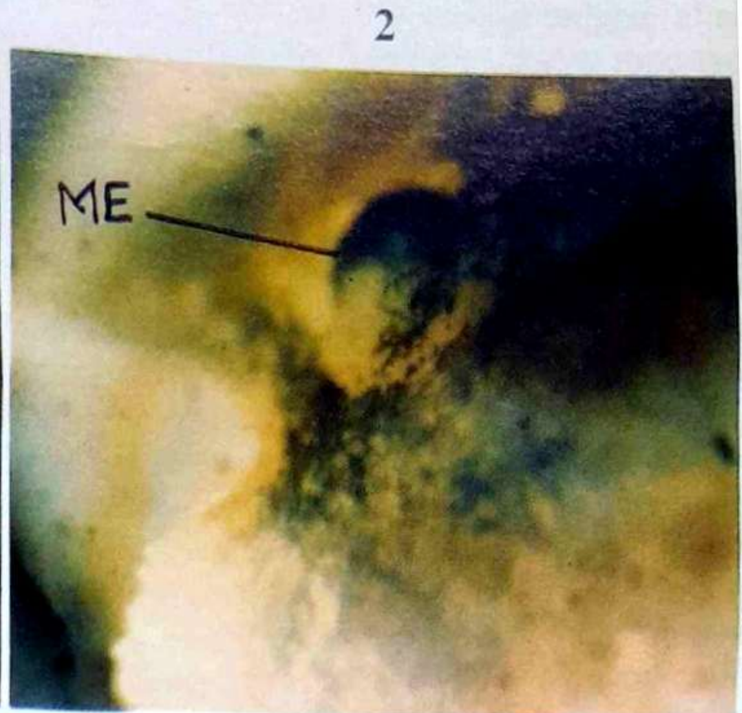
Table 1 :Transdifferentiation of pineal organ into median eye in *Bufo melanostictus*

Time after amputation	Total number of tadpoles	Trandifferentiated Median-eye		Fused	Two frontal eyes	Undifferentiated structure	Percentage
		Median protuberance	Median eye				
6 hrs	20	12[1]	-	-	02	06	70
Day -1	15	02	10	02	-	01	93.33
Day - 3	15	-	12	01	01	01	95
Day - 7	20	-	15	02	02	01	95

1.Number of tadpoles



1



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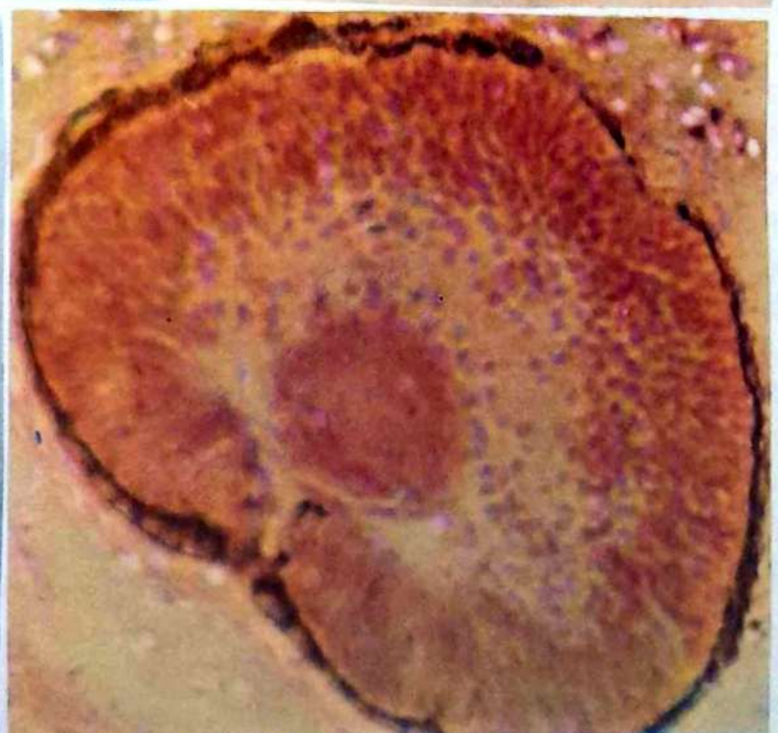

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Fig. 1 : Post-operated tadpole on day 7 showing well developed median eye ; **Fig. 2** : Close-up of the Median eye (ME) **Fig. 3** : Post-operated tadpole on day 7 showing fused medium eye (FE) ; **Fig. 4** : Transverse section of post-operated tadpoles on day 7 showing well developed median eye with normal lens.

DISCUSSION

The exact cause of transdifferentiation of pineal gland into median eye is not clear but a complete median eye developed at the site of brow spot of normal tadpole after removal of both lateral eyes in the external gill stage tadpoles. Holtfretor (1954) reported homeotic regeneration in urodeles. Recently, Mohanty-Hejmadi *et al.*, (1992), Mahapatra and Mohanty-Hejmadi (1994) have extended their findings to many Indian species of frogs. In their findings they observed the transformation of tail into limbs after vitamin A treatment. Maden (1993) succeeded in confirming these results in the European frog, *R. temporaria*.

In the present study, it was observed that the pineal eyes had normal eye components like lens, retina, choroid etc. A new structure, the stalk was also observed in these developing eyes. In some cases two stalked eyes developed in place of the pineal organ in amputated tadpoles. Trasdifferentiation during lens regeneration has been studied by several workers (Dumont and Yamada, 1972; Swami, 1992; Shekhawat, 1998). Removal of the lens initiates profound changes in the cells of the surrounding pigmented iris epithelium. They transform into new cell phenotype and synthesize lens proteins instead of pigment to regenerate a new lens. Biochemical study also indicates that various tissues like retina, cornea and iris have one or more antigenic components identical with lens antigens. This suggests that under normal conditions antigens identical with lens antigens are made by the cells and integrated into their structure. Under abnormal conditions, such as removal of the original lens in the embryo, the normal equilibrium in the cells of iris, pigment layer of the retina, and cornea may be disturbed and the lens antigens may become building stones for the formation of lens cells. Indeed, pigment layer of the retina, iris and cornea, which contain antigens identical with those of the lens, have the ability to form a lens. Histological study revealed that the pineal sacs develop by invagination of the diencephalic roof. The ciliated cells and supporting cells of the pineal gland become specialized to develop into photoreceptors. They show the same general plan as the paired eyes, but with no differentiated lens of dioptric apparatus. In recent years, melatonin binding sites have been demonstrated in the cells of pineal gland as well as in retinal cells and photoreceptors of retina and pinealocytes of the pineal gland share common components of signal transduction (Lolley *et al.*, 1992). Looking at these similarities, it can be speculated that removal of both lateral eyes might be a causative factor to transdifferentiate pineal gland into a complete median eye. It may be possible that mesenchymal cells of this cephalic region particularly eye field region might have the inductive ability and induce the epidermal cells to transform into the complete median eye.

Transdifferentiation capacity is found to be stage dependent. In the present experiment it has been found that only early stage tadpoles (external gill stage tadpoles) showed the transdifferentiation of pineal gland into median eye whereas the same result was not found in late stage tadpoles (Unpublished results).

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BREEDING AND DEVELOPMENT OF *POLYPEDATES MACULATUS* (ANURA: RHACOPHORIDAE)

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ABSTRACT

Polypedates maculatus is a seasonal breeder, the breeding occurring only in the rainy season. Pre-mating congregation is seen near breeding sites and the species is a solitary breeder. Egg laying takes place at night and also in the absence of amplecting males; the females construct foam nests and lay unfertilised eggs. The eggs are laid inside these foam nests. The foam is either circular or semicircular and the eggs are white, not allowing morphological identification in early developmental stages. The clutch size is 107-678 and hatching success is 90%. The species possesses two types of hatching stages; primary hatching (late tail bud inside foam) and secondary hatching stage (external gills stage when embryo hatches into water). Within 4 d of egg laying, the embryos hatch. The growth of tadpoles is typical of other anurans and the life history is completed within 40 d of egg laying. A total of 13 developmental stages have been identified and their characters analysed.

Key words: *Polypedates maculatus,* breeding, development, clutch size.

INTRODUCTION

The Indian tree frog, *Polypedates maculatus,* is a widely distributed rhacophorid. As is typical of most tropical anurans, the species is a seasonal breeder (Rath, 1994), the breeding activity being confined to the monsoon period (May-August). As interpreted by Duellman (1989), the reproductive strategy of anurans is an adaptation to local environmental conditions. Rhacophorids avail the opportunity of transitional aquatic

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habit during the monsoons and desiccation is a major factor for reduction of populations in tropical climates (Mohanty-Hejmadi and Dutta, 1988). Construction of foam nests by rhacophorids has been reported by Ikeda (1897), Okada (1928), Bhaduri (1932,1953), Mallick *et al.*, (1980), Mallick and Mallick (1982) and Mohanty-Hejmadi and Dutta (1988). Studies on the breeding habits of rhacophorids is very scanty. The genital organ is correlated with breeding habits, specifically with the formation of the foam nest. The female urinogenital organ is modified for this peculiar breeding habit (Bhaduri, 1932). Studies by Bhaduri (1932) report the shape and size of testis and their relationship to body size in several anurans. Studies by Spengel (1976) and Noble (1927) reported on ovarian lobes and their relationship to egg size. Another important aspect of breeding output of anurans is clutch size which is variable both at inter-and intraspecies level. Ratio of clutch size to body weight serves as a useful approximation of reproductive effort (Kuramoto, 1978). There are few publications reporting reproductive pattern and clutch size of rhacophorids (Villadolid and Rosario, 1930; Kiyasetuo and Khare, 1986 in *P. leucomystax;* Morgan-Davis, 1958 in *P. cruciger;* Alcala and Brown, 1982 in *Rhacophorus appendiculatus;* Mishra and Dash, 1984a; Mohanty-Hejmadi and Dutta, 1988 in *P. maculatus*). The present paper deals with the reproductive efforts *P. maculatus* in terms of behaviour, clutch size, development and larval growth.

MATERIALS AND METHODS

The breeding sites were examined during rainy nights to study the mechanism of foam nest construction. Amplecting pairs were observed at the site of oviposition. Nesting sites were monitored during one breeding season to find the site of foam nest construction. Various parameters (call of males, time of amplexus and egg laying) were considered to analyse the breeding and developmental strategies. To determine the shape and size of gonads mature males and females were sacrificed, the gonad removed and preserved in 4% formaldehyde for measurement and photography. A total of eight males and nine females were used for the study. Amplecting pairs and foam nests were collected from the wild. The snout-vent length (SVL) of amplecting pairs was measured. The eggs were removed from the nest using the method of Coe (1974) and counted. Hatching success was calculated on eggs collected from the wild and also obtained in the laboratory. Various stages (fertilised eggs through metamorphosed froglets) were preserved from a single clutch of egg, reared in the laboratory and the time interval of development was recorded. Each stage was measured in millimetre

(mm) either by a micrometer or a scale. Morphological characters were considered for description of developmental stages. The length of each stage was correlated with the time interval of development. Life history was analysed using the time intervals of development from egg laying to completion of metamorphosis.

A total of 13 developmental stages were identified from a single clutch reared in the laboratory. As the eggs are found embedded within the foam, 10 embryos of each stage were removed and the time period of development (age) recorded. Accordingly, embryos until primary hatching (late tailbud sage) were removed from the foam and once the embryos were ready to lead independent life (external gill stage), they were collected from the rearing medium for further study. As the eggs are white, it was not possible to identify early developmental stages (two-cell through morula). Hence, several early stages are missing in the study. Further, most of the important larval stages which are differentiated by age, size and external morphological characters, have been included in the study.

RESULTS (Tables 1-3)

Breeding strategy : The species is a seasonal breeder and breeding starts before the onset of monsoon (April) and ends during late monsoon (September). Two types of call can be heard at night. Fully inflated vocal sac produces a call that can be synthesised as a "tak-tak" and partially inflated vocal sac produces a "dodo-dodo" sound. The intermittent call of 30 seconds duration continues for over an hour. The species is quiet when not engaged in courtship and produces a "tak-tak", the former type of call. The second type call is produced during courtship. The frequency of this call reaches its peak when a female is approached by a male frog or in male congregations. Pre-mating congregation involves a group of males congregating at a breeding site and producing a high pitched buzzing note continuously. The females are also found sitting silently at the same locality. Any male from the congregation enters into amplexus with a female and there is no mating combat. Spawning site includes leafy twigs, overhanging water, grassy banks of pond, under stones and also on tree trunks. Occasionally, foam nests are found attached to each other and such nests are laid by several females on different nights.

At the time of construction of the foam nest, the female produces a jelly like substance without eggs. During this process, the vent of the female is widened by the toe of the male. Jelly substance is beaten to a foam by the hindlimbs of the female. The shanks remain crossed after the completion of this process. Foam is produced by crosswise movement of the female's feet. Eggs are released gradually; only a few at a time and at short intervals. Males are less involved in the nest construction. With its toes, male clears the vent of the female when the foam nest construction is completed. The nest is usually constructed during mid-night and sometimes egg laying continues till the morning. Generally, the foam nests are globular, but the shape varies with the nature of the substratum. The diameter of the foam varies from 95 mm to 120 mm. Occasionally during the breeding season, gravid females lay unfertilised eggs in the absence of amplecting males. Examination of ovaries of such females indicate no mature eggs, except developing oocytes. This suggests that the non-amplecting females, when ready to ovulate, deposit all the mature eggs. The amplecting females also release all mature eggs at the same time. In addition, the examination of ovaries of such females reveal similar result (released all the eggs) like that of non-amplecting females observed in nature. To verify the egg laying and foam formation by non-amplecting females, captive experiments suggested that, in the absence of males, gravid females also lay eggs.

Clutch size: Of 63 foam nests collected from the wild, the lowest number of eggs in a clutch was found to be 107 and the highest 678, with an average of 337.2. The SVL of amplecting females ranged from 42.5-47.0 mm and the average was 44.0 mm. Similarly, the SVL of amplecting females ranged from 60-69 mm, with an average of 63.7 mm. The SVL of amplecting males and females indicate no correlation (4 males, all with SVL of 46.0 mm were found in amplexus with females measuring 63.0, 68.0, and 65.0 mm). Of 20 egg clutches obtained from amplecting females, the maximum and minimum clutch sizes were 228 and 415 and the average was 357.5. Correlation between SVL of female and clutch size was significant. In comparison with clutches obtained from breeding grounds, the average was lower (337.2) for the clutches obtained from breeding grounds. However, the highest number of eggs (678) was counted from a clutch obtained from breeding grounds. This variation in clutch sizes of amplecting females (from which eggs were obtained in the laboratory) and field collected clutches, is probably due to variations in the number of clutches counted for both types of foam nests.

Hatching success: Out of seven clutches, the minimum and the maximum spoiled eggs were 9 and 34 respectively. Similarly, the minimum and the maximum numbers of eggs hatched were 299 and 475 respectively. The maximum hatching success (97.07%) was found with an egg clutch containing lowest number (308) of eggs. However, the maximum clutch size (509) had less hatching success (93.32%) than the minimum clutch size (308), which also had the maximum hatching success (97.07%). This suggested no correlation between clutch size and hatching percentage. The largest number of spoiled eggs were found appeared to be on the superficial layer of the foam nest, and such eggs being were subject to desiccation.

Development: Egg laying starts during the pre-monsoon period and the development is dependent on environmental temperature. Field studies indicate constraints associated with development of early developmental stages. One of the major constraints was the destruction of foam nests by rainfall. Occasionally, the foam nests are laid away from water and such foam nests are washed away due to excessive rain. If there is no rainfall after egg laying, the temporary rainwater pools close to the foam nests are dried off and the embryos can not escape into water. Subsequently, they die due to desiccation. The different developmental stages in *P. maculatus* are :

1. Fertilised egg (1.3-1.5 mm) : The eggs are white, hence the animal and vegetal poles are not distinguishable externally. As the eggs were deposited in the foam, which was formed while egg laying continued, several eggs were found on the surface layer of the foam.

2. Early tail bud (2.5-3.0 mm) : Within ca 28h of egg laying the eggs are transformed into early tailbud stage. During this stage, the developing embryo is seen semi-circular in shape, the other half being occupied by unusually large amount of yolk. Like the egg, early tailbud stage is also white.

3. Mid tailbud (3.0-4.3) : There is complete differentiation of head, tail and abdomen of the embryos. In addition, within ca 20h, early tailbud is transformed into mid tailbud stage.

4. Late tailbud (Primary hatching) (4.3-5.0 mm) : Within ca 8h, mid tailbud is transformed into late tailbud or primary hatching state. Embryos slightly larger than

previous stage due to increase in length of tail. Abdomen is elongated and tapering towards developing cloaca. First movement of embryo designated muscular movement stage. Primary hatching stage white.

5. External gill (Secondary hatching) (5.0-5.5 mm) : Embryos generally hatch out. During unfavourable conditions (no possibility of emerging from foam), embryos develop further (until feeding or operculum complete stage) inside foam. Pigmentation develops.

6. Operculum complete (7.5-10.1 mm) : Within ca 5 d of egg laying, embryos are transformed for feeding. Mouth opening completely re-structured and intestinal coiling develops. Cloaca develops as a small tube. Pigmentation more developed. Tail musculature and fin prominent. Occasionally found inside foam.

7. Feeding stage (10.1-11.2 mm) : Beak prominent and with well defined keratinized teeth rows and oral papillae. Intestinal coiling more developed. Within ca 6 d of egg laying, embryos start feeding.

8. Hind limb bud (18.2-19.5 mm) : Two prominent morphological variations observed between feeding and hind limb bud stages (which is ca 7.0-8.3 mm greater in hind limb bud than feeding stage) and development of hind limb in the form of a white round but at the base of cloacal tube.

9. Pre-metamorphic (24.6-26.2 mm) : Within ca 22 d of egg laying, embryos are transformed into tadpoles with hind limbs comprising of toe pads and webs. Fore limbs seen through transparent opercular area. Spiracular opening on left side of head, in the form of a small tube. Dorsum and tail fin deep brown pigmented.

10. Well developed hind limb (Pro-metamorphic) (39.6-41.6 mm) : Tadpole attains maximum length and growth exponential within ca 35 d of egg laying. No external morphological variations between pre-and prometamorphic stages, except the size.

11. Both limbs and tail (41.6-41.8 mm) : Emergence of both fore limbs. Left fore limb emerges through spiracular opening and within ca 6h, right forelimb emerges by rupturing opercular fold. Marks initiation of metamorphic events, including tail resorption.

12. Metamorphosing (41.0-18.2 mm) : Decrease in size of metamorphosing larvae. Shows tail loss after emergence of forelimbs. One of the characteristics is their ability to change colour. Once out of water, they change their colour from brown to yellowish-

white. During Gosner Stage 45, reduced tail a deep brown rounded stump at base of cloaca.

13. Metamorphosed froglet (15.3-16.1 mm) : Within ca 40 d of egg laying, larvae transformed into tiny frogs. From Gosner Stage 42 (both limb and tail) till Stage 46, metamorphosing larvae take ca 4 d for complete resorption of tail.

DISCUSSION

The breeding habits and reproductive strategy of the species *P. maculates* is typical of rhacophorids (Coe, 1974). The breeding period coincides with the monsoons which is characteristic of several other Indian anurans (Agarwal and Niazi, 1977, Dutta and Mohanty-Hejmadi, 1976, 1978; Dutta *et al.*, 1993; Mohanty-Hejmadi *et al.*, 1979a, 1979b; 1980; Platt, 1986; Roy and Khare, 1978). However, a few ranids such as *Euphlyctis cyanophlyctis* and *Limnonectes limnocharis* have the ability to breed round the year (Mohanty-Hejmadi *et al.*, 1983; Mohanty, 1994). The species has a prolonged breeding period simlar to that of *Ramanella variegata, Uperodon systoma, Tomopterna rolandae* and *Microhyla ornata* (Mohanty-Hejmadi *et al.*, 1979; Mohanty-Hejmadi *et al.*, 1980; Dutta *et al.*, 1991). The construction of foam nest in *P. maculatus* agrees with the observation made by Ikeda (1897) and Morgan-Davies (1958). However, formation of foam nest and laying of unfertilised eggs by non-amplecting females are unusual phenomena. As reported by Heyer and Rand (1977), some leptodactylid frogs also construct foam nests, but there are differences in the pattern of foam nest formation between *P. maculatus* and leptodactylid frogs which might be due to inter-familial difference. Clutch size of the species is dependent on body size. Terentjev (1960), Salthe (1969), Salthe and Duellman (1973), Salthe and Mecham (1974) and Matsui and Ota (1984) reported a positive or negative correlation between body size and clutch size. However, when the clutch size and body size of *P. maculatus* is compared with data from other species (Dutta and Mohanty-Hejmadi, 1976; Mohanty, 1994; Dutta *et al.*, 1993), there is no correlation between them. This might be due to seasonal breeding pattern of *P. maculatus* and capability of continuous breeding in the others.

Terentjev (1960) reported a correlation between body size and clutch size of temperate anurans. Inger and Bacon (1968) studied the annual reproduction and clutch size of rain forest frogs from Sarawak, Malaysia and using Terentjev's formula (1960) found that the observed value was significantly lower than the calculated value. In *L. limnocharis*, a small female (SVL:34.0 mm) laid 1203 eggs (Mohanty, 1994). Two other ranids, *H. tigerinus* and *H. crassus* show larger body sizes and clutch sizes than *L. limnocharis* (Dutta *et al.*, 1993; Dutta and Mohanty Hejmadi, 1976). In ranids, clutch size increase

with body size between species but not within a species. More eggs are seen in several microhylids which are smaller in body size than *P. maculatus*. This is due to the fact that, smaller species are more prone to predation and to severe physical environment. Accordingly, smaller species need a suite of adaptations for the uncertainty of the environment in which the larvae live. They therefore produce relatively larger clutches (Kuramoto, 1978).

Mohanty-Hejmadi and Dutta (1988) reported the maximum clutch size of *P. maculatus* as 719, with an average of 454, while Mishra and Dash (1984) reported clutch sizes as 622 and 367, respectively. In this study, the maximum clutch size was 678. The variations found are attributed to the number of clutches utilised for the study. Similar studies have been conducted by Alcala (1955,1962) and Alacala and Brown (1982) for *Rhacophorus appendiculatus* (300-400), *Rhacophorus pardalis* (44-50), *P. leucomystax* (150-225) and *P. macrotis* (300-400) from the Philippines. Hatching success in *P. maculatus* is over 95%. Previous studies by Mohanty-Hejmadi and Dutta (1988) reported the range of hatching success from 89 to 100% with a mean of 96.4%. Mishra and Dash (1984), reported the hatching success as 40.3%.

The white eggs of the species do not allow morphological differentiation of animal and vegetal poles. Further, early division of the eggs (until morulation) is not observable. However, the eggs of most of the Indian anurans are with distinct animal and vegetal poles (Agarwal and Niazi, 1977; Roy and Khare, 1978; Dutta and Mohanty-Hejmadi, 1976; Mehta, 1983 and Dutta *et al.*, 1991). The terms "Primary" and "Secondary" hatching for the embryos (Mohanty Hejmadi and Dutta, 1988) appear justifiable. The primary hatching stage (tailbud stage) of the species is comparable with the typical hatching stages (tailbud stage) of other Indian anurans. The secondary hatching stage of the species is comparable with late external gill stage of other anurans. Deposition of eggs in the foam away from water and subsequent hatching at a mobile stage (late external gill stage) are proposed as "indirect parental care" by the species. When the eggs are deposited inside the foam, the early embryos are protected from predation and desiccation. In addition, the jelly like foam keeps the embryo alive until the foam is completely dried. Occasional emergence of larvae at the feeding stage (beyond secondary hatching stage) from the foam nest supports the idea of avoidance of desiccation. Then mean time period for completion of life history is 45 d. Mohanty-Hejmadi and Dutta (1988) reported 55 d for completion of life history. However, 45 d is considered as the peak time when the majority of larvae from a single clutch metamorphosed. In addition, growth is dependent on food, temperature and also population densities in a given rearing medium (Dash and Hota, 1980; Dutta - personal observation).

Table 1: Size of amplecting males and females and clutch size of *Polypedates maculatus*.

Date of Collection	SVL of male (mm)	SVL of female (mm)	Clutch size
8.8.1978	42.5	60.0	228
8.8.1978	47.0	69.0	415
3.7.1979	43.0	61.0	318
3.7.1979	46.0	63.0	385
8.7.1980	46.0	68.0	410
9.7.1986	43.0	63.0	380
18.8.1987	45.0	66.0	351
19.8.1987	44.0	63.0	340
12.6.1988	43.0	61.0	332
20.6.1988	46.0	68.0	415
19.6.1989	43.0	60.0	287
10.7.1989	44.5	62.5	310
18.7.1990	43.0	61.0	290
18.7.1990	44.0	63.0	318
23.7.1990	46.0	65.0	417
23.7.1990	45.0	65.0	405
26.7.1991	47.0	67.0	409
26.7.1991	42.5	62.0	680
3.8.1991	43.0	64.0	375
6.8.1991	44.0	63.0	385

Table 2: Hatching success of *Polypedates maculatus* eggs

Clutch number	Spoil eggs	Number of eggs hatched	Clutch size	Hatching %
1	21	402	423	95.03
2	13	306	319	95.92
3	25	395	420	94.04
4	9	299	308	97.07
5	34	475	509	93.32
6	17	394	411	95.86
7	13	310	323	95.97

Table 3: Age and size of developmental stages of *Polypedates maculatus* (n=10)

Stages	Age	Gosner Stage	Size range (mm)
Fertilised egg	0*	1	1.3-1.5
Early tailbud	28*	(17-18)	2.5-3.0
Mid tailbud	48*	19	3.0-4.3
Late tailbud (Primary hatching)	56*	20	4.3-5.0
External gill (Secondary hatching)	90*	(21-23)	5.0-7.5
Operculum complete	5**	24	7.5-10.1
Feeding	6**	25	10.1-11.2
Hind limb bud	15**	(26-30)	18.2-19.5
Pre-metamorphic	22**	(31-40)	24.6-26.2
Well developed hind limb (Pro-metamorphic)	35**	41	39.6-41.6
Both limb and tail	36**	42	41.6-41.8
Metamorphosing	36-39**	(43-45)	41.0-18.2
Metamorphosed froglet	40**	46	15.3-16.1

* hrs ; ** days

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