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The emblem of Pranikee

The emblem "***NABAGUNJARA***" is a chimeric animal and a common motif of Odishan art and literature. It literally means "Nine form". This form has been described by poet Sarala Das in the Odia 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 form 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 his 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 a "Chakra" or the "stylized discus" of Lord Krishna. Chimeric forms are encountered in literature and art all over the world. However, a chimera of nine animals is uniquely Odishan. Therefore, it was considered to be an appropriate emblem for the Journal of Zoological Society of Odisha.

Padma Shri Prof.PriyambadaMohanty-Hejmadi
Former Editor

Editorial

The present edition (XXVIIth volume) is dedicated to the founder President of the Zoological Society of Orissa, Prof. Basant Kumar Behura who left for his heavenly abode on 16th February, 2015 for eternal peace.

This volume contains two parts. The first part includes Golden Jubilee lectures delivered during celebration of department. In the second part, research articles covering different aspects of Zoological Sciences have been incorporated.

Pravati Kumari Mahapatra

Zoological Society of Orissa

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FROM EGG TO LEG: THE STORY OF VITAMIN A

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ABSTRACT

Vitamin A has profound effects on development and regeneration. Out of all the effects the most surprising is its ability to convert amputated tails into limbs in anurans considered to be the first report of complete homeotic transformation in vertebrates. It has been acknowledged internationally that Niazi's laboratory in University of Rajasthan and our laboratory in Utkal University have contributed significantly to this breakthrough work, as far as vitamin A related regeneration research is concerned.

In the middle of the 18th century the phenomenon of regeneration was first observed with the discovery that Hydra, a freshwater polyp, could generate a complete body from a small piece by Trembley (Table 1). Subsequently, it was observed that a variety of amphibians could regenerate their legs and tails following amputation. Over the period of 200 years many substances have been applied to regenerating limbs. All of them without exception, either had no effect or inhibited the process of regeneration. Therefore, the discovery of the remarkable property of vitamin A to influence pattern formation in a specific manner is considered as one of the significant discoveries on regeneration studies in the field of developmental biology (Table 2).

The history of regeneration research took a dramatic turn with the discovery of Niazi and his group in University of Rajasthan that when the hind limb buds of the toad *Bufo andersonii* were amputated through the shank and treated with retinyl palmitate (vitamin A), instead of growing just the missing parts, the regenerates seemed to contain extra elements in the proximo-distal axis: often two regenerates appeared instead of one. It was later found that vitamin A and other retinoids can induce an alternation of pattern in both developing and regenerating limbs. In regenerating amphibian limbs, retinoids (RA) can lead to pattern duplication in the proximo-distal, antero-posterior and dorso-ventral axes. Local application of RA to the anterior side of a developing limb bud causes duplications in the antero-posterior axis of the limb. The diversity of retinoid effects on differentiation and morphogenesis reflects not only different effects on different tissues but also different effects on the same during different stages of development. The presence of endogenous RA in chick limb buds indicated that endogenous RA may actually be involved as a morphogen during limb development. Furthermore, the discovery of several nuclear receptors for RA belonging to the steroid hormone receptor family and homologous receptors with unknown ligands went in favour of endogenous RA levels leading to differential gene expression leading to pattern formation. An extensive list is now available of the limb bud expression patterns of retinoic acids and their binding proteins during limb development as described by Mohanty-Hejmadi and Crawford (2003). Further, the colinear response of Hox genes to RA and multiple examples of RA-induced alterations to Hox expression has been worked out in a variety of vertebrate embryos.

It is interesting to note that while extensive work has been done on the effects of vitamin A on the regeneration of limbs, regeneration of tail has received very little attention. This is inspite of the fact that Niazi started his work on tail regeneration. A positive relationship was later found between the inhibitory influence of vitamin A and the developmental stages of the regenerating tails of the in several species.

Subsequently, we showed that in addition to the inhibition of tail regeneration, limbs were generated at the site of amputation in *Uperodon systoma* (Mohanty-Hejmadi, *et al.*, 1992). This was the first clear demonstration of homeotic transformation in any vertebrate. Further, it is reported inhibition of tail regeneration and homeotic transformation in several species namely in *Polypedates maculatus; B. melanostictus, Microhyla ornata* and *R. tigerina* (Mahapatra and Mohanty-Hejmadi, 1994, 2000; and Mahapatra *et al .*, 2001). Skeletal and histological studies showed that the ectopic limbs (EHLs) at tail are comparable to the normal hind limbs although they developed away from the tissues normally associated with limb development. Obviously, vitamin A respecified the tail tissue into limb tissue probably by switching off the tail specific Hox genes followed by activation of limb specific Hox genes. The whole segment with axial elements, pelvic girdle and the limbs are induced. Therefore, this being the first repeatable homeosis in Batesonian sense, the model can be used for molecular and genetic studies of homeosis for the first time in vertebrates. A detailed analysis of this phenomenon and its implications have been discussed by Mohanty-Hejmadi and Crawford (2003). Some of the interesting aspects are presented here.

Table 1 Landmarks in studies on regeneration event

Serial Number		Year	
01	Abraham Trembley	1774	Regeneration in Hydra
02	Lezzaro Spallazani	1765	Regeneration in Salamander
03	Tweedy J. Todd	1823	Neutrophic Effect
04	Gustaff Wolff	1884	Regeneration of Lens from Iris
05	Eleanor G Butler	1933	Dedifferentiation of mesoderm as source of blastema
06	Goro Eguchi & Tokindo S. Okada	1973	Cell transformation *in vitro*
07	A.Niazi & S. Saxena	1978	Abnormal limb regeneration with Vitramin A
08	Mohanty-Hejmadi, Dutta & Mahapatra	1992	Homeotic transformation of tail into limb by Vitamin A
09	Maden	1993	Homeotic transformation by retinoids

Table 2 Landmarks in vitamin A/Retinoids Research in regeneration in amphibians

Serial number	Niazi & Saxena	1968	Tail abnormality	***B. andersonii***
01	Niazi & Saxena	1978	Pattern duplication (hind limb)	***B. andersonii***
02	Maden	1982	Pattern duplication (hind limb)	***A. mexicanum***
03	Tickle *et al*.	1982	Bead of RA in limb Bud duplicates ZPA	**Chick**
04	Scadding	1987	Inhibition of tail regeneration	***N. viridescens***, ***A. mexicanum, X. laevis***
05	Mohanty-Hejmadi, Dutta & Mahapatra	1992	Homeotic transform-ation (tail to legs)	***U. systoma***

Thus, regeneration is a fascinating biological process during which the missing structures are completely restored. Vitamin A and its derivatives, retinoids have remarkable effects on the development and regeneration of limbs of several vertebrates.

The Effects of retinoids (RA) are stage dependent, time dependent and different retinoids have different potencies. In limb regeneration, RA enhances the morphogenetic potential of the blastema radically changing the structural pattern of the regenerate. With the discovery of endogenous RA in limb buds there is significant evidence of RA being an endogenous morphogen. Limb buds synthesize retinoic acid *in situ* from its precursor retinol. Retinol is

distributed uniformly across the limb bud but there is an endogenous differential distribution of retinoic acid across the limb.

Since retinoids can change positional as well as regional specifications to generate normal limbs, it is reasonable to assume that retinoids activate the Hox series critical for limb development. The genes that play important roles in the establishment of axes and regeneration of limbs are Hox-4, Hox-3 and Hox-1 clusters. Effect of retinoids on H-4 has been demonstrated (Fig. 1).

In regenerating amphibian limbs, retinoids can lead to pattern duplicating in: Proximodistal (PD), Anteroposterior (AP), and Dorsoventral (DV) axes.

Several extrinsic factors influence regenerative processes in amphibians:

(a) X-irradiation inhibits by preventing cell division
(b) Nerves/extracts promote
(c) Vitamin A and its derivatives have paradoxical effects (inhibit/ promote/heteromorphosis)

Basic morphogenic rules for forming a limb appear to be the same in all tetrapods. Different cell types give rise to different organs namely chondrocytes form cartilage and osteocytes form bone matrices. Same cell types also form different patterns: digit at one end and a shoulder at the other end. Proteins play a role in the formation of limb axes. Proximo-distal is due to FGF, antero-distal due to Sonic hedgehog (shh) and dorso-ventral due to gene Wnt7a. Apoptosis or cell death signaled by gene BMP4 leads to formation of digits

There are two aspects that are remarkable in the homeotic transformation of tail into limbs, first that the EHLs are always hind limbs and the second that the whole pelvic segment with skeletal elements which normally do not occur in the tail, are generated.

First, the vertebrate limb is an extremely complex organ with an asymmetrical pattern of parts. It is formed by a complex series of interactions during development. Under normal circumstances limbs originate as a consequence of the differential growth of cells from the lateral plate mesoderm at specific axial level. Two sources of mesenchyme contribute to the formation of limb. Cells migrating from the somites give rise to all muscle cells. Cells from lateral plate mesoderm give rise to connective tissue and cartilage. Limb development begins when mesenchymal cells are released from the somatic layer of the limb field lateral plate mesoderm followed by cells from the somite. The cells migrate laterally and accumulate under the epidermal tissue of the neurula. The circular bulge on the surface of the embryo is called the limb bud. The initial stage of this proliferation appears to be under regulation of the mesonephros (see Gilbert, 1994). There is enough evidence that the three axes of limb (dorsoventral, proximodistal and anteriorposterior) are linked by the respective signals gene WNT7a, Fibroblast growth factor (FGF4) and Sonic hedgehog (Shh) during limb outgrowth and patterning (Yang and Niswander, 1995). In chick, ectopic limb induction by FGF family has shown that the phenotype of the ectopic limb depends on the somite level at which it is formed; limbs in the anterior flank region resemble wings, whereas those in the posterior flank resemble legs. Hox code and FGF expression in the lateral plate mesoderm specify and select the expression of Tbx5 and Tbx4/Pitx 1 in the prospective wing and leg buds , respectively. Pitx1 acts as a Tbx4 inducer, and Tbx 5 represses expression of

Tbx 4. Tbx 4 induces expression of Hoxd 9 which is specific for hind limb (Ohuchi *et al.*, 1998; Takeuchi *et al.*, 1999 and Rodriguez-Esteban *et al.*, 1999).

During homeotic transformation of tail into hind limbs, it is reasonable to speculate that under the influence of vitamin A, mesodermal cells in the tail region induce a limb bud in the presence of a mesenchymal FGF which in turn induces Pitx1 which acts as a Tbx 4 inducer to form the hind limbs. Pitx and Tbx proteins are transcription factors and their targets either direct or indirect, include genes of the Hox homeodomain cluster. Tbx 4 induces hind limb specific Hox genes (Hoxc9-Hoxc11) and suppresses the fore limb specific Hoxd9 genes (Niswander, 1999). Further, the genes that play important roles in establishment of axes and regeneration of limbs are the Hox-4, Hox-3 and Hox-1 clusters (Simon and Tabin, 1993). The Hox-3 cluster is expressed specifically in normal and regenerating posterior appendages and apparently, provides positional memory for differentiation of hindlimbs from forelimbs. So, it is reasonable to propose that a gene of the Hox-3 cluster may also be involved in the development of the tail to legs (Mahapatra and Mohanty-Hejmadi, 1994). The molecular mechanism relating to the role of retinoids in the regulation of Hox genes and influence in the Shh have been discused by Johnson and Scadding (1991). It will be interesting to see if the same processes are repeated during EHL formation from tail tissue.

Second, coming to the transformation of tail tissue into a pelvic or anterior segment under the influence of vitamin A, considerable information is available on the establishment of cranio-caudal axis which is pertinent here. Christ *et al.* (2000) have presented a comprehensive account of the segmentation of the paraxial mesoderm leading to somite formation and the role of Hox genes in cranio-caudal identity based

on observations in chick embryo applicable to other vertebrates. The vertebral column develops from somites, segmental units of the paraxial mesoderm. The regionalization of the paraxial mesoderm and the determination of the axial identity are achieved by Hox genes which include atleast 38 members representing 13 paralogous groups aligned in four clusters (a-d). Hox genes show a cranial-to-caudal pattern of expression with a sequence of cranial expression boundaries that corresponds to their alignment on the chromosomes. The development of vertebral column is a consequence of segment-specific balance between proliferation, apoptosis and differentiation of cells under normal circumstances.

During homeotic limb induction, the sources of the cells and their association are completely different. Cells differentiate away from the normal inducer, mesonephros. But, during this transformation, most noteworthy is the enlargement of the notochord in the treated group (Das and Mohanty-Hejmadi, 2003). Within 72 h the control group regenerates a normal notochord, but in the treated group there is considerable enlargement of notochord which remains in this condition for long time. This has also has been noted by Maden (1993). At times, a limb bud can be discerned within 72 h after treatment in the flank region of the tail . There is a strong possibility that the notochordal cells may play a role in induction of the pelvic segment leading to the formation of limb buds. In chick and mouse, notochord is known to be a source of polarising factors such as shh which in turn encodes a signal that is implicated in both short and long-range interactions that pattern the central nervous system, somite and limb (Marti *et. al.*, 1995). As reviewed by Christ *et al.* (2000), somite formation is not possible without the nerve cord and the notochord. At the amputated tail, both the nerve cord and

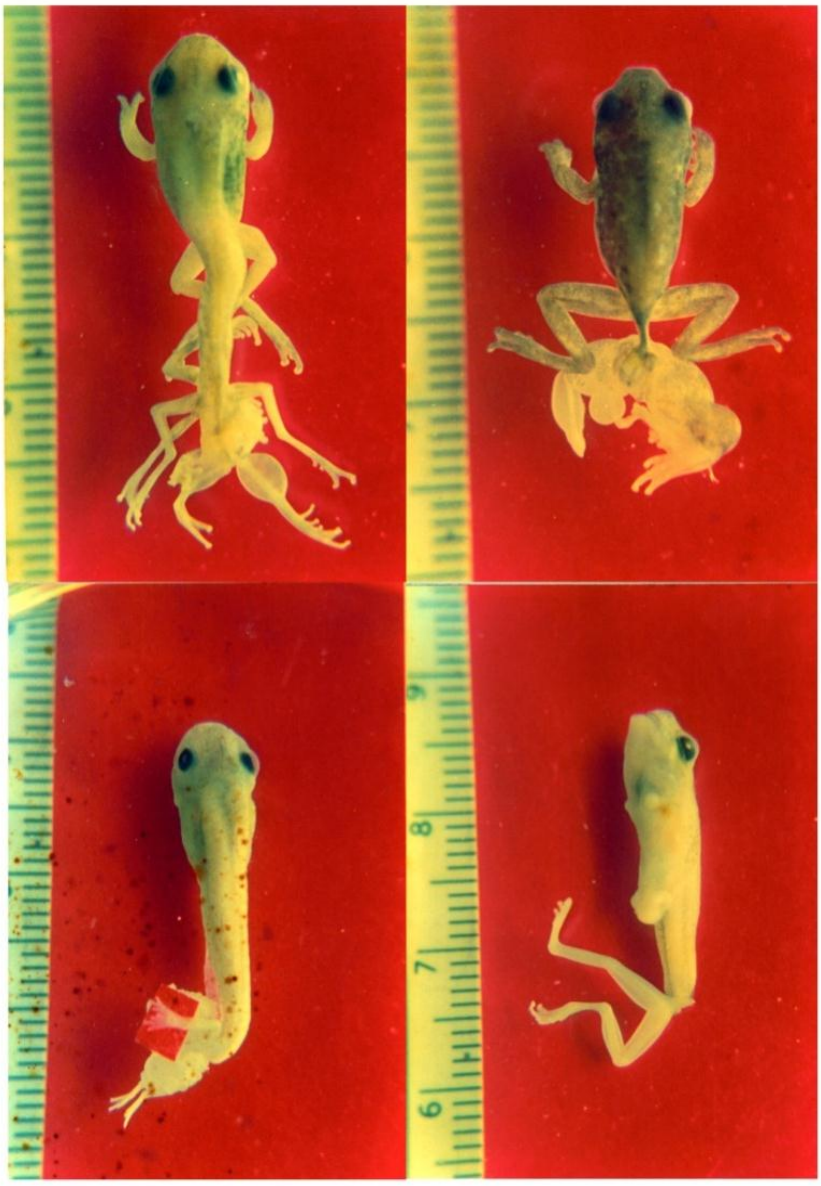
notochord are present. There is considerable enlargement of notochord after amputation and vitamin A treatment (Das and Mohanty-Hejmadi, 2003). Nerves grow out to innervate the EHLs. Therefore, it is reasonable to speculate that under the influence of vitamin A, notochord mediates the expression of the key Hox genes for the establishment of the pelvic region including the vertebral elements and nerve supply by re-specification and proximalisation, in the tail tissue. Interestingly, the epidermal cells of the tail of an anuran larva can transform into adult types (Yoshizato *et al*., 1993).

As pointed out by Maden (1993), during homeotic transformation, large amounts of transformed tail tissues are generated and molecular analyses of this tissue may lead to the identification of the genes involved in homeotic transformation. Probing the tissue during this unique transformation may lead to identification not only of the cells which contribute to limb formation, but also the mechanism by which tail specific genes are suppressed and limb specific genes are activated. Once the origin of the limb cells are traced in the tail region, these cells can be cultured to see if limbs can be induced *in vitro* which will have academic as well as applied implications.

Last but not the least, as pointed out by several workers (Maden, 1993; Okada, 1996 and Müller *et al.*, 1996) this phenomenon has considerable evolutionary significance and now provides an opportunity for genetic studies similar to the ones which have enriched our knowledge in pattern formation in invertebrates, for the first time in vertebrates. In describing our work, Maden (1993) says that this was the first clear demonstration of homeotic transformation in the sense Bateson originally described in vertebrates. As pointed out by Müller *et al*., (1996) this phenomenon is also indicative

of retension of segmentality as far as induction and pattern formation is concerned even in a downhill transitory structure like the tail. This is now a powerful model to work as we finally have segments on which we can work in vertebrates like those in invertebrates like *Drosophila*. People like Van Valen (1994) who tried to speculate on the Batesonian implication on *polymely* in anurans can be happy that in vertebrates we have a model to test out Batesonian *"Homeosis"*. It is expected that this model can be used for understanding *homeosis* from molecular to organismic levels in vertebrates. We agree with Okada (1996) that it has opened a way to investigate the most mysterious phenomenon known from early 20^{th} century in modern eyes, most probably in terms of epigenetic switch of homeotic genes.

Epilogue: Gods and Goddesses in different religions are presented with additional limbs as representative of valour. Now it is seen that extra limbs need not be mythical but reality too. It is also pertinent to mention that during a presentation in Sukuba, Japan, Dr Tosi Amano was kind enough to present me with a slide with the sketch of a rare 300 years old specimen showing legs at the tip of the tail labeled as a "three legged frog" of *Bufo japonicus* collected in Tokyo. This indicated that homeotic transformation can also occur in nature probably due to trauma although spotting them under natural circumstances in rare.



Homeotic Transformation of *Polypedates maculatus*

REFERENCE

Christ B, Huang R and J Wilting (2000). The development of the avian vertebral column. *Anat. Embryol*. 202 179-194.

Das P and Mohanty-Hejmadi P (2003). Vitamin A mediated homeotic transformation and histological changes in amputated tail tissues of tadpoles of *Rana tigerina. Curr. Sci.* 84(4): 557-562.

Gilbert Scott F (1994). Development of the Tetrapod Limb; in *Dev. Biol.* (4th edn) pp 600-714 (Sinauer Associates Inc., USA).

Johnson KJ and Scadding SR (1991). Effect of vitamin A and other retinoids on the differentiation and morphogenesis of the integument and limbs of vertebrates; C*an. J. Zool.* 69: 263-273.

Maden M (1982). Vitamin A and pattern formation in the regenerating limb; *Nature* 295: 272-275.

Maden M (1983). The effects of Vitamin A on limb regeneration in *Rana temporaria*; *Dev. Biol*. 90: 409-416.

Maden M (1993). The homeotic transformation of tails into limbs in *Rana temporaria*; *Dev. Biol.* 159: 379-391.

Maden, M (1994). Vitamin A in embryonic development; *Nutrition Reviews,* 52(2): 3-12.

Maden, M (1996). Retinoic acid in development and regeneration; *J. Biosci.* 21: 299-312.

Mahapatra P and Mohanty-Hejmadi P (1994). Vitamin A-mediated homeotic transformation of tail to limbs, limb suppression and abnormal tail regeneration

in the Indian jumping frog *Polypedates maculatus; Dev. Growth. Differ*. 36(3): 307-317.

Mahapatra P and Mohanty-Hejmadi P (2000). Suppression of normal limb development in the tadpoles of *Bufo melanostictus*, *Uperodon systoma* and *Polypedates maculates* following vitamin A treatment; *J. Zool.Soc. India.* 40/41: 67-74.

Mahapatra P, Mohanty-Hejmadi P and Dutta SK (2001). Polymelia in the tadpoles of *Bufo melanostictus* (Anura: Bufonidae); *Curr. Sci.* 80: 1447-1451.

Marti E, Takada R, Bumcroft DA, Sasaki H and McMohan AP (1995). Distribution of sonic hedgehog peptides in the developing chick and mouse embryos; *Development* 121 (8): 2537-2547.

Mohanty-Hejmadi P and Michael JC (2003). Vitamin A, Regeneration and Homeotic Transformation in Anurans. *Proc. Indian natn. Sci. Acad*. B69 No.5: 673-690.

Mohanty-Hejmadi P, Dutta SK and Mahapatra P (1992). Limbs generated at the site of tail amputation in marbled balloon frog after vitamin A treatment; *Nature* 355: 352-353.

Müller GB, Streicher J and Wurm R (1996). Homeotic duplication of the pelvic body segments in regenerating tadpole tails induced by retinoic acid; *Dev.Genes Evol.* 206: 344-348.

Niazi IA (1996). Background to work on retinoids and amphibian limb regeneration: Studies on anuran tadpoles-a retrospect; *J. Biosci.* 21(3): 273-297.

Niazi IA and Saxena S (1968). Inhibitory and modifying influence of excess vitamin A on tail regeneration in *Bufo* tadpoles; *Experientia* 24: 852-853.

Niazi IA and Saxena S (1978). Abnormal hind limb regeneration in tadpoles of the toad *Bufo andersonii*, exposed to excess vitamin A; *Folia Biol.* 26: 3-8.

Niazi IA and Saxena (1979). Relationship between inhibiting influence of vitamin A and developmental stage of tail in toad tadpoles (*Bufo andersonii*); *Ind. J. Exp. Biol.* 17: 866-868.

Ohuchi H, Takeuchi J, Yoshioka H, Ishimaru Y, Ogura K, Takahashi N, Ogura T and Noji S (1998). Correlation of wing-leg identity in ectopic FGF induced chimeric limbs with differential expression of chick Tbx 5 and Tbx 4; *Development* 125: 51-60.

Okada TS (19960. A brief history of regeneration research- For admiring Prof. Niazi's discovery of the effect of vitamin A on regeneration; *J. Biosci.* 21(3) : 261-271.

Rodriguez-Esteban, C, Tsukul T, Yonei S, Magallon J, Tamura k and Izpisua JC Belmonte (1999). The T-box genes Tbx4 and Tbx5 regulate limb outgrowth and identity; *Nature* 398: 814-818.

Simon Hans-George and CJ Tabin (1993). Analysis of Hox-4.5 and Hox- 3.6 expression during newt limb regeneration,differential regulation of paralogous Hox genes suggest different roles of members of different Hox clusters; *Development* 117: 1397-1407.

Spallanzani A (1768). *An essay on animal reproduction* (Translated from the Italian by M. Maty, London 1769).

Tabin CJ (1991). Retinoid, Homeoboxes, Growth Factors: Towards Molecular Models for Limb Development; *Cell* 66: 199-217.

Takeuchi JK, Koshiba-Takeuchi K, Matsumoto K, Vogel-Höpker A, Naitoh-Matsuo M, Ogura K, Takahashi N, Yasuda K and Ogura T (1999). Tbx5 and Tbx4 genes determine the wings/leg identity of limb buds; *Nature* 398 : 810-814.

Trembley A (1744). *Memoires*, *Pour Servir a L'histoire d'un genre de Polypes d'eau douce, A bras en forme de cornes* (Leide, Verbeek)

Van Valen L (1974). A natural model for the origin of some higher taxa; *J. Herp.* 8(2) :109-121.

Yang Y and Niswander L (1995). Interaction between the signalling molecules WNT7a and SHH during vertebrate limb development: Dorsal signals regulate anteroposterior patterning; *Cell* 80: 939-947.

Yoshizato KA, Nishikawa A, Izutsu I and Kaiho M (1993). Epidermal cells of the tail of an anuran larva are competent to transform into the adult-type cells; *Zool. Sci.* 10:183-187.

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SILKWORM *BOMBYX MORI* – AN ECONOMIC INSECT

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"Natural silk" a secretion of silk gland, is produced when a full grown silkworm larva spins its cocoon during pre-pupation. It is known as the "Golden Fibre" of the "Queen of textile" and is admired all over the world for its sleek and luster. Silk produced by the silkworm *Bombyx mori* is one among the natural fibers and considered the most desirable and sensuous natural fabric in existence and highly valued textile fiber of animal origin used for the production of high quality textiles. Its products are wonderfully light and soft but strong and smooth and universally accepted by the world top fashion designers for its elegance, colours, dyeing affinity, thermo tolerance and water absorbance. Silk is a way of life and an inseparable part of Indian culture and traditions. The wide range of weaving traditions and the artistic creations such as irresistible ikats of Odisha, bright brocades of Benares, temple designs of Kanchipuram, trendy tasars and many more are truly a boon to the Indian silk industry. Silk, considered as the queen of fibres, is proteinaceous in nature. The bulk of commercial silk is produced from the mulberry silkworm *Bombyx mori*, of which there are many strains ranging from the exotic high-yielding bivoltines to the sturdier, native multivoltines. The other silkworms commercially exploited for silk production are eri (*Philosamia* species), and tasar and muga (*Antheraea* species). India is unique in producing all these varieties of silk. India is home to vast variety of silk secreting fauna which also includes an amazing diversity of silkmoths. This has enabled India to

achieve the unique distinction of being a producer of all the commercial exploited varieties of natural silks namely mulberry and non-mulberry (tasar, oak tasar, eri and muga). Non-mulberry silkworms are also known as "vanya silkworms".

Mulberry: The bulk of commercial silk produced in the world is mulberry silk that comes from the domesticated silkworm, *Bombyx mori* L. which feeds solely on the leaves of the mulberry plant.

Tasar: Tasar is copperish colour, coarse silk mainly used for furnishings and interiors. Tasar silk is less lustrous than mulberry silk, but has its own feel and appeal. Tasar silk is generated by the silkworm, *Antheraea mylitta* which mainly thrive on the food plants Asan and Arjun. The rearings are conducted in nature on the trees in the open.

Oak Tasar: It is a finer variety of tasar generated by the silkworm, *Antheraea proyeli* J. in India which feeds on natural food plants of oak. China is the major producer of oak tasar in the world and this comes from another silkworm which is known as *Antheraea pernyi*.

Eri: Eri silk is the product of the domesticated silkworm, *Philosamia ricini* that feeds mainly on castor leaves. A multivoltine silkworm known for its white or brick red silk. Eri cocoons are open-mouthed and are spun. The silk is used indigenously for preparation of chaddars (wraps) for own use by these tribals. Eri culture is a household activity practiced mainly for protein rich pupae, a delicacy for the tribal.

Muga: This golden yellow colour silk is prerogative of India and the pride of Assam state. It is obtained from semi-domesticated multivoltine silkworm, *Antheraea assamensis* confined to north-eastern states of India, is the least understood and unique

species among saturnid moths. The silk proteins of these species have not been studied so far despite their unique properties of providing golden luster to the silk thread. These silkworms feed on the aromatic leaves of Som and Soalu plants. Muga culture is specific to the state of Assam and an integral part of the tradition and culture of that state. The muga silk, a high value product is used in products like sarees, mekhalas, chaddars, etc. The domesticated silkworm *Bombyx mori* L., a member of the family Bombycidae, is a well-studied lepidopteran model system with rich repertoire of genetic information on mutations affecting morphology, development, and behavior (Arunkumar et al., 2006). This species has been used as a main source of silk, and has lost some characteristics due to long-term breeding under artificial conditions. Though there are many types of silkworms, only mulberry silkworm has enjoyed a large share of the patronage of the global scientific community. As a result the mulberry silkworm has achieved the distinction of being a "lepidoteran model system".

The wild silkmoth, *Bombyx mandarina* M., is believed to be the ancestor of *B. mori* (Banno et al., 2004), as these two species can cross-breed and yield fertile hybrid offspring. *B. mandarina* includes significant variation within species (Yukuhiro et al., 2002). From the aspect of morphological and physiological characteristics, *B. mandarina* was very similar to *B. mori* (Astaurov et al., 1959; Yoshitake, 1968). The mulberry silkworm belongs to Phylum Arthropoda, Class Insect, Order Lepidoptera, Family Bombycidae, Genus *Bombyx* and Species *mori*. The domestic silkmoth, *Bombyx mori*, is a member of the family Bombycidae of about 300 moth species under the order Lepidoptera and vanya silkworms belong to family Saturniidae.

Silkworms are lepidopteron insects. The larvae are caterpillars, which, at the end of the larval stage, spin a cocoon of silk, and transform into pupae and finally into adult

moths. The silk is synthesized in the silk glands. Larvae possess a pair of silk glands, which are long complexly folded tubular structures located parallel to the gut, extending nearly throughout the entire length of the larvae, but much longer, may be up to seven times the length of the caterpillar, being coiled in the posterior region of silk glands. Silk gland can be divided into three morphologically distinct regions. They are anterior, middle and posterior silk glands. The gland is a tube made of glandular epithelium with two rows of cells surrounding the lumen. Mulberry silk fiber is mainly composed of a major silk protein known as fibroin heavy chain and two small silk proteins hexamerin and fibroin light chain. All of these are secreted by the posterior silk gland that is 1.5cm long comprises about 500 secretary cells. Silk proteins are stored, until spinning, in the lumen of middle region of silk gland comprising of 300 secretary cells producing sericins, or proteins which cement the fibroin threads of the cocoon. The middle region acts as a reservoir of fibroin and the fibroin matures in this region during the storage period. Glue proteins (sericins) are synthesized in the middle region and coat the fibroin in three layers, i.e., Sericin–I (inner most layer) secretion of posterior part of middle region, Sericin–II (Middle layer) secreted by the middle portion of middle region, Sericin–III (outer most layer) secreted by the anterior portion of the middle region. The two anterior silk glands, which serve as ducts, converge near the oral cavity, farming a scleroid structure, and open at the base of the median projection in the labium called spinneret which the silk is extruded as fibre. The hind part of the spinneret has muscles which contract, forcing silk into the tube-like front part of the spinneret. The caterpillar then moves its head to draw more silk outward. The threads of two sides are called the brins and the sercin layer binds the two brins into a single filament which is known as bave. “Spinning of the cocoon is accomplished by

movement of the head. The silk itself is a double-stranded, since it comes from two separate glands. Spinning of cocoon is completed in 2-3 days, at the end of which changes taking place with in the larva culminate in the transformation to a pupa. The cocoon is thus one long silk fibre, from about 600 meter in the local races to about 1,600 meters in high–yielding bivoltine races.

Silk fibre is produced at the end of the larval life of these insects and is woven in the form of cocoon in which the larva is metamorphosed into pupal form. Silk cocoon is woven by the insect larva to safeguard the sedentary, non-feeding phase of insect's life cycle. The life cycle of silkworm represents the most advanced form of metamorphosis, termed holometabolous. The silkworm completes life cycle through serial progression of four distinct stages of development; egg, larva, pupa and adult. The number of life cycles (generations, which is termed as voltinism) per year depends on the silkworm strain and it varies with the environmental conditions particularly temperature. Silkworm strains which go through multiple generations in a year are polyvoltines or multivoltines. These strains do not undergo egg diapause, which is an adaptation to tropical condition in which there is no severe winter. Under natural conditions, silkworm strains which undergo only one generation in a year are known as univoltines and two generations are known as bivoltines. This is an adaptation to overcome harsh winters in temperate countries. Artificially, these eggs which hibernate during winter are stored at 4^{o}C. After removal from cold storage to room temperature (25^{o}C), through intermediate temperature of 10-15^{0}C, about two weeks later ova in diapause eggs begin final development until hatching. The egg is tiny in size and measures 1 to 1.3mm in length and 0.9 to 1.2mm in width and resembles a poppy seed. The egg shell provides a protective covering for embryonic development. When first laid, an egg is light yellow

in colour. Fertile ova darken to a blue-grey before hatching. The larva is an elongated caterpillar, the only feeding stage in the life cycle. Newly hatched larva is black in colour and it has large head and the body is densely covered with bristles so that it looks like a hairy caterpillar. As larva grows, it becomes smoother and lighter in colour due to the rapid stretching of the cuticular skin during the different instars of the larval stage. The larva is monophagous, feeds only on mulberry (*Morus alba*). During larval life, the larva sheds its skin (molt) 4 times to accommodate growth. The period between successive molts is called an instar. At the end of final instar (fifth instar), larvae cease feeding, and their bodies become shorter, stouter, body becomes transparent and body colour changes to pale yellow in colour. These larvae are called mature larvae; the larva spins a silk cocoon of one continuous fibre within which it undergoes pupation. Silk cocoons are the commercial source of silk. Prior to spinning, a matured larva excretes semi-solid excretion and oozes a tiny droplet of silk to anchor its body to spin a cocoon. First it spins entangled floss layer the outer most of layer of cocoon to provide foothold. Then it spins main shell from where the silk is extracted, followed by thin pelade layer the inner most layer of the silk cocoon.

From the time larva hatches out from the egg up to the end of fifth instar grows about 10,000 times. Bred in captivity for thousands of years on trays of mulberry leaves, *B. mori* is fully domesticated and cannot survive without the assistance of man. The silk cocoon serves as protection for the pupa. Cocoons are shades of white, cream and yellow depending on silkworm variety. After a final molt inside the cocoon, the larva develops into a brown, chitin covered structure called the pupa. Metamorphosis of the pupa results in an emerging moth or adult. The moth is covered with heavy, round, furry scales and lacks functional mouth parts, so are unable to consume food. The forewing

has a hooked tip, which is a characteristic feature of this family; however it is flightless. Wings and body are usually white, but may vary in shades of light brown. Wingspan is 1.5 to 2.5 inches. It is the reproductive stage where adults mate and females lay eggs. Adult is the final stage in the life cycle of *B. mori* with short life span of 4-6 days.

Silk and silk fabric are the products of a series of stages involved in the production process derived from the cultivation of mulberry as a feed to the domesticated silkworm, *Bombyx mori* (Agriculture based activities) and post cocoon processing is industrial activity very much fit into the rural structure of India. During the caterpillar phase, the worm wraps itself in a liquid protein secreted by two large glands. This secreted protein hardens upon exposure to the air. The resulting filament is bonded by second secretion, sericin, which forms a solid sheath or cocoon. Under natural conditions, a moth eventually breaks through the cocoon. Production of cocoon the basic raw material for the silk industry is agriculture based activity. Then the cocoons are taken for silk extraction. To unwind the silk, the larva is killed in the cocoon by steam or hot air in the chrysalis stage before its metamorphosis. Sustained heat processing softens the hardened sericin so that the filament can be unwound. The silk filament is a continuous thread of great strength measuring from 500-1500metres in length. Single filaments are too thin for utilization; several filaments are combined for the production of the raw silk this process is known as "silk reeling ". Raw silk passes through series of industrial activities viz., twisting, warp making, weft making, dyeing, printing, fabric making, finishing etc to get silk fabric for marketing. A tiny cute worm is the basis of the silk industry which is providing livelihood for 6 million people in India in various stages of production process.

The silkworm is a small and highly domesticated insect and plays a main role in production of Queen of textiles. Currently, it is the major economic resource for more than 30 million families in countries such as China, India, Vietnam, and Thailand. The silkworm which has traditionally produced rich silks can now be manipulated as an ideal laboratory tool. *B. mori* possesses excellent characteristics as an experimental animal; it can be reared and bred under complete human control and is an ideal organism for research in various fields of sciences. Silkworm is the well studied insect model system because of its rich repertoire of well characterized mutations affecting virtually every aspect of the organisms' morphology, development and behavior and its economic importance. Well characterized and physical mapping of chromosome mutants in silkworm offers excellent opportunities for understanding basic concepts of cytological and molecular studies. The implications of physiological studies in silkworm have found their applications in pharmaceutical and medical fields. Silkworm is an excellent laboratory tool for conducting various bimolecular as well as toxicological studies. It is the genetically best known insect next to *Drosophila melanogaster.* Apart from this, an upcoming field where transgenic silkworm is used for production of life saving drugs in pharmaceutical industry deserves special and careful attention. The silkworm can now be manipulated to produce a vaccine for hepatitis B. Researchers at the Indian Institute of Science (IISC) in Bengaluru have been successful in making the silkworm produce an antigen for hepatitis B, a blood-borne viral infection whose rising incidence is a cause for concern in the country.

Silkworm genomics has been used to unravel the lepidopteron specific genes like novel insecticide target genes, pheromone binding genes, etc. which have found tremendous applications in the field of Agriculture. Apart from these, there are several other

frontiers where sericulture can be mended using modern tools of biotechnology to suit the needs of man and industry.

Some of the research reviews on the use of silkworm as a model are discussed. Not many are aware that silkworms can be used, as effectively in place of mice, or guinea pigs or higher vertebrates in the study of pathogens and drugs. Recently, the use of invertebrate models for study of infection has given exciting insights into host pathogen interaction for a number of bacteria and this has revealed important factors of the host response with parallels in higher organisms. The advances attained in understanding the virulence determinants of a major human pathogen, *Staphylococcus aureus* in relation to invertebrate models, the silkworm (*Bombyx mori*), the fruit fly (*Drosophila melanogaster)* and the round worm (*Caenorhabditis elegans*) are reviewed by Jorge Garcia-Lara et al., (2008). It was concluded that use of silkworms is technically convenient, ethically acceptable, a valuable first tool to discriminate molecules as well as speedy screening method for antimicrobials. Katio et al., (2008) of Graduate School of Pharmaceutical Sciences, University of Tokyo examined silkworms as an animal model of human infection with pathogenic bacteria. Barman et al., (2008) Indian researchers from the New Drug Discovery Research Unit of Ranbaxy Research Laboratories used the silkworm as an alternative animal model for testing the efficacy of novel oxazolidnones, which are used as antibiotics. The minimal lethal dose for *Staphyllococcous aureus* was 1.5×10^7 CFU per larva, exhibiting more than 90% mortality within 2 to 4 days post-infection. Treatment with vancomycin, linezid, and novel oxzolidinones, under development, showed survival in a dose-dependent manner. The antibacterial effect of the new molecules was compared with that of vancomycin and linezoilid, and the Effective Doses (ED^{50}) obtained. ED^{50} values showed a similar

trend in murine model (i.e., using mouse or similar animal) of infection. Owing to the small amounts of new chemicals were required to test their in vivo efficacy in this model. The silkworm model may, therefore, be used in the early stage of new discovery research.

With development of modern scientific technology silkworm is being used to develop products with functionality besides the traditional cocoon production. In recent years, more and more efforts have being made by the scientists to non-fibre utilization of silkworm. A series of functional products such as silkworm powder, pupal amino acids, silk cosmetics and gene engineering products of silkworm etc., have been developed. Gui et al., (2001) Chen et al., (2002) studied the effect of silkworm powder on type-2 diabetes mellitus for further development of anti-diabetic agent. The results showed that silkworm lowers blood-glucose of diabetes mellitus animals effectively by partially related to the inhibition of □- maltase glycosidase activity in the small intestine in order to delay of the absorption of glucose by blood or tissues. Silkworm powder has also some effects proved by therapy in immunization stimulation (T-lymphocyte transformation rate was elevated by 35%), lowering blood lipid, recovering fatigue and improving resistance of insulin etc, (Gui et al., 2001). It is believed that silkworm powder is one kind of natural and multi-effect anti-diabetic agent without side effect. Meanwhile, IGF-1 (Insulin Growth Factor-1) was successfully expressed in silkworm larvae with baculovirus expression system and used to lowering blood-glucose for diabetic patients.

Dried dead larvae or pupae of silkworm infected with *Beauveria bassiana* is widely used in traditional Chinese medicine with much advantage in detoxification, lowering

cholesterol, and effective to apoplexy, convulsions, parotitis mumps, tonsillitis, diabetes mellitus etc. It also inhibits respiration cancer cells (Jiang, 1996).

Silkworm faeces are utilized as the material for extraction of chlorophyll, which is widely used medical and food industries. It enhances metabolism, activates cells and inhibits bacterial growth in human body, which is helpful for treatment of some diseases such as hepatitis, gastric ulcer, nephitis and others, and also to increase the synthesis of haemoglobin. Silkworm pupa is a potential bio-resource with plenty of high quality protein to be exploited. The pupae protein is used to produce pupae cans and pupa amino acids for soft drinks and additives for the functional food and medicine. The chitin of pupa is one of the primary material for surgical line, vector of stationery enzymes, artificial skin etc. Fibroin, main protein of silk decomposed into oligopeptide or aminoacids by chemical and biological methods for further use in cosmetic material, functional food and medical substance. Because of its excellent properties in skin affinity, ultraviolet ray blockage, moisture retention, skin adhesion, silk fibroin is used as additives to produce many kinds of cosmetics. Scientists of Japan Science and Technology Corporation in Hiroshima inserted a human gene for collagen into silkworms. Genetically Modified Silkworms secrete the human protein collagen in their cocoons. The insects produced both silk and collagen, which is used to generate artificial skin and cartilage and in cosmetic surgery to fill out lips and wrinkles. The silkworm nuclear polyhedrosisvirus (baculovirus) has been used as a potential expression system for various proteins of pharmaceutical interest. The use of baculovirus BmNPV will be advantageous in expression of foreign product. The host insect cell infected with recombinant baculovirus carrying a foreign gene can produce gene products complete with post transitional modifications such as glycosylation,

phosphorilation, cleavage of signal peptides and proper folding of proteins. It has been demonstrated that the production cost can be greatly reduced by using caterpillars instead of culture cells. The host range of BmNPV is virtually limited to silkworm *Bombyx mori*. The larva grows at rapid rate and the body weight increases by 10,000 times in 20-25 days. Already, more than thirty five proteins of pharmaceutical interest have been expressed in a biological active and useable form in silkworm using baculovirus as vector signaling the importance of using silkworm as bioreactor. Silkworm produces a fine, glistening, fabulous proteinous silk thread the fabric made of which is regarded as the queen of textiles and has made great contribution to the human civilization, biologically, silkworm innovates as potential insect system for production of high cost proteins and currently silkworm is technically convenient, ethically acceptable a valuable laboratory animal model to discriminate molecules as well as screening antimicrobials, evaluating the efficacy of novel antibiotics for pathogenic bacterial infection in humans, screening of drugs etc. Still there is a vast scope to explore the tiny insect for the welfare of the mankind.

REFERENCES

Arunkumar KP, Metta M and Nagaraju J (2006). Molecular phylogeny of silkmoths reveals the origin of domesticated silkmoth, Bombyx mori from Chinese Bombyx mandarina and paternal inheritance of Antheraea proylei mitochondrial DNA. Mol. Phylogenet. Evol. 40: 419-427.

Astaurov BL, Golisheva MD, Roginskaya IS (1959). Bombyxmandarina M. withspacial reference to the problem of the origin of the domesticated silkworm, Bombyxmori L. Cytology, 1:327-332.

Banno Y, Nakamura T, Nagashima E, Fujii H, Doira H (2004). Mchromosome of the wild silkworm, Bombyxmandarina (n D27), corresponds to two chromosomes in the domesticated silkworm, Bombyxmori (nD 28). Genome, 47:96-101.

Chen, Y.G., Zi, M., Hai, L.N., Zhang, Y (2002). Analysis of amino acids from silkworm chrysalis. Yunnan Chem. Technol. 6, 22–23.

Garcia-Lara J, Needham AJ & Foster SJ (2005) Invertebrates as animal models for Staphylococcus aureus pathogenesis: a window into host-pathogen interaction. FEMS Immunol Med Microbiol 43: 311–323.

Gui, Z., Chen, J., Chen, W., Zhuang, D (2001). Effect of silkworm powder (SP) lowering blood-glucose levels in mice and its mechanism. Sci. Sericulture 27, 114–119.

Jiang SJ (1996). Effect of white muscardine silkworm on the cancer. *Edible fungus.* 18: 40.

Kaito C, Morishita D, Matsumoto Y, Kurokawa K & Sekimizu K (2006) Novel DNA binding protein SarZ contributes to virulence in Staphylococcus aureus. Mol Microbiol 62: 1601– 1617.

Yukuhiro K, Sezutsu H, Itoh M, Shimizu K, Banno Y (2002). Significant levels of sequence divergence and gene rearrangements have occurred between the mitochondrial genomes of the wild mulberry silkmoth, Bombyxmandarina, and its close relative, the domesticated silkmoth, Bombyxmori. Molecular Biology and Evolution, 19(8):1385-9.

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ALTERNATIVE MODELS IN GENOTOXICITY TESTING

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INTRODUCTION

In the present fast changing society man is in the mad rush to meet his demands. It seems he has forgotten the basic principle that is to weigh knowledgeably the cost of change against the benefits. In the last 7-8 decades there has been influx of huge number of agents to our environment to which we all are exposed. Although the credibility of technologies in modernizing our lives remains unquestionable, its products and byproducts may have harmful effects on our health and genetic material. A heavy mutational load hangs over the whole mankind. Any increase in this mutational load simply hastens the pace of the disaster in which not only the existing population will be affected but also many subsequent generations will be caught in its lingering shadow.

HISTORY

Almost all environmental agents especially chemical agents are capable of affecting our genetic material - a new subject "Genetic Toxicology" emerged. The first convincing evidence of the influence of environmental agents on the genetic constitution of future populations comes with the discovery by H. J. Muller (1927) - "Artificial transmutation of the gene". Immediately, of course during World War II, when mustard gas was shown to induce mutation in fruit flies. Our environment is abused by a large number of

chemical agents (medicines, pesticides, cosmetics, hair dyes, food additives, industrial effluents etc.). The situation is very alarming since environmental toxicants have varieties of adverse effects on biological systems including man. First successful work on chemical mutagens was done by Aurbach and Robson (1944) in animal and by Oelker (1943) in plants.

In this lecture/article I would concentrate on the genotoxic effects of chemicals since I devoted almost three decades to screen different test systems (mammalian and sub-mammalian) employing cytogenetic assays. Since large number of chemical agents to be screened for genotoxicity, it is very important to fix up the priority of the group of chemicals to be taken first for the said purpose. According to health Protection Branch (HPB) of genotoxicity committee (USA) the agents to which human exposure is high or wide spread merit the greatest initial concern. In India majority of population depend on agricultural product to earn their livelihood. In order to grow crops varieties of pesticides are being used and naturally large number of people are exposed to these harmful chemicals. This was back background of our work and some widely used pesticides were selected for genotoxicity testing in mouse system. Good numbers of pesticides (50 ±) have been screened for genotoxic potential employing cytogenetic assays–mainly chromosome aberration. Almost all pesticides produced positive results.

USE OF ALTERNATIVE MODELS (ETHICS AND COMPULSION)

As usual for all these experiments we had to sacrifice large number of mice. Such type of act of human beings is cruelty in the name of profit and knowledge. People from different sections of the society, including researchers in the area all over the world started advocating against animal cruelty and as a result many countries adopted

'Animal act". India is a member country of this act. Besides many Animal lovers' group and NGO's started campaign against animal cruelty.

Gradual random use of mouse became a serious problem. Options were very clear- to abandon research with mouse or to switch over to some scientifically sound system for the said purpose. In this confusing situation I started thinking about a dependable alternative test system. Immediate choice was chick test system. The reasons for opting chick as an alternative to mammal were: Easily available, Easy to handle, Cost effective, Large number of dividing cells in bone marrow and Animal act not strictly adhered to.

Before presenting our results, I would like to elaborate the basic principle of alternative research. The publication of legendary book by Russell and Burch (1959) gave new turn to the whole concept. The title of the book was "Principles of Human Experimental Technique" where the authors classified humane techniques under the headings of replacement, reduction and refinement now commonly known as 3Rs.

Replacement

Replacement may be of two types such as a) partial or relative replacement and b) absolute replacement. In "relative technique" animals are still required. Use of completely anesthetized animals (completely free from inhumanity; isolated cells, tissues or organs of vertebrates. Tissue culture forms a bridge to the next major division- "absolute replacement". Under this category, the techniques are study of metazoan parasites (nematodes, cestodes etc.) *in vitro*; use of higher plants etc. Because of time factor this method of replacement has gained little importance. And also use of microorganism. Lastly use of non- living physical and chemical system.

Reduction

This is achieved by alternative technique by trial and error negotiating with statistics, by reducing number of animals for a particular experiment. Avoidance of overlapping research experimentation; by demonstration of experiments through electronic media etc.

Refinement

The objective of refinement is simply to reduce the distress imposed on those animals that are still used. For example, animals of lower taxa can be used.

OUR WORK IN THE DEPARTMENT (CHICK SYSTEM)

As mentioned above, we stopped using mouse for genotoxicity testing and switched over to neonatal chicks and according to our ongoing project we tested some commonly used pesticides employing bioassays like bone marrow chromosome aberration and micronucleus test. All pesticides produced significant results and considered to be positive. So a new technique was standardized i.e., chick as a alternative to mammalian system (Bhunya and Jena, 1993: Mutation Research). All results were published in Mutation Research, successively, and also in other peer reviewed journals.

Along with chick system we extended our work using amphibian system and fish system. But in amphibian and fish bioassay like micronucleus assay from peripheral blood was done. And to our expectation these two test systems were proved to be dependable. In amphibia *Bufo melanostictus* and in fish *Heteropneustes fossilis* and *Anabas testudeneus* were used. Results were published in journals (Sahoo and Bhunya, 2004).

CONCLUSION

Lastly, I must and you all would agree with me that in 21st century for obvious reason, we shall have to depend on alternatives for biological researches and education, and genetic toxicity is not exception to that. We must foresee that researches in biomedical science with animals would be more humanely and as a result cruelty to animals will be reduced speedily.

REFERENCES

Auerbach C and Robson JM (1944). Production of mutations by allyl isothiocyanate. Nature 154: 81.

Bhunya SP and Jena GB (1993). Studies on the genotoxicity of monocrotophos, an organophosphate insecticide, in the chick in vivo test system. Mutat. Res. 292: 231-239.

Bhunya SP and Sahoo SN (2004). Genotoxic potential of carbaryl in the peripheral blood erythrocytes of *Anabas testudineus.*51 (4): 417-423.

Muller HJ (1927). Artificial transmutation of the gene. Science, 66 (1699): 84-87.

Oehlkers F (1943). Die Auslosung von Chromosomenmutationen in der Meiosis. Durch Einwirkung von Chemikalien. Z. Indukt. Abstammungs. Vererbungsl, 81: 313-341.

Russell WMS and Burch RL (1959). The principles of humane experimental technique. Wheat Hampstead (UK): Universities Federation for Animal Welfare.

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SEX COMB VARIATION IN NATURAL AND LABORATORY POPULATIONS OF *DROSOPHILA MELANOGASTER*

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ABSTRACT

Morphological trait variation and their evolution has become one of the prime interests of modern evolutionary biologists. Nature always acts as a showcase displaying various morphological traits among closely related species leaving some clues to understand the underlying mechanism of their diversity. Insects, especially *Drosophila*, are ideal model systems for studying this diversity and exploring more about their ecological and evolutionary consequences due to availability of a large number of species. One such sex based secondary sexual character in Drosophila is sex comb. The sex combs restricted to the forelegs of males are known to help in tight pairing with females during copulation and exhibit great divergence among the

species of *Drosophila*. Though there is evidence of a strong relation between the size of sex comb and choice of mating partner, it is highly species specific though the reason behind this is not yet known. To address this issue, a study on the sex comb teeth number (SCTN) variation both at intra-and inter population level involving natural and laboratory populations of *Drosophila melanogaster* was undertaken. The result clearly shows significant variation in the pattern of sex comb among natural vs laboratory populations, which is absent in natural vs natural populations. The high range of SCNT in laboratory population indicates that they are under sexual selection and are more successful reproductively.

Key words: Sex comb pattern, natural vs laboratory populations, *Drosophila melanogaster*

INTRODUCTION

Darwin introduced the concept of sexual selection as the competition among individuals of one sex to possess their sex partner. However, the definition has taken a new since when it is now known that selection arises from differences in mating success (Jones and Ratterman, 2009). Morphological traits play a major role in achieving mating success, which in turn is a driving force in the process of speciation, both in the formation of reproductive isolation and during ecological diversification (Bradshaw et al., 1998; Albertson et al., 2003). The sex comb is one of the most rapidly evolving male-specific traits consisting an array of modified black bristles (teeth) restricted to the male (foreleg region) in *Drosophila*, making it an attractive model to study morphological trait variation and the mechanism underlying its evolution. Sex comb is known to help in grasping the female's abdomen and genital during copulation (Lachaise and Chassagnard, 2002). Previous studies on sex comb

show that this trait is under rapid evolution and exhibits great divergence among the species of *Drosophila* (Kopp and Barmina, 2005). Hence it is widely used in identification of species especially where other taxonomic characters are either absent or less prominent. Males with the largest sex combs sired more offsprings than less ornamented individuals, which clearly indicates the role of the sex comb in reproductive success (Polak and Simmons, 2009).

In addition, it has been observed that the relation of sex comb size to mating success is very species specific (Kopp, 2011), a selective advantage for fewer sex-comb teeth when observed in *D. simulans*. It is just the reverse in *D. bipectinata* (Polak et al., 2004). Therefore it is clear that the individuals having more SCNT are not always more privileged and under sexual selection. However, due to scarcity of intra-specific data in different population and sufficient interspecific as well, it is difficult to reach any strong conclusion behind the variation existing both in inter- and intra- species level. Present study aims at observation of the SCNT and their range variation in males of natural and laboratory populations in *D. melanogaster*, which has a very clear sex comb on Tar 1 of the front legs, to understand the nature of effective selection acting on this trait.

MATERIALS AND METHODS

Samples of *Drosophila* used

Population samples of *Drosophila melanogaster* were collected during October-November, 2012 from two geographical regions of Northern India namely Kangra, Himachal Pradesh and Noida, Uttar Pradesh. Both isofemale and mass cultures were established in the laboratory and maintained at 25^{o} C with regular cornmeal-molasses-

agar culture media. In the present study, five isofemale lines of Kangra (KA7, KA25, KA26, KA27 and KA31) and two isofemale lines of Noida (NA1 and NA15) were considered as natural populations as the study was carried out within one month of the establishment of laboratory. A mass culture of hybrid population has also been established by mixing 10 males and 10 females from each isofemale lines of KA and NA and then maintained for two months only by mass transferring. In addition, a mass culture of *D. melanogaster* (ND07*),* collected from New Delhi (10 kms away from Noida) and established in laboratory in 2007, was used to represent the laboratory population.

OBSERVATION

20 male *Drosophila* flies from each isofemale lines of natural (NA, KA), hybrid (NA and KA) and laboratory (ND 07) population were separated out. Temporary slide of only one foreleg of each male was prepared by directly mounting with few drops of glycerol. The prepared slides were examined under stereoscope for their sex comb position and SCNT (Fig. 1).

Statistical analysis

Student's t-test (two tailed; independent samples) was performed to compare the SCNT among different populations of *D. melanogaster* as described above and the results were analysed at 38 degrees of freedom (df=N1+N2-2= 20+20-2) at both 95% and 99.9% confidence level. In case of KA (compiled result of all isofemale lines), the sample size considered the same 20 as have been taken average of each individual from each isofemale lines).

RESULTS AND DISCUSSION

During the present study, the size of sex comb has been analyzed in both natural and laboratory populations of *Drosophila melanogaster*. The natural population was collected from Kangra, a valley situated at an average elevation of 2000 ft and within Western-Himalayan sub-range and from Noida only 10 km away from New Delhi. These two places are located at a distance of 460km apart. The overall range variation in SCNT in KA lines and NA lines was observed to be 7-13 and 8-11 respectively (few of them has been shown in Figure1). Number of individuals carrying particular size of sex comb has been calculated for both natural and laboratory populations (Fig. 2A). The individuals having SCNT 9 and 10 are found to be most common in both the natural population. Earlier, the sex comb variation in natural population of *D. melanogaster* has been reported to be 7-14 (Kopp and Barmina, 2005; Kopp, 2011), which is very similar as per the present observation (7-13) in natural population. Sex comb pattern was also studied in few species belonging to the *melanogaster* subgroup, the result of which shows that the mean sex comb tooth number varies from 7 teeth in *D. teissieri* to 14 teeth in *D. mauritiana and D. melanogaster* itself is intermediate 127 between the two extremes with 10–11 mean sex-comb teeth/leg (Graze, 2007).

The average number of sex comb teeth was calculated for each isofemale line and comparisons was made both at intra- and inter- geographical population. The average number of SCNT calculated from all five isofemale lines of KA and two isofemales from NA are found to be 9.6 and 9.52 respectively which shows no significant variation exist between these two geographically separated population as the result obtained from student t test (Table 1). However, the range of SCNT is found to be

slight higher (7-13) in isofemales of Kangra than what observed (8-11) in the isofemale lines of Noida (Table 1). The highest range variation in KA population compared to NA population may be due to the variation in climatic condition. The temperature is very suitable throughout the year in Kangra except few winter months (Dec. to Feb.) and the temperature varies from 5-30^{0}C on average, where the temperature in Noida has a very higher range, i.e., 9-45^0 C on average. It is observed during the collection that *D. melanogaster* prefer cold environment and frequency distribution is quite high in Kangra than in Noida (unpublished data). Also it has been observed that *D. melanogaster* is available throughout the year in Kangra due to suitable environment, whereas, completely unavailable in Noida during summer. According to Druger (1967), the temperature affects the phenotypic trait with a positive response in both ways, i.e., when cultured in high and low temperature. He also opines that the mean number of scutellar bristles decreases with an increase in temperature. The narrow range found in NA populations may be due to environmental stress, the most favoured SCNT (9, 10 etc.) may got selected and the range narrowed down to 8-11. The average number of sex comb teeth of hybrid natural population (NA-KA mixed mass culture as mentioned above) is found to be 9.56, which corroborates our finding in individual population of KA and NA (Table 1, Fig. 2B). However, the range lies in between the observed range of both individual populations, i.e. 8-12. In compiling both the data of natural populations (NA and KA), the SCNT varies from 7- 13 and the average found to be 9.5. However, drastic change has been observed in laboratory population (ND 07), where the SCNT varies from 10-14 and the average comes to be 11.95 (Table 1). Significant variation was observed when SCNT has been compared between natural (compiled data of KA and NA) and

laboratory population (Table 1, Fig. 2B) and student t test result shows significant variation ($p<0.001$). The range of SCNT was also found to be narrowed down to 10-14 in comparison to the range 7-13 as observed in natural population. Most interestingly, individual wise comparison of sex comb size shows prominent difference in natural vs. laboratory population and individuals having 11-12 number of teeth were found in more frequency (Fig. 2A) in laboratory population, whereas, 9-10 sex comb teeth are most common in both natural population and few individuals found having SCNT 13-14, which strongly supports the earlier report that extra-large sex comb size(> 11) are not favoured and they might be acting as barrier during copulation (Ahuja & Singh, 2008; Kopp, 2011). There may be two possible reasons in explaining the variation observed in case of natural vs. laboratory population. First, the individuals having 10 and above SCNT are preferably more successful in mating as evidenced from individual result (Fig. 2A). So there is a positive selection pressure acting on selecting the high range of SCNT. Interestingly, not a single individual was found having SCNT below 10 in laboratory population. Frequency distribution of SCNT among individuals was quite similar in both the natural populations (Figure 2A). However, the bottleneck effect cannot be ignored completely in laboratory population due to its small size in compared to the natural population, though large number of flies (>50) are transferred each time during maintaining the culture. Interestingly, in conservation genetics, it has been reported that small population size might actually experience an increase rather than a decrease in the rate of adaptation (Hoffmann et al., 2003), which is very much evident in the present result. In our opinion, there is a positive selection pressure on selecting higher number of SCNT which is clearly observed in laboratory population and no such observation is found

in natural population. This may be due to the selection which is more effective in laboratory population because of small population size. Very recently, study has been undertaken to estimate the benefits of this highly diverged trait in sexual selection in two species of *Drosophila* and result obtained does not support the earlier report as there is lack of evidence of female biasness for the size of the sex comb present in males (Snook et al., 2013). However, it has also been reported that SCNT influences fertilization success (Ahuja and Singh, 2008). So still, the role of sex comb and the underlying mechanism of their rapid evolution remains a mystery. This study is the first to analyse the range variation of this secondary sexual trait among natural and laboratory population, which are planned to be with some more populations including the South Indian populations in future. This may give a clear picture on the slope of the rate–temperature relationship with respect to this trait. Although it is difficult to draw strong conclusions regarding the causative mechanism due to controversial opinion of some workers about their association with mating success, the data set can inspire to extend this study with more natural population and in more species and finally can help in driving our thought process to future theoretical and empirical exploration.

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Fig 1 Foreleg of *D. melanogaster* with different SCNTs on the tar 1 (in decreasing order; 13, 11, 9, 8).

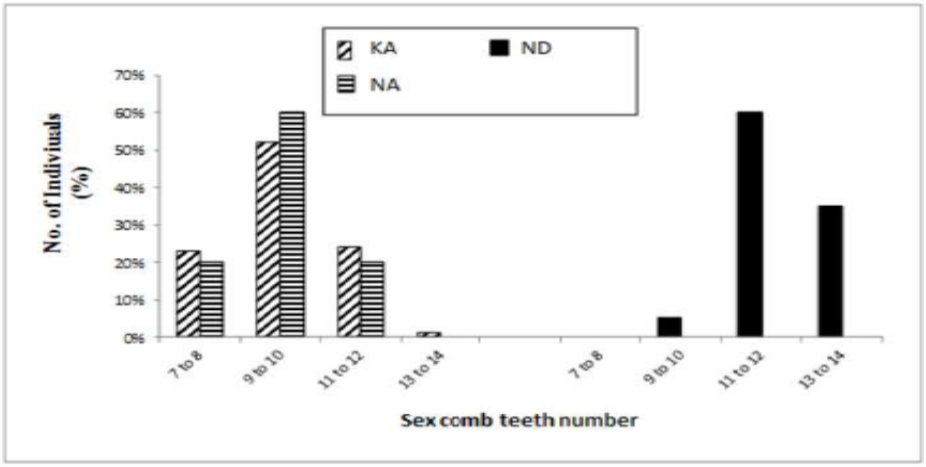

Fig 2A No. of individuals (%) with different SCNT in natural and laboratory population.

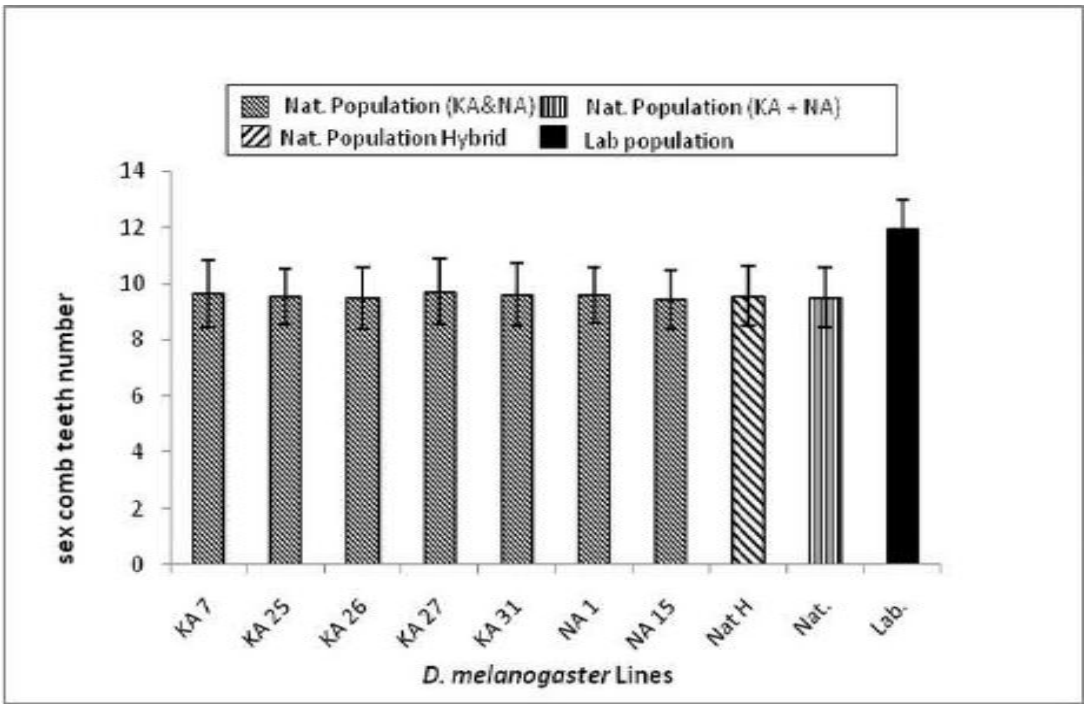

Fig. 2B: Average (**± SE)** SCNT in all KA and NA isofemale, hybrid, Nat. (combined KA and NA) and ND lines of *D. melanogaster*

Table1. Average number of sex comb teeth and their range variation in natural and laboratory population and student t test results

Drosophila Population	**N (No. of flies observed)**	**Range (sex comb teeth no)**	**Avg.± S.E**	**Student's t-test between populations**	**t values**
Natural Population				KA7-KA25	0.802
KA(5 Isofemale lines)	20*5	7-13	9.60 ± 1.10	KA25-KA26	0.889
				KA26-KA27	0.625
				KA27-KA31	0.816
				KA 31- KA7	0.910
NA (2 Isofemale lines)	20*2	8-11	9.52± 1.02	NA1-NA15	0.653
Natural Population (NA+KA)	140	7-13	9.50 ± 1.06	Nat.Vs. Nat. NA - KA	0.747
KA x NA hybrid	40	8-12	9.56 ±1.06	-	-
Laboratory Population ND 1	20	10-14	11.95 ± 1.02	Nat.Vs. Lab. ND 1 - (KA+NA)	7.480*

REFERENCES

Ahuja A and Singh SR (2008) Variation and Evolution of Male Sex Combs in *Drosophila*: Nature of Selection Response and Theories of Genetic Variation for Sexual Traits. *Genetics,* 179, 503–509.

Albertson RC, Streelman, JT and Kocher TD (2003) Directional selection has shaped theoral jaws of Lake Malawi cichlid fishes. *Proc. Natl. Acad. Sci.USA* 100, 5252–5257.

Bradshaw HD *et. al*. (1998) Quantitative trait loci affecting differences in floral morphology between two species of monkey flower (Mimulus). *Genetics,* 149, 367–382.

Druger, M (1967) Selection and the effect of temperature on scutellar bristle number in Drosophila. Genetics, 56(1), 39-47

Graze MR *et al*. (2007) New Candidate Genes for Sex-Comb Divergence between *Drosophila mauritiana* and *Drosophila simulans*. *Genetics,* 176, 2561 –2576.

Hoffmann AA, Hallas RJ, Dean JA and. Schiffer M (2003) Low potential for climatic stress adaptation in a rainforest Drosophila species. *Science,* 301: 100–102.

Jones GA and Ratterman LN (2009) Mate choice and sexual selection: What have we learnt? Since Darwin? *PNAS,* 106, 10001-10008.

Kopp A (2011) *Drosophila* sex combs as a model of evolutionary innovations. *Evolution & Development,* 13, 504–522.

Kopp A and Barmina O (2005) Evolutionary history of the *Drosophila bipectinata* species complex. *Genet Res.,* 85, 23–46.

Lachaise D and Chassagnard M (2002) Divergence of sex comb phenotypes in the *Drosophila fima* species group and radiation on Afrotropical Ficus, including

five new species from East Africa and Madagascar (Diptera: Drosophilidae). *Ann. Soc. Entomol. Fr. (N.S),* 38, 79–99.

Polak M and Simmons WL (2009) Secondary 228 sexual trait size reveals competitive fertilization success in *Drosophila bipectinata* Duda. *Behav Ecol.,* 20, 753–760. Snook R, Gidaszewski AN, Chapman T (2013) Sexual selection and the evolution of secondary sexual traits: sex comb evolution in *Drosophila*. *J. Evol. Biol*., 26, 912-917.

Polak M, Starmer WT and Wolf LL (2004) Sexual selection for size and symmetry in a diversifying secondary sexual character in *Drosophila bipectinata*. *Evolution,* 58, 597–607.

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HEMATOLOGICAL AND CARCINOGENIC BIOMARKERS OF INDUSTRIAL EFFLUENT INDUCED TOXICITY IN AQUATIC SPECIES

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ABSTRACT

The present research work aims to study the toxicity due to pollution of Chambal River (Nagda, M.P.India) at three different habitats of *Mystus tengara* (cat fish). Water from three different habitats such as upstream and downstream of Chambal River were collected in different seasons during 2011-12 and carcinogenic and hematobiochemical parameters were assessed. Fish were sampled in all seasons from all sampling stations. Blood was assayed for selected hematological parameters like haematocrit, hemoglobin, red blood cell counts, white blood cell counts, erythrocyte sedimentation rate, total plasma protein and plasma glucose and circulating nucleic acids of carcinogenic origin (DNA, RNA). The derived hematological indices of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated. Result showed that the value of haematocrit and Erythrocyte Sedimentation Rate (ESR) values were upregulated in fish collected from polluted area.

ESR is negatively correlated with RBC count. Compared to those of the normal fish, the Hb content and RBC count of the diseased fish, *Mystus tengara* was much low. Significant and positive correlation was noticed between the hemoglobin and packed cell volume. The value of MCV, MCH and MCHC of infected fish were also seen to fluctuate compared to those of the normal fish. Results clearly indicate that total protein, DNA and RNA content were low in the fish of station I as compared II and III. The influence of stress due to pollution on *Mystus tengara* was correlated with water quality and blood indices. This study can be used to determine the ecologicalstatusof River water along with dwelling organisms. It was concluded that the industrial and sewage contamination of Chambal River induced several biochemical, hematologic and mutagenic alterations in *Mystus tengara*. We recommend these parameters as indicator of toxicity related stress.

Keywords-Effluent, circulating nucleic acids, hematology, Biomarkers

INTRODUCTION

To define and measure the effects of pollutants on aquatic system both hematological and carcinogenic biomarkers have attracted a great deal of interest. The pollutants can become a major threat to the health of the aquatic ecosystem due to their accumulation in the tissues of various species. (Newman, 1998; Walker et al., 2004). Also, the pollutants disperse through the biomagnification into higher trophic levels across the food chains reaching areas of significant human activity (Galloway et al., 2002; Clark, 2006). Pollutants may induce carcinogenesis directly or indirectly at different trophic levels. Consumption of these fish and fish products may induce different types of cancer in nearby human habitat. In order to evaluate

the environmental impact of these pollutants on the ecosystem, it has become important for a rapid assessment of their toxic and cancerous effects on the aquatic organisms.

The use of hematological, biochemical and mutagenic biomarkers in aquatic organisms is very important in order to address the broad spectrum of industrial and agricultural chemicals entering the environment (Reddy and Baghel, 2010; Reddy and Renusingh, 2011; Reddy and Baghel, 2012). The blood indices, changes in circulating nucleic acids, total protein, liver and kidney function tests etc. are commonly used in assessment studies of pollution. Such eco- toxicological studies can establish the risk of environmental pollution to key components in the ecosystem. Both laboratory and field studies have shown that pollutants present in water can modify biochemical and immune system functions (Dick and Dixon, 1985; Hutchinson and Manning, 1999). Assessment of toxicity in sensitive species like fish can be used as an early warning of population decline and as an ecologically relevant endpoint (Carlsson, 2007).

Most of the DNA and RNA in the body is located within cells. But a small amount of nucleic acids can also be found circulating freely in the blood. The term **"circulating nucleic acid"** (CAN) refers to segments of DNA or RNA found in the bloodstream. Available evidence suggests that these molecules are released in circulation in the form of nucleosomes through apoptosis and necrosis. Detection of CNA in blood pull is a new dimension in early cancer detection. For diagnosis of breast cancer CNAs can also be a valuable tool where it is difficult to get the tissue samples (Schwarzenbach, 2013). As unlike mammalian red blood cells, fish erythrocytes contain nucleic acids and the detection of CNA in blood pool can light

into the carcinogenic effluents in river ecosystem. Even though CNAs are shown to have promising diagnostic utility as biochemical and genetic biomarkers for a variety of pathologies especially cancer, there is deficiency in our knowledge about the functional significance of CNAs. Currently, the biological effects of CNAs are unknown and this area has left largely un-touched. Therefore, attempt has been made to estimate serum circulating nucleic acids hematologic alternations biomarkers in catfish *Mystus tengara* at different sites of Chambal River, in different seasons of the year at Nagda.

MATERIAL AND METHODS

Study Area: Chambal River in Nagda is very close to Tropic of Cancer at 23’27 N and 75’25 and 517 meters above sea level (MSL). Chambal River at Nagda receives water from different units of industrial complex including municipal sewage from the town. Water samples were assessed for various parameters in three different research stations and in three different seasons of the year during 2012-13. Physico chemical analyses of water like pH, electrical conductivity (EC), total dissolved solids (TDS), total hardness (TH) dissolved oxygen (DO), biochemical oxygen demand (BOD) and chemical oxygen demand (COD) were detrmined (Anonymous, 1985). Healthy specimens of *Mystus tengara* (Ham.) of approximately same size (irrespective sex) were collected from different stations in different seasons. Blood was collected from the caudal region by puncturing the peduncle and processed for various blood parameters. Haematocrit and packed cell volume (PCV) were estimated by the use of a micro capillary reader. Erythrocytes (RBC) were counted immediately after blood collection in hemocytometer (Improved Neubauer. Weber

Scientific Ltd.) (Wintrobe, 1934). Hemoglobin concentration was measured by the cyanmethaemoglobin method (Houston, A. H. 1997) using a commercially available kit (Span, India). Mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), and Mean corpuscularvolume (MCV) were calculated using the formulae (Larsen and Snieszko,) for estimating the circulating nucleic acid as tool of carcinogenic biomarker. Circulating DNA and RNA was estimated by the methods as described by Plummer (). Plasma total protein was estimated according to Lowry et al. (1951).

RESULTS

Water analysis: The results of physico chemical parameters of water obtained from Chambal River for all the three different stations are measured (Table.1). Results clearly indicate that the physico chemical parameters monitored in station 2 and 3 showed high levels of BOD, COD, TDS, TSS, EC and low DO in all seasons and exceeds the limits WHO standards. This must have been as a result of the nature of effluents discharged from the industries. However, pH and temperature did not show much variation in all sampling stations in all seasons.

Table 1 Changes in physico chemical parameters of Chambal River at Nagda in different stations and different seasons of the year

SESSION		**RAINY**			**WINTER**			**SUMMER**		
Sl. No	Parameter	S 1	S 2	S3	S1	S2	S3	S1	S2	S3
01	**BOD, mg/L**	11.3± 0.01	24.2± 1.1	25.43± 1.1**	10.0± 0.01	54.4± 4.2**	51.2± 4.8	12.2± 0.09	45.5± 3.8**	46.2± 4.1
02	**COD mg/L**	1.8± 0.01	15.1± 1.1**	14.0± 0.9	12.0± 1.4	38.4± 2.6**	32.0± 1,8	10.0± 0.9	34.0± 2.1**	24.1± 1.8
03	**DO mg/L**	7.2± 0.1	6.9± 0.02	6.8±** 0.09	7.5± 0.1	8.8± 0.8	8.9±** 0.07	7.9± 0.4	8.5±** 0.8	9.2± 4.3
04	**EC,umho/cm**	88± 7.6	80.2± 9.7	50± 7.6**	85± 2.3	198± 12.1	419± 13.2**	62± 2.3	310± 11.4	650± 23.6**
05	**pH**	7.2± 0.1	8.7± 0.1	8.9±** 0.1	7.1± 0.09	9.4±** 0.02	8.8± 0.9	6.8± 0.43	9.8± 0.7**	9.9± 0.64**
06	**TDS mg/L**	32.13± 0.87	162.0± 3.3**	122.1± 9.9	110.10± 5.3	448.1± 6.3**	392.1± 3.2	112.1± 4.5	410.1± 12.1**	415.5± 13.4
07	**Temperature ‘C**	24.1± 0.1	24.0± 0.1	24.2± 0.09	22.4± 0.33	23.1± 0.44	22.8± 0.67	30.1± 0.23	31.0± 0.21	30.5± 0.54
08	**Total hardness**	250± 12.1	233± 18.4**	239± 16.1	200± 9.67	1400± 34.3**	1380± 34.3	280± 8.11	1900± 46.7**	1780± 39.8
09	**TSS mg/L**	44.1± 1.2	42.1± 5.3	44.0± 3.3NS	26.1± 2.2	148.1± 9.6**	140.2± 8.76	21.1± 7.6	128.0± 5.6**	126.0± 6.6

Table 2 Annual changes in hematological and bio chemical parameters in *Mystus tengara* of Chambal River in different segments and different seasons of the year

SESSION		**RAINY**			**WINTER**			**SUMMER**		
Sl No	Parameter	S 1	S 2	S3	S1	S2	S3	S1	S2	S3
01	DNA (mg/L)	2.1± 0.3	3.1±0. 2	4.7±0. 1*	1.8±0 .1	8.1±2. 1**	9.1±0.3 *	2.2±0 .1	12.1± 0.4*	13.9 ±0.5
02	RNA (mg/L)	22.1 ±1.1	26±1. 8	24.1±0 .2NS	16.1± 1.1	29.3±1 .1**	44.1±2. 1*	21.1± 0.6	34.1± 3.1*	46.1 ±4.1 **
03	Total protein.m g/L	3.4± 0.32	2.95± 0.11*	1.89±0 .37**	3.5±0 .34	3.0±0. 28NS	2.4±0.2 2*	3.40. 22±	2.8±0. 41*	2.9± 0.44 *
04	Plasma Glucose (mg/L)	234. 5±6. 1	174.3 ±2.3	168.1± 3.4***	239.1 ±6.1	186.1± 2.3	164.5± 3.4***	241.4 ±6.1	164.3 ±2.3	168. 1±3. 4***
05	Haematoc rit (%)	31.1 ±0.9 4	24.8± 0.25	19.2±0 .18**	31.1± 0.94	24.8±0 .25	19.2±0. 18**	31.1± 0.94	24.8± 0.25	19.2 ±0.1 8**
06	Hemoglob in(g/100m l)	13.3 ±0.8	9.71± 0.21	8.11±0 .42**	13.3± 0.8	9.71±0 .21	8.11±0. 42**	13.3± 0.8	9.71± 0.21	8.11 ±0.4 **
07	RBC(106 mm-3)	1.82 ±0.1 2	2.22± 0.89	2.23±0 .23**	1.82± 0.12	2.22±0 .89	2.23±0. 23**	1.82± 0.12	2.22± 0.89	2.23 ±0.2 3**
08	ESR (mm/h)	21.8 0±1. 00	18.63 ±1.1	19.1±0 .9	21.80 ±1.00	18.63± 1.1	19.1±0. 9	21.80 ±1.00	18.63 ±1.1	19.1 ±0.9
09	MCHC (%)	29.1 1±2. 11	25.1± 1.0	23.3±2 .11**	29.11 ±2.11	25.1±1 .0	23.3±2. 11**	29.11 ±2.11	25.1± 1.0	23.3 ±2.1 1**

10	MCH (g)	78.1 ±9.1 1	72.1± 3.8	59.8±6 .51**	78.1± 9.11	72.1±3 .8	59.8±6. 51**	78.1± 9.11	72.1± 3.8	59.8 ±6.5 1**
11	MCV (ug)	178. 3±12 .2	154.3 ±4.3	148.4± 5.3	178.3 ±12.2	154.3± 4.3	148.4± 5.3	178.3 ±12.2	154.3 ±4.3	148. 4±5. 3

= P<0.05 * = P<0.01 *** = P<0.001

Biochemical analyses: The circulatory DNA and RNA content in station 1 is significantly lower than other stations across the seasons. Both the values were found to be in a significantly increasing order at station 2 and 3 during winter and summer as compared to rainy season.The protein and glucose contents at Station 2 and 3 were having lower values (P <0.05) with respect to station 1 in all seasons. But only in station 3 there is significant increase in protein content from rainy to winter and summer with respect to station 1 in all seasons. The cDNA and cRNA content of fish was low from station I when compared to other research stations (P>0.001) and it was significantly higher in the fish of station II and III in all seasons of the year. The glucose value of fish from station II and III were significantly decreased in both winter and summer (P <001). The hematological indices of MCHC, MCH and MCV were similarly decreased in both station II and III in all seasons. But station 3 shows significant reduction with respect to station 1 for above parameters. The hemoglobin and haematocrit value also followed similar trend. However, there was an increase in red blood cell and decrease in erythrocyte

sedimentation rate of fish of station II and III compared to the station I of control fish (Table 2).

DISCUSSION

Physiological stress indicators such as hematological parameters could be useful to evaluate the effects of contaminant heavy metals on fishes. Several studies on different condition of experiments have confirmed that the hematological system as a whole is affected by exposure to pollutants, though the extent of hematological system is dependent on the type of pollutants, duration of exposure, species and even the strain of fish used. Results clearly indicate that there are seasonal variations in hematological parameters in different stations. Many factors like age, sex, pollution load, spawning time and genetic variation are responsible for these changes (Larson et al, 1984). Results also indicate that PCV value is positively correlated to the hemoglobin and erythrocyte sedimentation rate (ESR) while it is negatively correlated to the RBCs. Stress due to capture; handling and sampling procedures may also lower fish blood values (Hatting and Van Pletzen, 1974). Nutritional deficiency and secondary infections can also affect the erythropoiesis (Kelly, 1979) as nutrients like iron, copper, folic acid, thiamin, riboflavin and cynacobalamin are essential for bone marrow function. Reduction in the RBCs along with the reduction in hemoglobin are observed.

Even though blood index have proven useful for numerous diagnostic applications, the molecular diagnosis of diseases requires advanced technology. For these reasons, finding of circulating cell-free DNA in the blood of healthy and diseased individuals has gained increasing attention during the last year (Holdenrieder et al.,

2005). Most of the nucleic acids are located within cells, but a small amount can also be found circulating freely in the blood. These DNA, RNA and small RNA molecules are thought to come from dying cells that release their contents into the blood as they break down. Therefore, free circulating nucleic acids (CNAs) offer unique opportunities for early diagnosis of clinical conditions.

The results clearly indicate that DNA and RNA content in the fish from polluted stations II and III of Chambal River significantly increased ($P>0.01$) both in winter and summer but not in rainy season. It may be due to the over flow of the river which reduced the pollution load in all research stations. The increased levels of plasma CNA have also been reported in a number of clinical disorders like cancer, stroke, trauma, myocardial infarction, autoimmune disorders, and spawning – associated complications. (Vishnu Swaroop and Rajeswari, 2007). It is known that the products of DNA/RNA in plasma actually arise from cell death or by lysis of tumor cells which was also evidenced by histopatholoigcal alterations in various organs (Reddy and Renusingh, 2011). The presence of DNA and RNA in the plasma of *Mystus tengara* in the present investigation may be due to break down of blood cells, break down of any pathogens, e.g. bacteria or viruses, leucocyte surface DNA, apoptosis or necrosis of various organs (Holdenrieder et al., 2005). Necrosis or apoptosis is also a cause for the origin of Circulating Nucleic acids in Plasma and Serum (CNAPS) (Schwarzenbach, 2013). Some pollutants may act as carcinogen which is causing proliferation of erythroid progenitor cells and reflecting as increase in the TEC count in station 2 and 3 where the effect of pollution is more. Simultaneously the station 2 and 3 is signifying the reduced bioavailability of essential micronutrient requisite for mitosis of erythroid line reflecting as

microcytosis (low MCV) and synthesis of hemoglobin as that of microcytic hypochromic anaemia. Hematobiochemical alternation and increasing CNA can be cause of concern for public health issue as a predisposing factor for carcinogenesis in consumers level.

CONCLUSIONS

The investigation presented here, focuses on about the quality of the water in Chambal River, but it is equally important to extend the study to include properties of soil and microbial analysis. The other concern is the presence of carcogenic biomarkers in the water. The same in the long run may force the population to suffer different types of cancer which is dependant on the water of Chamble River. It is clear that comparison of circulating nucleic acids and blood indices can be used to identify and confirm the presence of xenobiotics and other toxic agents of fish.

REFERENCES

Anonymous (1985). Standard methods of for the examination of water and waste water. American Public Health Association (APHA), Washington DC, pp1244.

Carlsson and Gunnar (2007). *Fish and amphibians as test organisms for evaluation of effects caused by chemicals.* Sveriges lantbruksuniv., Acta Universitatis agriculturae Sueciae, 1652-6880 ; 2007:61 ISBN 978-91-576-7360-2

Clark RB (2006). Marine Pollution, 5th edition. Oxford University Press, Oxford

Dick PT and Dixon DG (1985) Changes in circulating blood cell levels of rainbow trout, *Salmo gairdneri* Richardson, following acute and chronic exposure to copper. J Fish Biol 26:475–481

Galloway TS (2002). Biomarkers in environmental and human health risk assessment. Mar Poll Bullet 53:606-613, Goetz, W. (1980). Diagnostik. Von Lebererkran- Kungen (In German). G.l.T. Verlag Ernst Giebeler, Darmstedt. Germany.

Hattingh HJ and Van Pletzen AJ (1974).The influence of capture and transportation on some blood parameters of fresh water fish, Comp.Biochem.Physical.4TA, 607-609.

Holdenrieder S, Stieber P, Chan LY, Geiger S, Kremer A, Nagel D and Lo YM. (2005). Cell-free DNA in serum and plasma: comparison of ELISA and quantitative PCR. Clin Chem. 2005 51(8): 1544-1546.

Hutchinson TH, Field MDR and Manning MJ (1999). Evaluation of immune function in juvenile turbot (*Scopthalmus maximus* L.) exposed to sediments contaminated with polychlorinated biphenyls. Fish. Shellfish Immunol. 9, 457–472.

Kelly WR (1979). Vetrerinary Clinical Diagnosis. 4thEdn, balliere Tindall London.

Larson AC, Haux and Sjobeck ML (1984). Fish Physiology and metal pollution, results and experience from laboratory and field studies. Ecotoxicol.Environ.Safety, 9:250-281.

Lowry OH, Rosebrough NJ, Farr AL and Randall RI (1951). Protein measurement with folin phenol reagent. J. Biol. Chem. 193: 265-275.

Newman MC (1998) .Fundamentals of Ecotoxicology. Ann Arbor Press, Chelsea, MI, persistent organic pollutant risk to human and wildlife. *Human Ecol Risk Assess* 9:303-324,

Plummer, D (1962). In 'Practical biochemistry'. Academic Press. London,.

Reddy PB and Baghel BS (2010). Impact of the industrial effluent on physico chemical characteristics of Chambal River at Nagda, M.P. India .Nature. Pollu. Tech (9), 3, 519-526.

Reddy PB and Renusingh (2011). Biomarker responses of industrial effluent in cat fish. In' Proc. Int. Conference. Green Tech. Envi.Conserv.265-269.

Reddy PB and Baghel BS (2012). Impact of Industrial waste water on the Chambal River and Biomarker responses in fish due to pollution at Nagda.M.P.India. DAV Int. J.Sci. -1, (1) 86-91.

Schwarzenbach H (2013). Circulating nucleic acids as biomarkers in breast cancer. Breast Cancer Res.15 (5):211.

Vishnu S and Rajeswari MR (2007). Circulating (cell-free) nucleic acids – A promising, non-invasive tool for early detection of several human diseases. Circulating (cell-free) nucleic acids – A promising, non-invasive tool for early detection of several human diseases. FEBS Letters 581, 795–799.

Walker CH, Hopkin SP, Sibly RM and Peakall DM (2004). Principles of Ecotoxicology, 3rd edition. Taylor and Francis, New York.

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EFFECT OF FERRIC CHLORIDE ON REDUCED GLUTATHIONE AND LIPID PEROXIDATION LEVEL OF LIVER OF *Hemidactylus leschenaultii*

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ABSTRACT

Hemidactylus leschenaulultii were collected locally from the campus of North Orissa University, Baripada. These were divided into four groups and treated with ferric chloride at different time interval (24 h, 48 h and 72 h) against the control. The protein content, reduced glutathione level (GSH) and lipid peroxidation (LPX) level were measured in the liver of *Hemidactylus* in both control and treated group. Variation of different parameteres of liver was observed at different time interval. On the basis of the results, it is concluded that ferric chloride at low dose altered the biochemical parameters and induces oxidative stress.

Key words: Ferric chloride, Reduced glutathione and lipid peroxidation, *Hemidactylus leschenaulultii*

INTRODUCTION

Oxidative stress, the imbalance between the production of prooxidant substances and antioxidant defences (Halliwell, 2007), is considered one of the most important ecological and evolutionary forces (Metcalfe and Alonso-Alvarez, 2010). Aerobic

metabolism implies the production of pro-oxidant substances (reactive oxygen/nitrogen species, RONS) (Finkel and Holbrook, 2000), which may react with molecular components of the cell such as lipids, proteins and nucleic acids, producing damages in cells' machinery (Halliwell, 2007). Organisms are protected against oxidative damage by enzymatic and non-enzymatic antioxidant defences,which work to maintain RONS levels at equilibrium and minimize RONS damages in the organism (Blokhina et al.,2003).

In 2010, 28% of the reptiles evaluated by the International Union for the Conservation of Nature and Natural Resources (IUCN) were enlisted as Critically Endangered (CR), Endangered (EN) or Vulnerable (VU) (IUCN, 2010) and environmental pollution has been recognized as one of the main contributing factors (Lange et al., 2009; Todd et al., 2010). Despite consistent calls for greater emphasis on research on reptilian ecotoxicology, the information regarding the response of reptiles to contaminants is inadequate (Sparling et al., 2010). In the present paper, Ferric Chloride was treated orally to *Hemidactylus leschenaulultii* and oxidative stress paramaters (lipid peroxidation and reduced glutathione) were measured and compared at different time interval.

MATERIALS AND METHODS

Animal

The lizards (*Hemidactylus leschenaulultii)* of various size (body weight ranging from 15-18g) for the experiment were collected locally from the campus of North Orissa University, Baripada, Mayurbhanj, Odisha from the month of October to March, 2015.

The lizards were kept inside the labelled plastic containers with small holes to allow air to pass into it. They were acclimatized for two days before the experiment.

Hemidactylus leschenaulultii (n=12) were divided into four groups of three animals each. Group I of controlled animals were treated with distilled water; Group II-IV as experimental were treated orally with 5µg ferric Chloride dissolved in distilled water. The treated animals were sacrificed after the time intervals of 24 hour, 48 hour and 72 hour (Group II-IV) whereas the control animals were sacrificed immediatetly (0h). The liver of both control and experimental group was dissected out quickly and kept at 0^0C. A 20% homogenate was prepared with phosphate buffer (pH 7.4). The liver homogenate was centrifuged at 4000 rpm for 10 minutes.

Protein estimation

Protein of the liver samples was estimated according to Lowry et al. (1951). To 0.1ml suitably homogenate of tissue, 0.4ml of distilled water was added. Then 5 ml of biuret reagent (containing alkaline Na_2CO_3, 0.5% $CuSO_4$ solution and 1% Sodium potassium tartarate solution in the ratio 100:2:2) was added and properly mixed up. After 10 minutes of incubation at room temperature, 0.5ml of Folin Ciocalteau phenol reagent (the commercial reagent diluted three times with distilled water) was added and incubated at 37^0C for 30 minutes at room temperature. Absorbance was measured at 700 nm against an appropriate blank. Absorbance was measured at 700 nm against an appropriate blank. Protein content was expressed as mg/g wet weight of the tissue and aqueous Bovine Serum Albumin (BSA) was taken as standard protein.

Reduced Glutathione

Reduced glutathione of the sample was estimated as per the method of Ellman (1959) method. 0.7ml of the tissue homogenate was added to 0.7ml of TCA. Then the substances in the tubes were centrifuged at 4000 rpm for 10 minutes. 0.5ml supernatant was added to 2.5ml of DNTB (DNTB 30 mM) diluted in PO_4 buffer 100 times. The absorbance was taken at 412 nm between 5 to 30 minutes against an appropriate blank.

Lipid Peroxidation

Lipid peroxidation of the sample was estimated as thiobarbiturate acid reacting substance (TBARS) by thiobarbituric acid (TBA) according to the method of Ohkawa et. al. (1979). 3.8ml of TBA reagent contain (2ml of 8.1% SDS , 1.5ml of 20% acetic acid of pH 3.5, 1.5ml of 0.8% aqueous solution of TBA, 5ml of distilled water and 1ml of BHT) was added to 0.2ml of suitably diluted post nuclear supernatant. After mixing thoroughly, the substance of the test tube was boiled in water bath for one hour. The tubes were cooled down to the room temperature. Then the substance of the tube was centrifuged at 4000 rpm for 10 minutes. The absorbance of the supernatant was measured at 532 nm against an appropriate blank.

RESULTS AND DISCUSSION

The natural colour of the lizard is brown. But the colour was observed to vary gradually with the treatment of $FeCl_2$ for 24, 48 and 72 hours respectively (Figs. 4-7).

Protein content (mg/g tissue) in the liver of treated *H. leschenaultii* was 49.014± 11.523mg/g tissue , 49.156± 3.222 mg/g tissue , 55.583 ± 6.914mg/g tissue and 44.503 ± 4.890mg/g tissue at 0 h, 24 h, 48 h and 72 h respectively (Fig. 1).

One way ANOVA revealed that the protein content at different time intervals in the liver of *Hemidactylus* was significant [F(3,11)=1.160,P=0.383]. Post Hoc analysis revealed that the protein content in the treated liver of *Hemidactylus* was significant at different time interval with respect to control ($P < 0.05$; LSD).

The LPX level (n mol TBARS /mg protein) in liver tissue of *H. leschenaulultii* treated with ferric chloride was 5.945±1.185n mol TBARS/mg protein , 7.610 ± 2.403 n mol TBARS/mg protein, 8.688± 1.064 n mol TBARS/mg protein, 7.227± 1.142 n mol TBARS/mg protein at 0 h, 24 h, 48 h and 72 h respectively (Fig. 2).

One way ANOVA revealed that the LPX level at different time intervals in the liver of *Hemidactylus* was significant [F (3,10)=1.604, P= .273]. Post Hoc analysis revealed that the LPX level in the liver of treated *Hemidactylus* at different time interval was significant in comparison to control ($P < 0.05$; LSD).

GSH content (µmol/g) tissue in liver of treated *H. leschenaultii was* 0.00204 ± 0.0004µ mol/g tissue , 0.00203± 0.0011 µ mol/g tissue, 0.00656± 0.0031 µ mol/g tissue and 0.00588± 0.0018µ mol/g tissue at 0 h, 24 h, 48 h and 72 h respectively (Fig. 3).

One way ANOVA revealed that the GSH level at different time intervals in the liver of *Hemidactylus leschenaultii* is significant[F(3,11)=4.412,P=.041]. Post Hoc analysis revealed that the protein content in the treated liver of *Hemidactylus leschenaultii* at different time interval was significant with respect to control ($P < 0.05$; LSD).

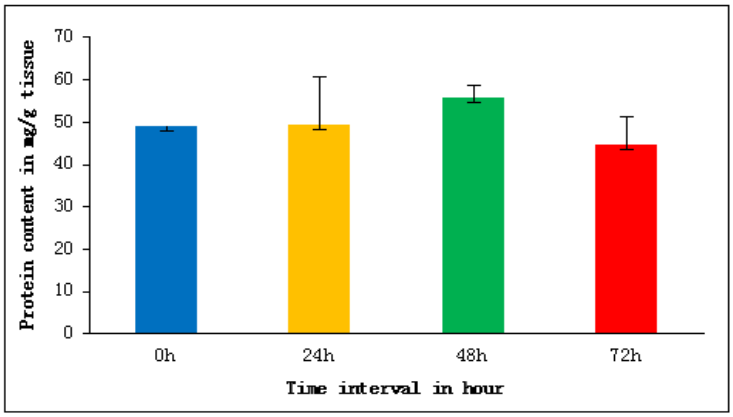

Fig. 1 Comparisons of protein content in liver tissue of *Hemidactylus leschenaultii* treated with ferric chloride at different time interval.

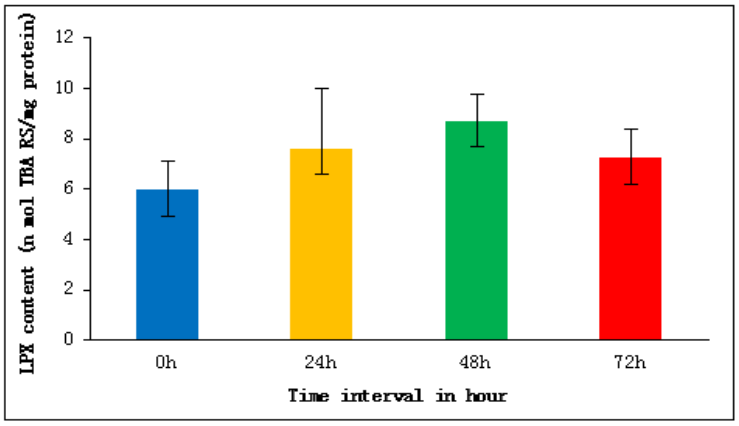

Fig. 2 Comparisons of LPX in liver tissue of *Hemidactylus leschenaultii* treated with ferric chloride at different time interval.

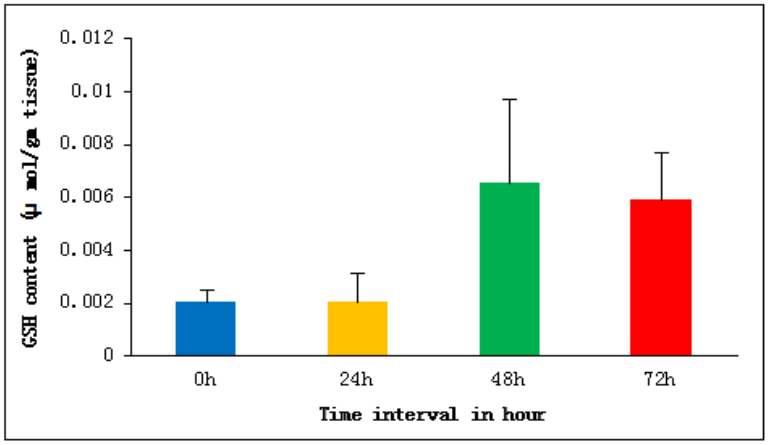

Fig. 3 Comparisons of GSH content in liver tissue of *Hemidactylus leschenaultii* treated with ferric chloride at different time interval.

Fig. 4 *Hemidactylus leschenaultii* with normal body and patches.

Fig. 5 *Hemidactylus leschenaultii* treated with $FeCl_2$ at (24 h) lighter in body colour and patches.

Fig. 6 *Hemidactylus leschenaultii* treated with $FeCl_2$ at (48 h) faint in body colour and patches.

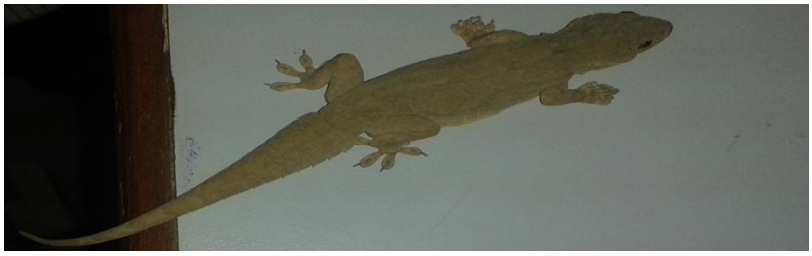

Fig. 7 *Hemidactylus leschenaultii* treated with $FeCl_2$ at (72 h) more faint in body colour and patches.

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REFERENCES

Anonymous (2010). IUCN, 2010. IUCN Red List of Threatened Species.

Blokhina, O., Virolainen, E., Fagerstedt, K.V., 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann. Bot. 91, 179–194.

Ellman, G.L., 1959. Tissue sulphydryl groups. Arch. Biochem. Biophy. 82:70-77.

Finkel, T., Holbrook, N.J., 2000. Oxidants, oxidative stress and the biology of ageing. Nature408, 239–247.

Halliwell, B., 2007. Biochemistry of oxidative stress. Biochem. Soc. Trans. 35, 1147–1150.

Lange, H.J.D., Lahr, J., Van der Pol, J.J., Wessels, Y., Faber, J.H., 2009. Ecological vulnerability in wildlife: an expert judgment and multicriteria analysis tool using ecological traits to assess relative impact of pollutants. Environ. Toxicol. Chem. 28, 2233–2240.

Lowry, O.H., Resbrough, N.J., Farr, A.L and Randoll, R.J. 1951. Protein measurement with the Folin-phenol reagent. J.Biol. Chem.19: 265-275.

Metcalfe, N.B., Alonso-Alvarez, C., 2010. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Funct. Ecol. 24: 984–996.

Ohkawa H., Ohishi, N. and Yagi, K. 1979. Assay of Lipid Peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. Physiol.118C (1):33-37.

Sparling, D.W., Linder, G., Bishop, C.A., Krest, S.K., 2010. Recent advancements in amphibian and reptile ecotoxicology. In: Sparling, D.W., Linder, G., Bishop, C.A., Krest, S.K. (Eds.) Ecotoxicology of Amphibians and Reptiles. Taylor and Francis, New York, pp 1–14.

Todd, B.D., Wilson, J.D., Gibbons, J.W., 2010. The global status of reptiles and causes of their decline. In: Sparling, D.W., Linder, G., Bishop, C.A., Krest, S.K. (Eds.), Ecotoxicology of Amphibians and Reptiles. CRC Press, New York, pp. 69–104.

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ROLE OF MELANOCYTES DURING CELL DEATH IN THE RESORBING TAIL OF ANURAN TADPOLES

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ABSTRACT

Thyroid hormone mediated metamorphosis in anurans involves programmed cell death in various tissues including tail. Cell death of tail tissues is followed by phagocytosis and degradation of cell debris by lysosomal enzymes in the macrophages. Recently, melanocytes have been found to play a pivotal role in degrading tail tissues during tail resorption in tropical anurans. Present study describes differential distribution of melanocytes in two species of anurans, *Polypedates maculatus* and *Duttaphrynus melanostictus* which also differ in the duration of larval period and tail regression. Variations in the activities of two lysosomal enzymes, acid phosphatase and cathepsin D in the two species seem to be associated with differences in the distribution of melanocytes.

Key words: Anura, tail, lysosomal enzymes, melanocytes.

INTRODUCTION

Thyroid hormone induced tadpole tail resorption is the earliest recognized example of programmed cell death (Brown and Cai, 2007). Tail resorption occurs by apoptosis, which involves death of cells followed by phagocytosis of cell debris by macrophages and other cells (Kerr et al., 1974; Gilbert and Frieden, 1981). Macrophages are activated by some primary autolytic changes in the tail tissue which is followed by neosynthesis of lysosomal enzymes in the macrophages. The lysosomal enzymes finally degrade the cell debris engulfed by the macrophages (Weber, 1968; Elinson et al., 1999; Mahapatra et al., 2001).

Recently, presence of large number of melanocytes around the degenerating tissues of regressing tail was reported in tadpoles of tropical anurans (Divya et al., 2010; Mahapatra and Mahapatra, 2011; Mahapatra and Mahapatra, 2012). Increase in number of melanocytes is due to the migration of the epidermal melanocytes to the dermis (Yasutomi, 1987). Melanocytes contain specialized lysosomes called melanosomes that contain a broad array of lysosomal hydrolases (Diment et al., 1995). Cytochemical staining for the activity of acid phosphatase demonstrated its presence within primary and compound melanosomes (Seiji and Kikuchi, 1965; Wolf and Schreiner, 1971). Thus, melanocytes seem to cause degradation of tail tissues in tropical anurans by acting as a source of lysosomal enzymes just like macrophages have been reported to do during tail resorption in other species of frogs. It is also possible that since melanocytes are present in large numbers in regressing tail of tropical anurans, they may be assuming the role of macrophages in those species in addition to macrophages.

However, such abundance of melanocytes has not been reported earlier in the regressing tail of other widely studied anurans like *Rana catesbeiana* (Komukai et al., 1986), *R. japonica* (Kinoshita et al., 1985) *Xenopus laevis* (Nishikawa et al., 1998) and *X. tropicalis* (Wang et al., 2008).

In anuran tadpoles metamorphosis begins (Sasaki et al., 1988) at Taylor and Kollros stage XX (Taylor and Kollros, 1946). The larval period consists of the pre-metamorphic (stages I-VIII) and pro-metamorphic periods (stages IX-XIX). Stages XX- XXV comprise the climactic stages during which tail is completely resorbed. At stage XX, the fore-limb protrudes out and marks the beginning of climax. Tail reduction is visible only at stage XXI. By TK stage XXIII, more than half of the tail regresses and by TK stage XXV, the tail is completely lost. However, the time period for complete resorption of tail (Stage XX to XXV) varies from species to species (Duellman and Trueb, 1985).

Remarkable variation has been observed in the larval period as well as duration of tail regression in two species of anurans *Polypedates maculatus* (Rhacophoridae) and *Duttaphrynus melanostictus* (Bufonidae). Eggs in the form of foam nests are laid by *P. maculatus* only during the rainy season but *D. melanostictus* is a perennial breeder that lays egg strings throughout the year. In laboratory conditions, the tadpoles of *P. maculatus* take 40 – 45 days for completion of larval period and 40-48 hours for complete tail resorption, whereas the larval period of *D. melanostictus* is completed in 22 – 30 days and regression of tail takes 18-24 hours. Variations in the larval periods are regulated by the thyroid hormones (Denver et al., 1998; Shi, 2000 and Rose, 2005) and recently, variations in the lysosomal enzyme activities have been reported to cause inter-population and inter-specific variations in duration of larval period and duration of

tail regression (Mahapatra et al., 2011; Mahapatra et al., 2012). However, a detailed analysis of the lysosomal activities in the pre-, pro- and metamorphic periods has not been undertaken in the above studies.

Since, melanocytes are a source of lysosomal enzymes and variations in enzyme activities cause the variations in the duration of tail regression, the present study investigates the role of melanocytes in causing variations in the duration of tail regression in above two species of anurans.

MATERIALS AND METHODS

1. Tadpoles

Foam nests of Indian tree frog *Polypedates maculatus* and egg strings of common Asian toad *Duttaphrynus melanostictus* were collected from around the campus of the Utkal University, Bhubaneshwar (20^0 21'N 85^0 53' E), Odisha, India during the month of July and April, respectively in the during 2013-2014. Tadpoles were reared in the laboratory following the standardized procedure (Mohanty-Hejmadi, 1977). In laboratory conditions, water availability (i.e., water levels in the rearing tubs) and quality and quantity of food was kept constant. The tadpoles were fed with boiled *Amaranthus* leaves and boiled egg yolk *ad libitum* throughout rearing.

2. Amputation of tail

The tadpoles were anesthetized with MS 222 (Tricaine methane sulphonate) prior to tail amputation. Amputation of tail was performed by keeping the specimens laterally on a pre-sterilized porcelain plate. For estimating enzyme activity of acid phosphatase and cathepsin D, tails were amputated through the base with the help of a sharp

sterilized blade and the whole tail was taken for temporal analysis. For spatial analysis, tails were amputated through base and then cut from the middle to have two equal halves. The portion of tail closer to the trunk of the tadpole was designated as the proximal half, while the other half as the distal. Both the halves were considered for analysis. Whole tails of stages XXI and XXIV were taken for histology.

3. Chemicals used

Chemicals used in the study were of the analytical grade. Bovine serum albumin (BSA) was obtained from Sigma Chemicals Co.,USA., para-Nitrophenyl phosphate (pNPP), para-Nitrophenol (pNP) and folin-phenol reagent were obtained from Sisco Research Laboratory, Mumbai, India. Haemoglobin was obtained from Himedia, Mumbai, India. Haematoxylin and eosin were obtained from Lobachemie, Mumbai, India. Stains for Mallory's trichrome staining, i.e., acid fuchsin, phosphomolybdic acid, aniline blue and Orange G were obtained from Ranbaxy, Gurgaon, Haryana, India. All other chemicals were of the highest purified grade available.

3. Biochemical investigation of tail

Estimation of specific activity of acid phosphatase and cathepsin D in different developmental stages was undertaken to investigate the trend of the specific activity of the enzymes throughout the larval period. For investigation, different stages of tadpoles, i.e., pre-metamorphic stages I, III, V, VII and VIII, pro-metamorphic stages X, XII, XIII, XIV, XV and XIX, and climactic stages XX, XXI, XXII and XXIV were selected (Fig.1) (Stages according to Taylor and Kollros, 1946). A pool of amputated whole tails from tadpoles was taken for temporal estimation of specific activity. For spatial estimation of the acid phosphatase in stages VIII, XIX, XX, XXI and XXII, the

proximal and the distal halves of the tails were taken separately. Tail pieces were kept on ice and a 10% homogenate (w/v) was prepared with 0.25M sucrose. The homogenate was centrifuged at 4°C for 10 minutes at 8000×g. The protein estimation was estimated according to Lowry et al. (1951). Acid phosphatase activity was estimated according to Guha et al. (1979) using p-nitrophenyl phosphate as substrate. Cathepsin D activity was determined according to Barrett and Heath (1977) using haemoglobin as substrate. Assays for both the enzymes were performed five times for each stage to obtain five values of specific activity.

4. Histological investigation of tail

For histological analysis, sections of climactic stages XXI and XXIV tadpoles were taken to analyse the extent of degeneration of tissues in the regressing tadpole tail. Following tail amputation, the tail tips were fixed in neutral buffered formalin for 24 hours. Paraffin embedded blocks of the tails were prepared as well as transverse and longitudinal sections of the tail pieces of 5µ thickness were cut using a rotary microtome machine. The sections were then stained following eosin – hematoxylin and Mallory's triple staining method. Photographs of clearly stained sections were taken using a compound microscope (Hund, H500) and Pentax camera and Photoshop 8.0 (Adobe Systems Inc., San Jose, CA, USA) was used to label images and insert scale bars.

5. Statistical analyses

The significant difference in the levels of acid phosphatase and cathepsin D in the various developmental stages (temporal estimation) and levels between the proximal and distal halves of the tail (spatial estimation) of different developmental stages of *P.*

maculatus and *D. melanostictus* were performed by ANOVA and Duncan's multiple range test using SPSS package. For comparing the level of acid phosphatase and cathepsin D in a particular stage between the two species, t- test was followed using Microsoft Excel. The same superscripts against the specific activities (Table 1) and over the bars (Fig. 4) represent the data that are not significantly different (data to be considered separately for each species).

RESULTS

The specific activity of acid phosphatase and cathepsin D (temporal) in fifteen developmental stages of *P. maculatus* and *D. melanostictus* are presented (Table 1) alongwith a comparative analysis of the same (Figs. 2 and 3). The levels of acid phosphatase in the proximal and distal halves of tails (spatial) in five different stages are discussed (Fig. 4).

1) Biochemical analysis

a) Acid phosphatase

i) Temporal estimation

The specific activity of acid phosphatase fluctuated between 2.46 ± 0.13 to 3.25 ± 0.12 from stage I to XIX. Pro-metamorphic (stage XIII) tadpoles had the lowest level of acid phosphatase in the tail. A significant increase in the activity of the enzyme was observed in the climactic stage XX as compared to the pre- and pro-metamorphic stages although the activity increased by only 1.08 fold. There was significant rise in the activity of acid phosphatase in stage XXI by 1.52 fold from stage XX. The activity further increased by 1.62 and 2.26 folds, respectively in stage XXII and XXIV than stage XX. The levels of acid phosphatase were significantly higher in stages XXI, XXII

and XXIV from rest of the stages. The maximum level of acid phosphatase was observed in stage XXIV where the tail was in the form of stump.

In tadpoles of *D. melanostictus*, the level of acid phosphatase was very high in stage I which was significantly higher than even the climactic stages XX and XXI (Table 1). The lowest level of acid phosphatase was observed in stage VII. The level fluctuated between 3.83 ± 0.061 to 4.75 ± 0.173 from stages VIII to XIX. From stage XX the level increased significantly and maximum was observed in stage XXII. There was decrease in the levels by 1.26 folds in stage XXIV than XXII but it was higher than the level observed in stage I.

The specific activity of acid phosphatase in each stage of *P. maculatus* was significantly different from its corresponding stage in *D. melanostictus* (Fig.2).

ii) Spatial estimation

In the pre-metamorphic stage VIII of *P. maculatus*, the level of acid phosphatase remained low both in the proximal and distal half of the tail than the four metamorphic climactic stages, i.e., XIX to XXII. In stage VIII, the level remained higher in the proximal half than the distal. But in the stage XIX, the level of acid phosphatase was 1.11 folds higher in the distal as compared to the proximal half. In the remaining three stages, i.e., XX to XXII, in the distal halves the levels were higher by 1.16, 1.24 and 1.38 folds, respectively than their proximal halves. Differences in the specific activity of acid phosphatase between the distal and proximal halves were significant in stage XX, XXI and XXII (Fig.4).

In *D. melanostictus*, similar lower levels of acid phosphatase were observed in stage VIII tadpoles (Fig. 4) where, in the proximal half the level was 1.08 times more than

the distal half. In stages XIX, XX and XXI, the level remained 1.03, 1.63 and 1.17 folds higher in the distal halves, respectively. However, in stage XXII, the level in the proximal half was higher by 1.02 folds than the distal half. Difference in the level of acid phosphatase was significant for stage XX and XXI while in stage XXII though the level reached its maximum, there was no significant difference between the proximal and distal halves of the regressing tails.

Comparative analyses of levels of acid phosphatase in the proximal and distal halves of different developmental stages of *P. maculatus* and *D. melanostictus* showed that the levels in *P. maculatus* in any half and any stage was always lower than *D. melanostictus* and the difference was significant at 5% level (Fig. 4).

b) Cathepsin D

In *P. maculatus*, the activity of cathepsin D was significantly high in the early stages I and III than the rest pre- and pro-metamorphic stages (Table 1). The activity fluctuated between 2.65 ± 0.25 to 3.72 ± 0.24 in stages V- XIX. The lowest activity was observed in stage XIV. The activity increased significantly in stage XX than the pro-metamorphic stages. The highest activity was observed in stage XXII. In *D. melanostictus*, however, the activity was the lowest in stage III. The activity fluctuated between 3.05 ± 0.4 to 6.66 ± 0.58 from stages I – XIV. There was significant increase in the activity in stage XV where the rise was 1.2 fold. Thereafter, the activity increased significantly in subsequent stages and the highest activity was observed in stage XXIV. The activity at stage XXIV was 3.28 fold higher than stage XIX. The specific activity of cathepsin D in *P. maculatus* was significantly different from that of *D. melanostictus* in all stages (Fig.3).

2) Histological analysis

Histological sections through the regressing tail of *P. maculatus* and *D. melanostictus* show similar structures. Hence, only the sections of *P. maculatus* are enumerated here and visible differences in sections of *D. melanostictus* have been described. The transverse section of stage XXI through tail of *P. maculatus*, showed the basic arrangement of various tissues just when tail regression begins (Fig. 5A). A single layered epidermis (e) lined the section. The dorsal (dtf) and ventral (vtf) tail fins were elongated and rounded at the tip. The muscle bundles (mb) were intact and degeneration had not yet begun. Spinal cord (sc) was present dorsally and two blood vessels (bv) were present ventrally on either side of the notochord (n). A well defined notochordal sheath (ns) surrounding was prominent and the notochord. melanocytes (m) were scantily seen.

The magnified longitudinal sections of stage XXIV of *P. maculatus* (Figs.5B-E) showed a clear representation of degenerating tissues. Towards the distal end of the regressing tail, notochordal cells had lost their integrity and notochordal sheath was not discernible in the degenerating notochord (dn). Melanocytes (m) were abundant in the degenerating tail fin (dntf) (Fig. 5B). The epidermis (e) was multi-layered and wavy and lined by a basement membrane (bm). Melanocytes (m) were also present just below the basement membrane (Fig. 5C). Melanocytes (m) were found to line the degenerating blood vessels (dbv) (Fig. 5D) and degenerating spinal cord (dsc) (Fig. 5E).

In the longitudinal sections of tail of stage XXIV of *P. maculatus* (Figs. 6A-C), the epidermis (e) was thick and there was no trace of basement membrane. All muscle

bundles were degraded and the notochord (dn) had lost its integrity. The lateral longitudinal sections were marked by presence of degenerated muscle and epidermis and mid- longitudinal sections consisted of the degenerating notochord alongwith muscle and epidermis. In the mid-longitudinal section, the notochordal sheath (ns) lining the degenerated notochord was thick (Fig. 6B). In the distal portion, degradation of the notochord was visible without trace of notochordal sheath and notochordal cells. Melanocytes were abundant in the lateral longitudinal sections (Figs. 6A and C) as compared to the mid-longitudnal section (Fig. 6B). Basement membrane was faintly seen in the lateral sections (Figs. 6A and C).

In the longitudinal sections of tail of stage XXIV of *D. melanostictus* (Figs.6D-F), similar degenerating structures were seen. The lateral longitudinal sections were marked by presence of degenerated muscle, epidermis and traces of degenerating notochord and mid- longitudinal sections consisted of the degenerating notochord alongwith muscle and epidermis. In the mid-longitudinal section, the epidermis (e) and the basement membrane (bm) were thick. A wavy notochordal sheath (ns) lined the notochord (n) (Fig. 6E). A large number of melanocytes (m) were found between the epidermis and notochord in lateral (Figs. 6D and F) as well as mid-longitudinal section (Fig. 6E). Basement membrane was faintly seen in the lateral sections (Figs. 6D and F). In the distal part of the tail, a wavy notochordal sheath lined the notochord and the notochordal cells were not intact. No trace of muscle bundles were noticed but degenerating muscle (dm) were present on the either side of the notochord. The notochord (n), spinal cord (sc) and blood vessels (bv) were visible in patches and not continuous throughout the tail.

DISCUSSION

Release of acid hydrolases from preformed lysosomes is the primary cause of tail atrophy and an increase in activity of lysosomal hydrolase is related to the progressive release of hydrolytic enzymes from preformed lysosomes (Weber, 1968). Acid phosphatase, an index of lysosomal activity (Locke and Collins, 1968; Collins, 1975), has already been reported to be involved during tail regression in *Polypedates maculatus* (Mahapatra et al., 2001) and *D. melanostictus* (Mahapatra et al., 2011). Tail resorption is mainly characterized by increases in total activity (per tail) and specific activity (per unit of protein) of acid phosphatase, as well as changes in the pH optimum and heat sensitivity of the enzyme (Robinson, 1972). Similarly, cathepsin D has also been found to be involved in the degradation of tail tissues in *P. maculatus* (Mahapatra and Mahapatra, 2011) and *D. melanostictus* (Mahapatra and Mahapatra, 2012). In the present study, temporal analysis showed the presence of the enzymes in all the stages of both the species. A higher level of the enzymes at stage I followed by decrease in later stages with intermediate fluctuation till stage XV was observed in both the species. From stage XIX, the level increased up to stage XXII in both but at stage XXIV acid phosphatase increased by 1.39 fold in *P. maculatus* while decreased by 1.26 fold in *D. melanostictus*. The pattern of the level of acid phosphatases, i.e., high in the first half of tail regression and then decline observed in *D. melanostictus* corroborates to the finding in *Xenopus laevis* (Robinson, 1970). But, in *P. maculatus* there was gradual rise in the level of acid phosphatase till the maximum level was observed in stage XXIV. The present study, thus, shows a high level of the lysosomal enzymes, acid phosphatase and cathepsin D in the tail of the anuran tadpole in the early stages and low levels throughout the pre-metamorphic and pro-metamorphic stages before regression. Thus,

it contradicts the earlier proposed theory of pre-formation of the lysosomes before tail resorption and proposes that there is presence of the lysosomal enzymes throughout the larval period without such phenomenon of pre-formation of lysosomes before tail regression. There is certainly neo-synthesis of the enzyme in the regressing stages, which was confirmed by the high specific activities of both the lysosomal enzymes in the regressing stages. In fact, in *D. melanostictus*, cathepsin D activity increases much before the visible signs of tail regression indicating the initiation of degradation of tail much before the visible signs of tail regression. In both the species, significant increase in cathepsin D activity (stage XV in *D. melanostictus* and stage XX in *P. maculatus*) has been observed before increase in acid phosphatase activity (stage XX in *D. melanostictus* and stage XXI in *P. maculatus*). This suggests that cathepsin D acts upstream of acid phosphatase during tail regression. Further, the activity of both the enzymes in *P. maculatus* is always lower than *D. melanostictus* which again confirms previous studies by Mahapatra et al. (2012) that higher levels of enzymes are characteristic of shorter larval periods and may be responsible for bringing about faster tail regression.

Spatial examination of the level of acid phosphatase showed no significant difference between the proximal and distal parts of the tail in stage VIII (pre-metamorphic) and stage XIX (climactic) but in stage XX and XXI, the level became significantly higher in the distal halves than the proximal halves in both the species. In *P. maculates*, similar difference in the level of acid phosphatase between the distal and proximal halves was also observed in stage XXII while in *D. melanostictus* there was no such difference. The difference between the level of acid phosphatase in the proximal and

distal halves from stage XX to XXII showed a gradual increase (1.17>1.24>1.41 fold) in *P. maculatus* while in *D. melanostictus* the difference in the levels decreased (1.64<1.18<1.02 fold) in the corresponding stages. This shows that the levels of the enzyme attained uniformity throughout the tail in *D. melanostictus* whereas in *P. maculatus* the enzyme levels became higher in the distal halves with the progress of tail regression. The uniformity in the levels of the enzyme may be causing degradation of tissues uniformly throughout the tail which can be correlated to faster rate of tail regression in *D. melanostictus* than *P. maculatus*.

The degradation of various tail tissues during tail resorption in *P. maculatus* and *D. melanostictus* has already been described in detail (Mahapatra and Mahapatra, 2011; Mahapatra and Mahapatra, 2012). The present study focuses on the differences in the distribution of melanocytes. The climactic stage XXI showed no remarkable degradation of tail tissues and melanocytes were less in number. However, in the climactic stage XXIV degradation of tissues was evident in both the species. Melanocytes were found associated with degrading tail tissues as already reported by Divya et al. (2010), Mahapatra and Mahapatra, (2011) and Mahapatra and Mahapatra, (2012). Remarkably, in *P. maculatus*, the melanocytes were distributed more laterally as compared to the middle portion of tail. But, in *D. melanostictus*, these were found abundantly in the resorbing tail uniformly in lateral as well as middle portion of tail. Since, melanocytes are also a source of acid phosphatase (Seiji and Kikuchi, 1965) uniform distribution of the enzyme in the tails of *D. melanostictus* can be correlated with the uniform distribution of melanocytes.

The two anurans selected for the present study show great variations in the duration of larval period and tail regression which may be an adaptive trait to enable them to adjust to the varied environment they live in. *D. melanostictus* is a perennial breeder whose tadpoles are subjected to varying water availability while the other anuran *P. maculatus*, breeds only in the rainy season. Hence the tadpoles are not subjected to stress related to desiccation. Moreover, in *D. melanostictus* the eggs are laid in strings and the only covering of egg is the jelly coat whereas in *P. maculatus*, the eggs are laid inside the foam, which prevents the eggs from desiccation. In the laboratory conditions, with similar water availability, tadpoles of *D. melanostictus* showed a shorter larval period and faster rate of tail regression than *P. maculatus*. As expected, higher levels of both acid phosphatase and cathepsin D were observed in the tails of the tadpoles of *D. melanostictus* than *P. maculatus* throughout the larval period as well as in regressing stages. Remarkably, uniform distribution of melanocytes and acid phosphatase in the tails of *D. melanostictus* as compared to *P. maculatus* is suggested to cause shorter duration of tail regression in tadpoles of *D. melanostictus.* Thus, higher levels of both the enzymes in the regressing stages, uniform levels of acid phosphatase and distribution of melanocytes in the regressing stage seem to cause faster rate of tail regression in *D. melanostictus* and can be described as a physiological adaptation in response to stress related to desiccation.

Table 1. Specific activity of acid phosphatase and cathepsin D in different developmental stages of *Polypedates maculatus* and *Duttaphrynus melanostictus*.

ENZYMES	ACID PHOSPHATASE		CATHEPSIN D	
STAGES	*Polypedates maculatus*	*Duttaphrynus melanostictus*	*Polypedates maculatus*	*Duttaphrynus Melanostictus*
I†	3.16 ± 0.202^{b}	10.11 ± 0.513^{g}	5.316 ± 0.349^{b}	6.46 ± 0.558^{bc}
III	2.65 ± 0.208^{a}	5.939 ± 0.054^{e}	5.323 ± 0.124^{b}	3.059 ± 0.4^{a}
V	3.13 ± 0.188^{b}	4.382 ± 0.515^{c}	3.517 ± 0.715^{a}	3.465 ± 0.769^{a}
VII	3.13 ± 0.361^{b}	3.485 ± 0.03^{a}	2.882 ± 0.524^{a}	3.712 ± 0.285^{a}
VIII	3.111 ± 0.272^{b}	4.613 ± 0.176^{cd}	2.989 ± 0.806^{a}	6.047 ± 0.705^{bc}
X	2.827 ± 0.237^{b}	4.402 ± 0.236^{c}	3.722 ± 0.248^{a}	4.942 ± 1.041^{ab}
XII	2.81 ± 0.104^{b}	3.983 ± 0.172^{b}	3.44 ± 0.413^{a}	5.003 ± 0.142^{ab}
XIII	2.469 ± 0.139^{a}	3.837 ± 0.061^{b}	3.109 ± 0.67^{a}	4.546 ± 1.124^{ab}
XIV	3.127 ± 0.224^{b}	4.629 ± 0.023^{cd}	2.657 ± 0.25^{a}	6.666 ± 0.58^{bc}
XV	2.986 ± 0.375^{b}	4.6 ± 0.166^{cd}	2.945 ± 0.379^{a}	8.09 ± 0.717^{c}
XIX	3.255 ± 0.127^{b}	4.752 ± 0.173^{cd}	3.184 ± 0.184^{a}	10.397 ± 1.209^{d}
XX	3.503 ± 0.249^{c}	5.52 ± 0.288^{e}	6.034 ± 1.283^{b}	13.386 ± 1.978^{e}
XXI	5.34 ± 0.535^{d}	6.368 ± 0.861^{f}	9.664 ± 0.956^{c}	15.292 ± 0.573^{e}
XXII	5.682 ± 0.388^{d}	15.24 ± 3.635^{i}	12.878 ± 2.485^{d}	22.772 ± 2.355^{f}
XXIV	7.943 ± 0.578^{e}	12.07 ± 0.754^{h}	16.069 ± 1.246^{e}	34.271 ± 3.361^{g}

† - Stages according to Taylor and Kollros, 1946

Similar superscripts against the specific activities represent values that are not significantly different from each other. The superscripts for *P. maculatus* to be considered separately from *D. melanostictus*.

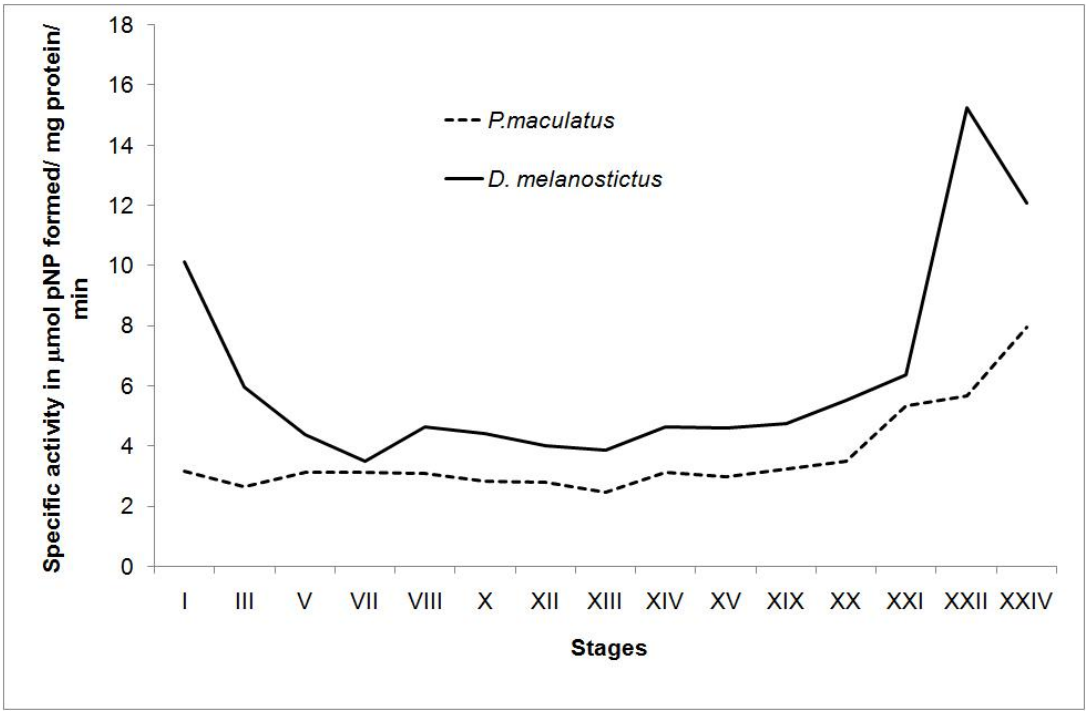

Fig.2. A comparative account of the levels of acid phosphatase in tails of different developmental stages of *Polypedates maculatus* and *Duttaphrynus melanostictus*.

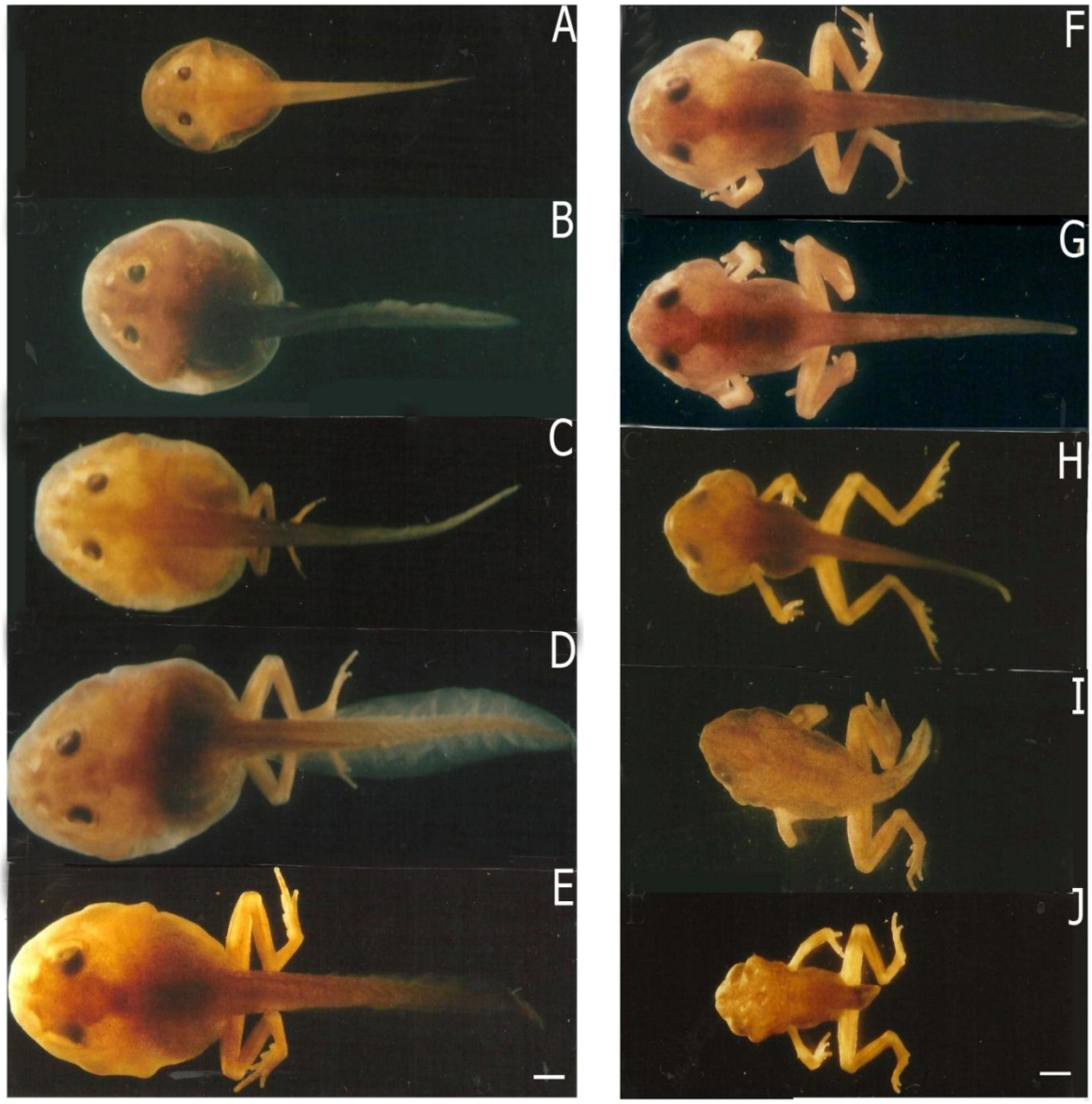

Fig.1 Different developmental stages in an anuran tadpole. A- Stage I, B- Stage V, C- Stage XIV, D- Stage XIX, E- Stage XX, F- Stage XXI, G- Stage XXII, H- Stage XXIII, I - Stage XXIV. (Bar= 1cm) (Stages according to Taylor and Kollros, 1946)

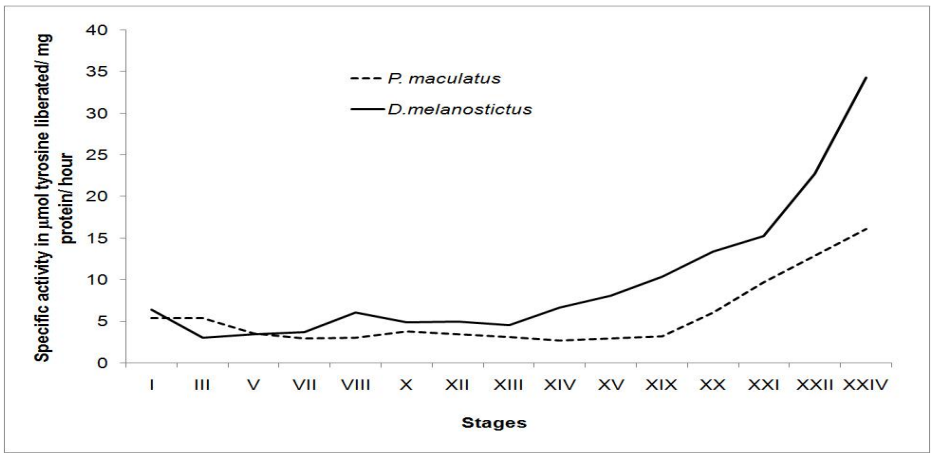

Fig.3 A comparative account of the specific activity of cathepsin D in tails of different developmental stages of *Polypedates maculatus* and *Duttaphrynus melanostictus*.

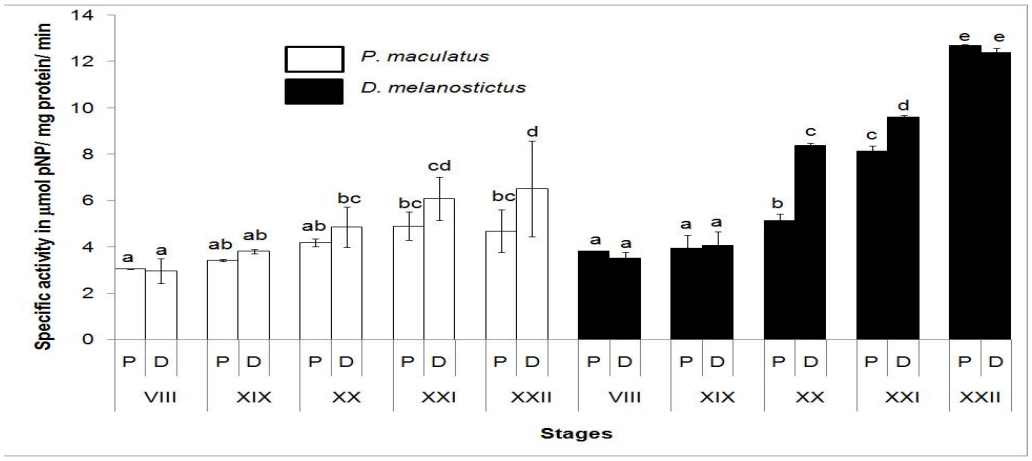

Fig.4. Levels of acid phosphatase spatially in the tails of five different developmental stages of *Polypedates maculatus* and *Duttaphrynus melanostictus.* (Same superscripts over the bars represent the values that are not significantly different. The superscripts for *P. maculatus* to be considered separately from *D. melanostictus*).

P= Proximal half of tail, D= Distal half of tail.

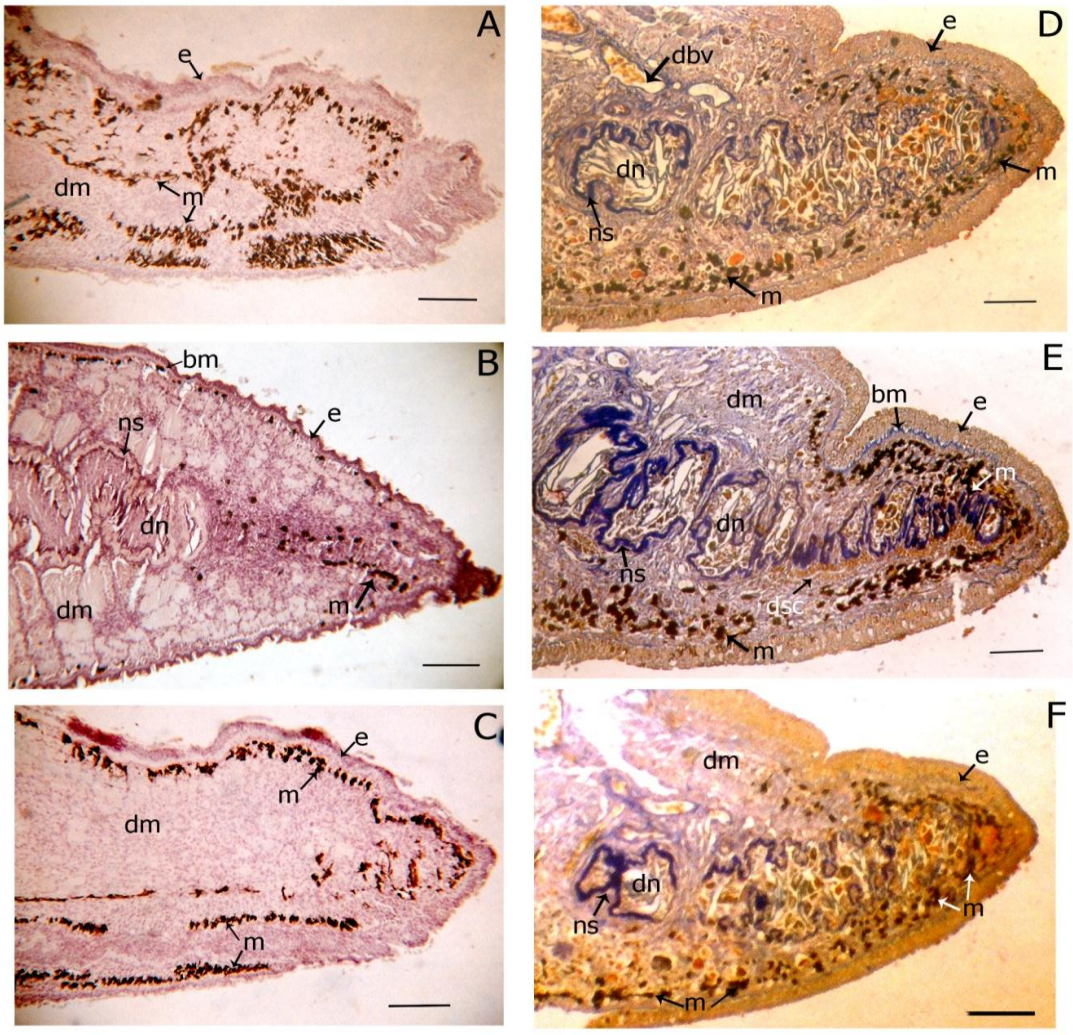

Fig 6. Comparative account of distribution of melanocytes in the tail of stage XXIV of *P. maculatus* and *D. melanostictus*.A and C- Lateral longitudinal sections (L. S.) of *P. maculatus,* B- Mid L. S. of *P. maculatus,* D and F- Lateral L. S. of *D. melanostictus*, E- Mid L. S. of *D. melanostictus*. dbv- degenerating blood vessel, dm- degenerating muscle, dn- degenerating notochord, e- epidermis, m- melanocytes,ns- notochordal sheath,bm- basement membrane, dsc- degenerating spinal cord. (Bars A-F= 63µm)

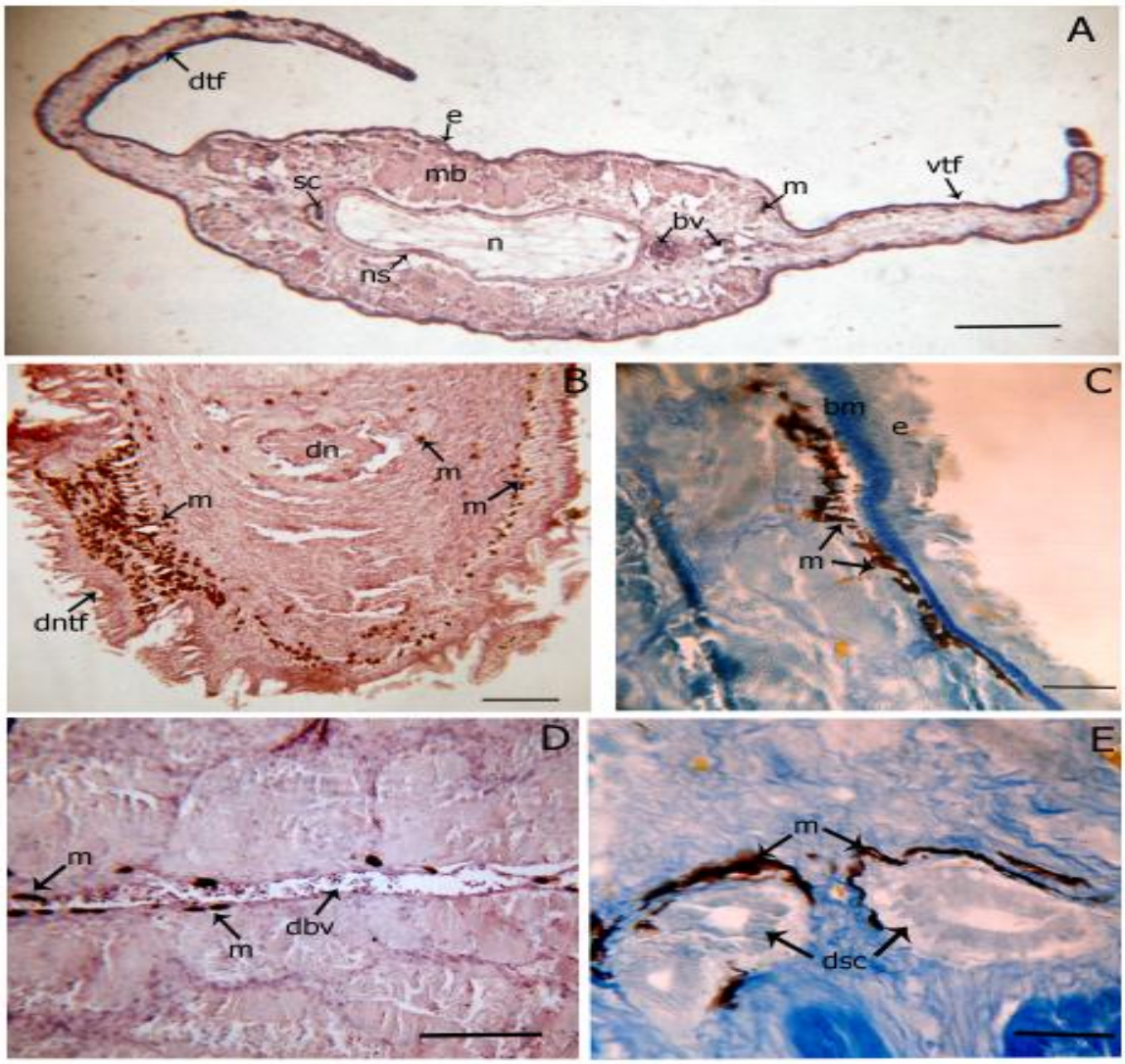

Fig.5 Distribution of melanocytes in the tails of stage XXI and XXIV tadpoles of *P. maculatus*. A: Transverse section (T. S) through tail of stage XXI, B-E: Longitudinal sections (L.S) through tails of stage XXIV tadpoles showing melanocytes near degenerating notochord and tail fin (B), below basement membrane (C), lining degenerating blood vessel (D) and degenerating spinal cord (E).bm-basement membrane, e- epidermis, m- melanocytes, n- notochord, ns- notochordal sheath,dtf- dorsal tail fin, vtf- ventral tail fin, sc- spinal cord, bv- blood vessels, mb- muscle bundle, dn- degenerating notochord,dntf- degenerating tail fin, dsc- degenerating spinal cord, dbv- degenerating blood vessel (Bars A, B and D=63 μm, C and E= 13 μm)

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REFERENCES

Barrett AJ and Heath MF (1977). Lysosomal enzymes. In: Lysosomes: A laboratory Handbook, Dingle JT, ed., North Holland Publishing Co. Amsterdam. pp 19-127.

Brown DD and Cai L (2007). Amphibian metamorphosis. *Dev Biol*., 306(1): 20–33.

Collins JV (1975). Soluble acid phosphatase of the hemolymph and fat body of *Calpodes ethlius* (Stoll) and the control of protein storage by fat body. *Canadian Journal of Zoology*, 53: 480-489.

Denver RJ, Mirhadi N and Phillips M (1998). Adaptive plasticity in amphibian metamorphosis: response of *Scaphiopus hammondi* tadpoles to habitat desiccation. *Ecology*, 79: 1859–1872.

Diment S, Eidelman M, Marcela Rodriguez G and Orlow SJ (1995). Lysosomal hydrolases are present in melanosomes and are elevated in melanizing cells. *The Journal of Biological Chemistry*, 270 (9): 4213-4215.

Divya L, Beyo RS, Sreejith P, Akbarsha MA and Oommen OV (2010). Skeletal muscle–melanocyte association during tadpole tail resorption in a tropical frog, *Clinotarsus curtipes* Jerdon (Anura, Ranoidea). *Zoology (Jena)*, 113: 175-83.

Duellman WE and Trueb L (1985). Biology of Amphibians. McGrawHill Book Company, USA, pp

Elinson RP, Remo B and Brown DD (1999). Novel structural element identified during tail resorption in *Xenopus laevis* metamorphosis. Lessons from tailed frogs. *Developmental Biology*, 215(2): 243-252.

Gilbert LI and Freiden E (1981). Metamorphosis; A problem in Developmental Biology. Phenum Press, New York, pp

Guha K, Karkkainen RR and Vanha-Perttula T (1974). Testicular acid phosphatase of the mouse. *Medical Biology*, 57: 52-57.

Kerr JF, Harmon B and Searle J (1974). An electron microscope study of cell deletion in anuran tadpole tail during spontaneous metamorphosis with special reference to apoptosis of striated muscle fibres. *Journal of Cell Science*, 14: 571-85.

Kinoshita T, Sasaki F and Watanabe K (1985). Autolysis and heterolysis of the epidermal cells in anuran tadpole tail regression. *Journal of Morphology*, 185 (2): 269-275.

Komukai M, Kobayashi K and Horiuchi S (1986). Autolysis in the tail muscles of metamorphosing tadpoles. *Comparative Biochemistry and Physiology B*, 85 (1): 55-59.

Locke M and Collins JV (1968). Protein uptake into multivesicular bodies and storage granules in the fat body of an insect. *Journal of Cell Biology*, 36: 453-483.

Lowry DH, Rosebrough NJ, Farr AL and Randall RJ (1951). Protein measurement with Folin phenol reagent. *Journal of Biological Chemistry*, 193: 265-275.

Mahapatra PK, Mohanty-Hejmadi P and Chainy GBN (2001). Changes in oxidative stress parameters and acid phosphatase activity in the pre-regressing and regressing tail of Indian Jumping frog *Polypedates maculatus*

(Anura:Rhacophoridae). *Comparative Biochemistry and Physiology Part C,* 130: 281-288.

Mahapatra C, Dutta SK and Mahapatra PK (2011). Inter-populational differences in activity of acid phosphatase in the tails of the tadpoles of *Duttaphrynus melanostictus* (Anura: Bufonidae). *Russian Journal of Herpetology*, 18 (2): 105-110.

Mahapatra, C and Mahapatra PK (2011). Involvement of cathepsin D during tail regression in tadpoles of the common Indian tree frog, *Polypedates maculatus* (Anura: Rhacophoridae). *Acta Histochem*., 113: 756-762.

Mahapatra PK, Jamdar SN, Mahapatra C, Dutta SK and Harikumar P (2012). Interspecies variation in activity of lysosomal cathepsins during tail regression in the tadpoles of three sympatric anurans. *Russian Journal of Herpetology*, 19 (3):232-238.

Mahapatra C and Mahapatra PK (2012). Immunohistochemical localization of cathepsin D and a possible role of melanocytes during tail resorption in tadpoles of a tropical toad. *Journal of Morphology*, 273 (7): 791–800 .

Mohanty-Hejmadi P (1977). Care and management of amphibian embryos. *Prakruti-Utkal University Journal of Science*, 11: 81-87.

Nishikawa A, Murata E, Akita M, Kaneko K, Moriya O, Tomita M and Hayashi H (1998). Roles of macrophages in programmed cell death and remodeling of tail and body muscle of *Xenopus laevis* during metamorphosis. *Histochemistry and Cell Biology* 109 (1): 11-17.

Robinson H (1970). Acid phosphatase in the tail of *Xenopus laevis* during development and metamorphosis. *Journal of Experimental Zoology*, 173 (2): 215-224.

Robinson H (1972). An electrophoretic and biochemical analysis of acid phosphatase in the tail of *Xenopus laevis* during development and metamorphosis. *Journal of Experimental Zoology*, 180 (1): 127-139.

Rose CS (2005). Integrating ecology and developmental biology to explain the timing of frog metamorphosis. *Trends in Ecology and Evolution*, 20: 129 - 135.

Sasaki K., Kinoshita T, Takahama H and Watanabe K (1988). Cytochemical studies of hydrogen peroxide production in the tadpole tail of *Rana japonica* during metamorphic climax. *Histochemical Journal,* 20: 99-107.

Seiji M and Kikuchi A (1969). Acid phosphatase activity in melanosomes. *Journal of Investigative Dermatology*, 52: 212-216.

Shi YB (2000). Amphibian Metamorphosis: From Morphology to Molecular Biology. Wiley – Liss, New York, pp

Taylor A and Kollros J (1946). Stages in the normal development of *Rana pipiens* larvae. *Anatomical Record*, 94: 7-23.

Wang X, Matsuda H and Shi YB (2008). Developmental regulation and function of thyroid hormone receptors and 9-*cis* retinoic acid receptors during *Xenopus tropicalis* metamorphosis. *Endocrinology*, 149(11):5610 –5618

Weber R (1968). The Biochemistry of Animal Development. Academic Press, New York, pp

Wolff K and Schreiner E (1971). Melanosomal acid phosphatase. *Arch. Dermatol. Forsch.*, 241: 255-272.

Yasutomi M (1987). Migration of epidermal melanophores to the dermis through the basement membrane during metamorphosis in the frog, *Rana japonica*. *Pigment Cell Research*, 1(3): 181.

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HAEMATOLOGICAL PROFILE OF DIFFERENT SEXES OF JAPANESE QUAIL *(Coturnix coturnix japonica)*

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ABSTRACT

The study is to compare the basic haematological parameters and differential leukocyte count of Japanese quail reared in the farm of the Central Poultry Development Organisation, Bhubaneswar. The haematological profile includes RBC, WBC, PCV, haemoglobin (Hb%) and differential leucocyte count (DLC) which includes lymphocytes, monocytes neutrophils, eosinophils, and basophils. Blood plays an important role in assessing clinical and nutritional health status of animals. The mean value of Hb and MCV differ significantly ($p<0.05$) between both the sexes of Japanese quail. MCHC ($p<0.01$) indicate significant difference between male and female. Samples are compared by using variance (ANOVA) to assess the significance of difference at $P<0.05$ and $P<0.01$ by the help of Paleontological statistics (PAST) version 2.17.

Keywords: Japanese quail, Blood Cell, Haematological parameters, Erythrocyte indices

INTRODUCTION

Poultry production has been identified as one major means of solving the problem of low animal protein intake. The term poultry refers to all domesticated birds kept for egg or meat production and it includes chickens, turkeys, ducks, geese, ostriches, quails etc. (Awobajo et al., 2007). The Japanese quail *(Coturnix coturnix japonica*) is the smallest avian species farmed for egg and meat production and it has assumed

worldwide importance as a laboratory animal (Baumgartner, 1993; Minville, 2004). Quail possesses excellent disease resistant variety of poultry bird than that of chickens (Deka and Borah, 2008). The Japanese quail belongs to the order Galliformes, family Phasianidae, genus Coturnix and species *japonica*. The scientific designation for Japanese quail is *Coturnix coturnix japonica* which is different from common quail "*Coturnix coturnix*" (Thear, 1998; Mizutani, 2003). Blood plays an important role in maintaining the nutritional balance in the body. However, blood represents a means of assessing clinical and nutritional health status of animals (Olorode and Longe, 2000). The haemato-biochemical profiles are most commonly used in nutritional studies for Japanese quail (Arora, 2010) and other birds like chickens (Adeyemi et al., 2000), pigeons (Pavlak et al., 2005), guinea fowl (Onyeanusi, 2007), bronze turkey (Schmidt et al., 2009) etc. Changes in the haematological profile in breeding and sexual maturation of Japanese quail were studied by Mihailov et al. (1999). Since information on haematological and differential leucocyte count on quails in particular is scanty, the present study was designed to evaluate the various blood cell profiles of Japanese quails of different sexes.

METHODOLOGY

The present investigation was conducted on Japanese quail being maintained at Central Poultry Development Organization (CPDO), Eastern Region (ER), Government of India, Bhubaneswar, Odisha. Blood samples were collected from 20 birds, i.e., 10 male and 10 female species of Japanese quail in the month of September with temperature and relative humidity 23.2–43.4^0 C and 92 % respectively. Blood was collected in the morning hours to avoid diurnal variation. Samples were collected by puncture of wing or ulnar vein of different sexes with the proper restraint method by a 5ml disposable syringe and was transferred immediately into the anti-coagulated vacutainer tubes containing ethylene diamine

tetra-acetic acid (EDTA) and mixed properly. Blood smears were prepared at site on clean grease free slides (Blue Star Pic-2, Polar Industrial Corporation, Mumbai, Maharashtra, India), air dried and fixed in methanol (Qualigens Product No.34457, Thermo Fisher Scientific India Pvt. Ltd., Mumbai, Maharashtra, India) for 2 minutes followed by staining (Sonia et al., 2012).

Blood cell analysis, i.e., hemoglobin (Hb) was estimated by using Sahil's haemometer (Sonia et al., 2012) and packed cell volume (PCV) was determined with microhematocrit tube at 3,500 rpm for 15 minutes by centrifuge machine (Remi Centrifuge, Catalogue No. C852 7/94, Serial No. GCLC-1632, Remi Motors, Bombay, Maharashtra, India). Total erythrocyte count (TEC) and total leukocyte count (TLC) were undertaken with the help of Neubaurs Haemocytometer and Turk's (HiMedia RO16-500ML, W.B.C. Diluting Fluid, HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India) and Hayem's fluid (HiMedia RO13-500ML, R.B.C. Diluting Fluid (Hayemis), HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India] respectively by using Hund Wetzlar Microscope. From these basic data, erythrocytic indices, i.e., mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were estimated by the standard procedure(Latimer and Bienzle,2010). The entire data are presented as mean± standard error and compared between males and females by using t-test for significance differences at $P<0.05$ and $P<0.0$. All these statistical analyses were obtained by statistical software Microsoft office excel 2007.

RESULTS AND DISCUSSION

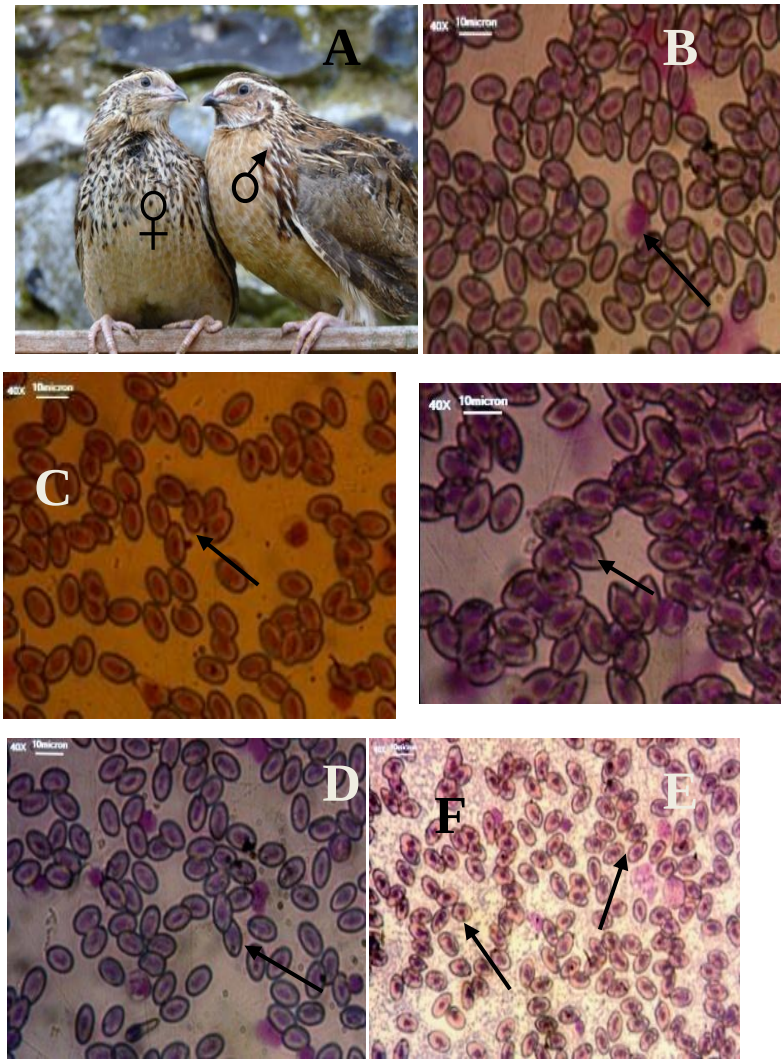
The result of the haematology profile of Japanese quail of both male and female shows a significant difference. It is observed that the parameters namely Hb, MCV and MCHC are significant at $P<0.05$ and $P<0.01$. Hematological values are influenced by various factors including breed, sex, age, reproductive status, circadian rhythm, handling procedure and nutritional status of anaemia (Adi, 2004).

Table 1 Haematological parameters of male and female Japanese quail (means ± SEM)

Sl No	Parameter	Male	Female
1	Hb (g %)	12.21±0.23*	11.64±0.32*
2	RBC (10^6/mm^3)	1.81±0.056	1.66±0.07
3	WBC (1,000/m^3)	6135±401.52	8285±805.77
4	PCV (%)	36.4±1.62	34.2±0.98
5	MCV (m3)	204.93±4.29*	213.83±11.83*
6	MCH (pg)	68.31±1.43	71.14±3.72
7	MCHC (%)	34.34±1.40**	32.64±0.92**
8	LYMPHOCYTE (%)	52.5±3.30*	54.7±3.31*
9	MONOCYTE (%)	7.7±0.76^{NS}	9.1±0.81 ^{NS}
10	HETEROPHIL (%)	24.6±2.22 ^{NS}	19.3±1.87 ^{NS}
11	EOSINOPHIL (%)	12.7±2.59**	13.7±1.97**
12	BASOPHIL (%)	2.5±0.54*	3.2±0.69*

Note: * significant at p<0.05, ** Significant at P<0.01, Non Significant (NS).

The red blood cell count, haemoglobin amount and haematocrit value has been observed to be increased with the advancement of age. This is lowest in chicks and highest in adults, which is corroborated with Nirmalan and Robinson (1971).



.Fig. 1 Blood cells of and Japanese quail (A) Japanese quail (B) Lymphocyte (C) Monocyte (D) Eosinophil (E)Heterophil and (F) Basophil , 40X with scale length 10 micron.

There is no significant difference in red blood cell (RBC) count, white blood cell (WBC) count between males and females of Japanese quail. Packed cell volume or haematocrit determines the percentage of volume of red blood cells in whole blood. It was recorded to be highest in males than females.

RBC, Hb and PCV are higher in males than females. In general, number of RBCs and haematocrit of birds are influenced by species, age, sex, hormonal factors, hypoxia, environmental factors and diseases. PCV and MCV have been observed to increase with age which gets corroborated with Islam et al. (2004) and Campbell (2012).

According to Hadzimusic et al. (2010), PCV and MCV value increase depending on temperature and storage duration. MCV, MCH and MCHC were calculated on the basis of PCV, RBC and Hb as studied by Campbell (1995). Erythrocyte indices with high and low MCV value are due to low haemoglobin concentration in blood. In the present study, MCH value does not show significant difference in both sexes. MCHC shows significant difference at $p<0.01$ between males and females. MCV and MCH value are higher in females than males. MCV measures the average size of individual red blood cell (Aina and Ajibade, 2014). Accelerated erythropoiesis can also cause an increase in MCV value when iron is abundant in surrounding. Erythropoietin enables the release of large amount of reticulocyte into the circulation and consequently increasing the MCV values. This increase in MCV is usually preceded by acute or severe haemolytic anaemia (Ali et al., 2012). The DLC reflects the increased value for lymphocyte. Basophil percentage in males and females shows significant difference at $p<0.05$ where as eosinophil reveales highly significant difference at $p<0.01$ level. The monocytes and heterophils do not show any significant difference.

CONCLUSION

This investigation is an attempt to develop a database for quail farmers, poultry industries, ornithologist, naturalists, pathologists, biologists and more specifically for veterinarians. This analysis is believed to answer the summative health status of the quail which may help in treatment, care and development of production quantum. Further, this also can be an effective and efficient tool in developing breeding programmes in Japanese quails.

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REFERENCES

Adeyemi OA, Fasina OE, Balogun MO (2000) Utilization of full fat jatropha seeds in broiler diet: effect on haematological parameters and blood chemistry. Proc. 25th Conf. Nigerian Soc. Anim. Prod. held at Michael Okpara University of Agriculture, Umudike, Nigeria, March 19-23, pp 108-109

Adi AAAM, Winaya IBO, Utama IH, Suarsana N, Erawan IGNK, Apsari IAP, Hayashi Y (2004) Pathological abnormalities of Bali ducks sampled from traditional farming system in Bali. *Journal Veteriner* 5(2): 40-46

Aina OO, Ajibade T (2014) Age-related changes in haematologic parameters of cage-raised Japanese quails (*Cortunix japonica*). *J. Vet Med. Ani. Health* 6(4): 104-108

Ali MA, Hmar L, Inaotombi LD, Prava M, Lallianchhunga MC, Tolenkhomba TC, (2012) Effect of age on the haematological and bio-chemical profile of Japanese quails (*Coturnix japonica*). *Int. Multidiscip. Res. J* 2(8): 32-35

Arora KL (2010) Differences in hemoglobin and packed cell volume in blood collected from different sites in Japanese quail (*Coturnix japonica*). *Int. J. Poult. Sci.* 9(9): 828-830

Awobajo OK, Skinrolabu AA, Mako AO, Igbosanu AO, Olatokunbo OT (2007) The mortality rate of two different breeds of broilers after brooding stage to maturity. *Middle-East J. Sci. Res.* 2(1):37-42

Baumgartner J (1993) Japanese quail production, breeding and genetics. Proceedings of the 10th International Symposium on current problems of avian genetics. Nitra, Solvakia, pp 101 – 103

Campbell T (1995) Avian Haematology and Cytology. Iowa State University Press, Ames

Campbell TW (2012) Hematology of birds. In: Thrall MA, Weiser G, Allison RW, Campbell TW (eds) Veterinary Hematology and Clinical Chemistry, 2nd Edition. Wiley, Chichester, 238–276

Deka K, Borah J (2008) Haematological and biochemical changes in Japanese quails (*Coturnix coturnix japonica*) and chickens due to *Ascaridia galli* infection. *Int. J. Poult. Sci.* 7: 704-710

Hadzimusic N, Katica M, Muharemovic Z , Musanovic (2010) Effect of temperature storage on haematological parameters of avian turkey blood. *Int.J.Col. Res.* 2(5): 158-166

Islam MS, Lucky NS, Islam MR, Ahad A, Das BR, Rahman MM , Siddiui MSI (2004) Haematological parameters of Fayoumi, Assil and local chickens reared in Sylhet region in Bangladesh. *Int.J.Poult.Sci*.3(2):144-147

Latimer KS, Bienzle D (2010) Chaper III. Schalm's Veterinary Hematology. 6th ed. / editors, Douglas J. Weiss, K. Jane Wardrop, pp 345

Mihailov R, Lasheva V, Lashev L (1999) Some hematological values in Japanese quails. *Bulg. J. Vet. Med*. 2: 137-139

Minvielle F (2004) The future of Japanese quail for research and production. *World Poult. Sci. J.* 60: 500-507

Mizutani M, (2003) The Japanese Quail , Laboratory Animal Research Station , Nippon Institute for Biological Science , Kobuchizawa, Yamanashi, japan, pp 408

Nirmalan GP, Robinson GA (1971) Hematology of Japanese quail (*Coturnix coturnix japonica*). *Br. Poult. Sci*. 52:457-482

Olorode BR, Longe OG, (2000) Effect of replacing palm kernel cake with shear butter cake on quality characteristics, haematology and serum chemistry of laying hens. *Nigerian J. Anim. Prod*. 27: 19-23

Onyeanusi BI, (2007) Calcium and phosphorus levels in Nigerian guinea fowls. Int. J. Poult. Sci. 6(8): 610-611.

Pavlak M, Vlahovic K, Jarcic J, Dorc A, Zupancic Z (2005) Age, sexual and seasonal differences of haematological values and antibody status to Chlamydophila sp. in feral and racing pigeons (*Columba livia formadomestica*) from an urban environment (Zagreb, Croatia). *European J. Wildlife Res*. 51(4): 271-276

Schmidt EMS, Paulillo AC, Martins GRV, Lapera IM, Testi AJP, Junior LN, Denadai J, Fagliari JJ (2009) Hematology of the bronze turkey (*Meleagris gallopavo*): Variations with age And gender. *Int. J. Poult. Sci*. 8(8): 752-754.

Sonia C, Asha Rajini R, Vairamuthu S (2012) Haematological parameters of pearl guinea fowl influenced by rearing system, age and sex. *Ind. J. Poult. Sci.* 47(3): 395-397

Sonia C, Rajini RA, Babu M, Vairamuthu S (2012) The effect of age, sex and rearing system on differential count in Guinea fowl. *Ind. J. Poult. Sci.* 47 (2): 251-3

Thear, K (1998) Keeping quail (a guide to domestic and commercial management), Third Edition, Broad Leys Publishing Company, London E9 5EN.*Veterinary Clinical Pathology*. Williams & Wilkins, Baltimore

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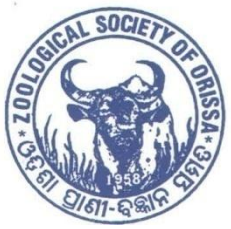

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