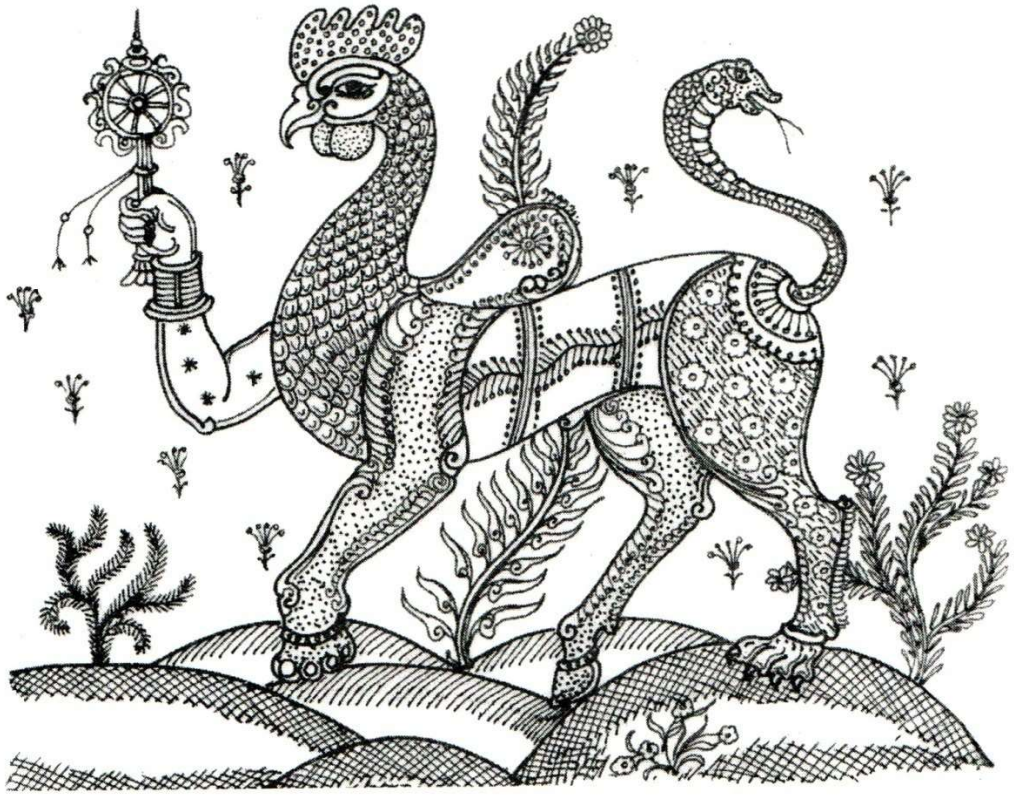
ISSN 0970-4450

PRANIKEE

Journal of Zoological Society of Orissa



Volume- XXXII
2020

ISSN 0970-4450

PRANIKEE

Journal of Zoological Society of Orissa

Department of Zoology
Utkal University, Vani Vihar
Bhubaneswar - 751 004, Odisha, India

Volume XXXII, 2020

ISSN 0970-4450

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. Priyambada Mohanty-Hejmadi

Former Editor

From the Editor's desk

The present edition of the Journal (Volume XXXII) was prepared during the trying period of the COVID-19 pandemic and is ready for circulation. This volume carries eight research articles which cover different aspects of Zoology including modes of reproduction in frogs, toxic and teratogenic effects of vitamin A on frog tadpoles, new records of some Chalcididae from Meghalaya, impact of cadmium chloride and lead nitrate on the major carp *Catla catla*, immune response of mice to *Escherichia coli*, effect of dietary replacement of fish meal on *Labeo rohita* fingerlings, oral apparatus of four species of anuran tadpoles and haematological profile of the tadpoles of the Asian toad. I believe that this volume will contribute to the expanding knowledge in Zoology.

PK Mahapatra

PRANIKEE
Journal of Zoological Society of Orissa
Abbreviation: Pranikee
Volume 32, Year - 2020

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Volume XXXII, 2020, pp 1-15

DIVERSE REPRODUCTIVE MODES OF SOME ANURANS IN A TEMPORARY HABITAT IN THE SOUTHERN SLOPES OF MEGHALAYA, NORTH EAST INDIA

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ABSTRACT

Among vertebrates, anurans have the most diverse reproductive modes.The high levels of rainfall and consequent relative humidity in Mawsynram, a region in the Northern ranges of the Southern slopes of Meghalaya support diverse reproductive modes of anurans. In the present investigation, diversity of reproductive modes of anurans from a breeding habitat at Mawsynramhas have been recorded. Environmental variables including rainfall, relative humidity, air and water temperature of selected breeding habitats of anurans have been recorded during the year 2018 and 2019. The study revealed seven reproductive modes among eight anuran species belonging to four families. Members of the family, Rhacophoridae demonstrate the greatest number of reproductive modes adapted to a terrestrial life by constructing foam nest. However, a newly recognized reproductive mode from the area of study is revealed by the rhacophorid *Kurixalus naso* which oviposit eggs in the soil surface and burrows without foam formation. Other anuran species belonging to families, Bufonidae, Hylidae and Dicroglossidae showed a common reproductive mode in lentic habitat. High levels of rainfall, relative humidity and optimal range of temperature of the selected breeding habitats may help to sustain more reproductive modes of anurans than the drier sites.

Key words: Anurans, Reproductive modes, Breeding habitat, Meghalaya

INTRODUCTION

Anurans breed in diverse habitats such as temporary rain-fed ponds, permanent ponds, cemented tanks, streams and rivers. In addition, they prefer those sites that have vegetation cover, that provides shelter and calling sites. Anurans have the highest diversity of reproductive modes among all vertebrate taxa (Duellman and Trueb, 1986). The classification of reproductive modes in anurans is based on the oviposition site, egg characteristics, rate and duration of development, stage and size of hatchling and type of parental care (Salthe and Duellman, 1973; Toledo et al., 2012). The diversity of reproductive modes from different areas varies according to altitudinal and climatic factors (Vasconselos et al., 2010). Availability of water at the breeding site strongly determines the level of diversification of the reproductive strategies of amphibians (Duellman and Trueb, 1986; McDiarmid, 1994; Vences and Kohler, 2008). A breeding habitat with high precipitation throughout the year supports more number of anuran reproductive modes than those sites with low precipitation and seasonal climates, where only species with specialized reproductive modes can thrive in such habitats to resist desiccation (Rodrigues da Silva et al., 2012).

The highest diversity of reproductive modes in anurans is found in the New World wet forests (Wells, 2007). Duellman and Trueb (1986) reported 29 reproductive modes from the Neotropics. Haddad and Prado (2005) updated 10 more modes in addition to that proposed by Duellman and Trueb (1986). Therefore, 39 reproductive modes have been recorded so far as revealed from the available literature. Recent studies on the breeding activity of anurans in Meghalaya, have been contributed by Iangrai (2007), Rangad and Hooroo (2013), Tron et al. (2015), Khongwir et al. (2016) and Shangpliang et al. (2020). Meghalaya, falling in the Indo-Myanmar biodiversity hotspot, is marked by numerous hills, valleys, streams, rivers and drainages that receive exceptionally high levels of rainfall on the southern slopes. Therefore, it is important to understand the species specific ecological requirements including the reproductive strategies of anurans that are still unexplored in the southern slopes of the State of Meghalaya. This may help to promote conservation measures of the breeding habitats so as to sustain the long term persistence of the anurans and the amphibian community as a whole.

MATERIALS AND METHODS

Investigation was conducted during the years 2018 and 2019 at the breeding sites of Mawsynram (between 25° and 18′ N latitudes; and 91° and 35′ E longitudes; 1400 m asl., Garmin Etrex 30 GPS). Field surveys were conducted from the selected habitats of adult anurans during the breeding period from 08.00 to 23.00 hour in a temporary rain-fed pond measuring 335sq m (Fig. 1) through Visual Encounter Survey. During night time, study plots were surveyed with the help of head lamps and torch lights. The egg characteristics and the microhabitats of the breeding sites of anurans for oviposition were noted. Few specimens from the study sites were collected and brought to the laboratory for morphometric measurements (using a dial calliper, Mitutoyo series No. 505-671). Few specimens were sacrificed through anesthetization with Tricane Methane Sulfonate- MS 222 solution and then fixed in 4% formaldehyde for preservation for future studies where as others were released back into the respective natural habitat. The preserved specimens were identified with the help of the monograph key prepared by Chanda (1994) and with the help of Zoological Survey of India, Shillong.

The breeding activities of the observed anuran species were photo documented at the study site using Nikon D3400 and Nikon D850 cameras. The reproductive modes were followed by Duellman and Trueb (1986), and Haddad and Prado (2005). In addition, we provide data on the breeding period, breeding habitat (lentic and terrestrial), oviposition sites and egg characteristics of the anuran species studies.

Environmental variables including rain fall, relative humidity, air and water temperature of the selected breeding habitats of anurans were also recorded at weekly intervals during the study period.

RESULTS

The present study revealed that the breeding site is surrounded by vegetation mainly grasses (*Cynodon dactylon, Erianthus fulvus*), shrubs and trees (*Castonopsis indica, Quercus glauca* and *Myrica esculenta*). Among the various anuran species observed in the habitat, members of the family Rhacophoridae (*Rhacophorus maximus, Rhacophorus bipunctatus, Polypedates teraiensis, Polypedates himalayensis and Kurixalus naso*) demonstrate the greatest number of reproductive modes adapted to a terrestrial life by constructing foam nest. Species belonging to the family Bufonidae (*Duttaphrynus melanostictus*), Hylidae (*Hyla annectans*) and Dicroglossidae (*Fejervarya nepalensis*) showed a common reproductive mode in lentic water during monsoon.

A select breeding habitat harbored eight anuran species from four families representing seven reproductive modes (Table 1). The rhacophorids of the area revealed six reproductive modes. They showed the common strategy that involved the construction of foam nests for egg deposition in both aquatic and terrestrial environments except for *Kurixalus naso*. The rhacophorid, *Rhacophorus maximus* exhibitd three reproductive modes, whereby the amplecting pair (Fig. 2A) deposited eggs by constructing foam nest floating on pond (mode 11; Fig. 2B), subterranean surfaces (mode 30; Fig. 2C) and on vegetation on the humid forest floor (mode 28; Fig. 2D). Exotrophic tadpoles were found in all these three modes. *Rhacophorus bipunctatus* (Fig. 3A) represented two modes (mode 28 and mode 33), where foam nests were constructed on the humid forest floor (mode 28; Fig. 3B) and on leaves (mode 33; Fig. 3C). The exotrophic tadpoles after hatching dropped into lentic habitat. *Polypedates himalayensis* (Fig. 4A) and *Polypedates teraiensis* (Fig. 5A) demonstrated a common reproductive mode (mode 28; Fig. 4B and Fig. 5B, respectively) whereby their eggs in foam nests were found to occur in thick vegetation cover on the humid forest floor about half a meter away from the water body with exotrophic tadpoles in pond. A newly recognized reproductive mode from the area of study shown by rhacophorid, *Kurixalus naso* revealed early emergence during the pre-monsoon after the first shower of rain and oviposited eggs in burrows (mode 17; Fig. 6A) and occasionally in the open soil surface on the ground (mode 18; Fig. 6B) with no foam formation. With the progression of the monsoon, the rain triggered the hatching process and further development of the tadpoles.

Duttaphrynus melanostictus eggs were deposited in the form of strings encased in a transparent jelly layer (Fig. 7) while amplecting pair of *Hyla annectans* (Fig. 8A) deposited eggs which spread as a surface film on the air water interface (Fig. 8B). *Fejervarya nepalensis* eggs (Fig. 9A) were found as clumps attached on the small vegetation about 5cms below the air water interface (Fig. 9B). However, *Duttaphrynus melanostictus, Hyla annectans and Fejervarya nepalensis* showed similar and the most common reproductive mode (mode 1) in lentic water with exotrophic tadpoles in ponds.

Ecological factors of the study site recorded during the study period revealed that the mean air temperature ranged from 15.5˚ C to 27˚ C and 14.4˚ C to 26˚ C in 2018 and 2019, respectively.

There was a gradual increase of air temperature with the progression of the monsoon (May-August) and then decline during the pre-monsoon (January-April) and the post-monsoon (September-December) (Table 2 and Fig. 10).

The mean water temperature had been recorded only from April to October in both the years as the breeding site was a temporary rain fed pond. The recorded mean water temperature ranged from 19˚ C to 23.6˚ C in 2018 and 18˚ C to 24.5˚ C in 2019. It was observed that there was no rainfall during the month of January in both the years. In the year 2018, the average daily rainfall ranged from 2.85mm in November to 129.46 mm in July while in 2019 it ranged from 13.44mm in November to 156.22mm in August. The relative humidity recorded at the breeding site ranged from 62% to 100% in 2018 and 57% to 100% in 2019 (Table 2 and Fig. 10).

Fig. 1 Breeding habitat at Mawsynram, Meghalaya, North East India.

Fig. 2 (A): *Rhacophorus maximus* amplecting pair; (B) reproductive mode 11; (C) reproductive mode 30; and (D) reproductive mode mode 28.

Fig. 3 (A): *Rhacophorus bipunctatus* amplecting pair; (B) showing reproductive mode 28 and; (C) reproductive mode 24.

Fig. 4 (A) *Polypedates himalayensis*; (B) showing reproductive mode 28.

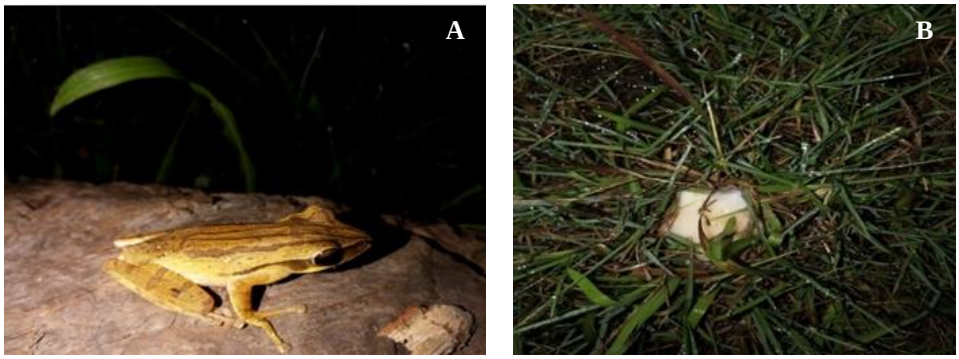

Fig. 5 (A) *Polypedates teraiensis*; (B) showing reproductive mode 28.

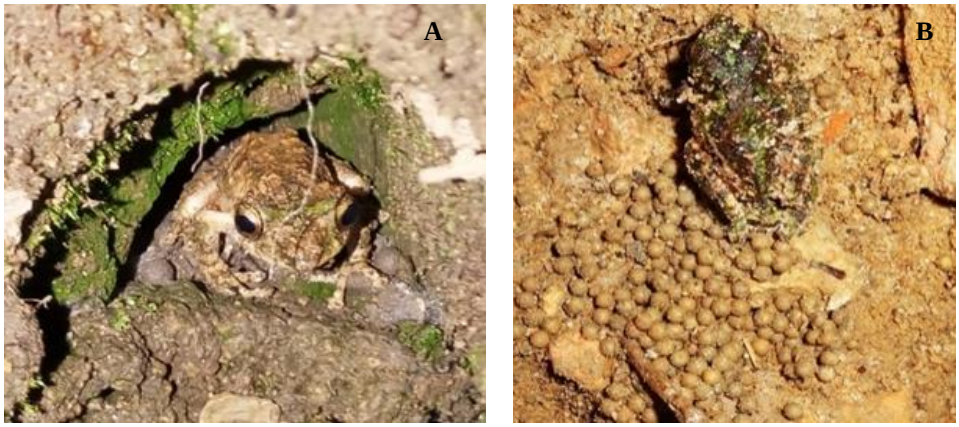

Fig. 6 (A) *Kurixalus naso* showing reproductive mode 17; (B) *Kurixalus naso* amplecting pair showing reproductive mode 18.

Fig. 7: *Duttaphrynus melanostictus* during oviposition showing reproductive mode 1.

Fig. 8 (A) *Hyla annectans* amplecting pair; (B) showing reproductive mode 1.

Fig. 9 (A) *Fejervaya nepalensis* amplecting pair; (B) showing reproductive mode 1.

DISCUSSION

The diversity and coexistence of eight anurans showing different reproductive modes in the same breeding habitat at Mawsynram suggest that it may be due to the availability of more microhabitats. The major ecological factors such as temperature, humidity, rainfall and vegetation structure may be of critical importance influencing the microhabitats selected for breeding by anurans. Further, it may be mentioned that the choice of oviposition site by *Rhacophorus maximus, Rhacophorus bipunctatus, Polypedates teraiensis and Polypedates himalayensis, Kurixalus naso, Duttaphrynus melanostictus, Fejervarya nepalensis and Hyla annectans* also depend on the availability of water in the breeding habitat for their survival as their eggs hatched into exotrophic tadpoles that completed their development to terrestrial adults in water. Since the breeding habitat was a rain fed pond chances of desiccation induced larval mortality was high (Smith, 1983; Newman, 1988). Hence, water availability acted as the directional cue for choice of breeding site by anurans (Lin et al., 2008).

Alternative reproductive strategies observed in case of *Rhacophorus maximus* (mode 11, mode 28 and mode 30), *Rhacophorus bipunctatus* (mode 24 and mode 28) and *Kurixalus naso* (mode 17 and mode 18), with more than one reproductive modes correspond with the finding that populations of the same species may exhibit alternative reproductive strategies (Toledo et al., 2012). A trend towards terrestriality was revealed by these anurans wherein they deposited their eggs in a foam nest away from the water body which might provide protection to the embryos from desiccation at the early stages (Duellman and Trueb, 1986). Similarly, Duellman and Trueb (1986) also suggested that laying eggs away from water in a foamy mass, in which tadpoles develop up to a pre-metamorphic stage before falling into water, is an alternative life history strategy. Oviposition of eggs in the moist soils and burrows by *Kurixalus naso* is of ecological significance and may help to offer protection from desiccation, and probably helps avoid predation by camouflaging with the soil as foam nest formation is absent (Shangpliang et al., 2020). Frther, these reproductive strategies may act as controlling

mechanism of an evolutionary trend towards terrestriality, allowing anurans to reproduce in terrestrial habitats (Salthe and Mecham, 1974, Duellman and Trueb, 1986).

The most common and phylogenetically widespread reproductive mode (mode 1) documented in *Duttaphrynus melanostictus, Hyla annectans and Fejervarya nepalensis* was also recorded in the diverse frog genera such as *Xenopus, Pelobates, Rana and Gastrophyne* (Duellman and Trueb, 1986). The ecological significance of such a mode is that it might be one of the adaptive features to meet the oxygen demands of the anuran embryos (Moore, 1940).

Different reproductive modes of anurans including their choice of oviposition sites appear to influence the general health and survival fitness of the embryos. Ecological factors such as air and water temperature together with rainfall and humidity appear to act in concert in preparing the suitable site for oviposition of anurans with different reproductive modes. Exceptionally high rainfall during the breeding season not only initiated the breeding activity of anurans (Shahriza et al., 2010) but also played a critical role on the hatching process of the eggs (Shangpliang et al., 2020). Rainfall provides water to the seasonal rain fed ponds to prepare the breeding ground suitable for completion of development of the observed anuran species. The high humidity level during the breeding period at the breeding site may also play an important role to support the diversified reproductive modes as amphibians are very susceptible to water loss. Reproduction, egg development and growth, adult activity and mortality requires moist habitats which act as an important limiting ecological factor (Duellman and Trueb,1986; Haddad and Prado, 2005). Haddad and Prado (2005) stressed more on the importance of humidity and moist habitats for anuran reproduction as most of them are disappearing when such sites become drier and seasonal. Temperature of the breeding site is another important ecological factor for development of the anurans (Duellman and Trueb, 1986). For normal development, temperature ranges vary among species. The temperature ranges during the breeding period observed in the present study appears to fall within the normal range for the survival of the eight anuran species. It may be suggested that increase in air and water temperature during the breeding period is responsible to support faster embryonic development of the eight anuran species at the breeding site. Similarly, Duellman and Trueb (1986) also mentioned that the development of the anuran embryos is much faster at the upper limit than at temperatures near the lower limit.

To summarise the present investigation, one seasonal breeding pond support seven diverse reproductive modes of eight anuran species. The climatic variables such as temperature, humidity and high rainfall of Mawsynram in the Southern slopes of Meghalaya influence survival, growth and completion of development of the eight anuran species. Furthermore, in addition to the studied ecological factors, presence of thick vegetation cover, burrows and open moist soil surfaces, longer hydric period at the habitat seems to be the major factors that prepare the site to harbour the diversified reproductive modes in the area. Data on reproductive modes of anurans may prove to be useful in understanding the species specific ecological requirements and this may support more realistic conservation strategies including creation of new protected areas that cover high diversity of anurans.

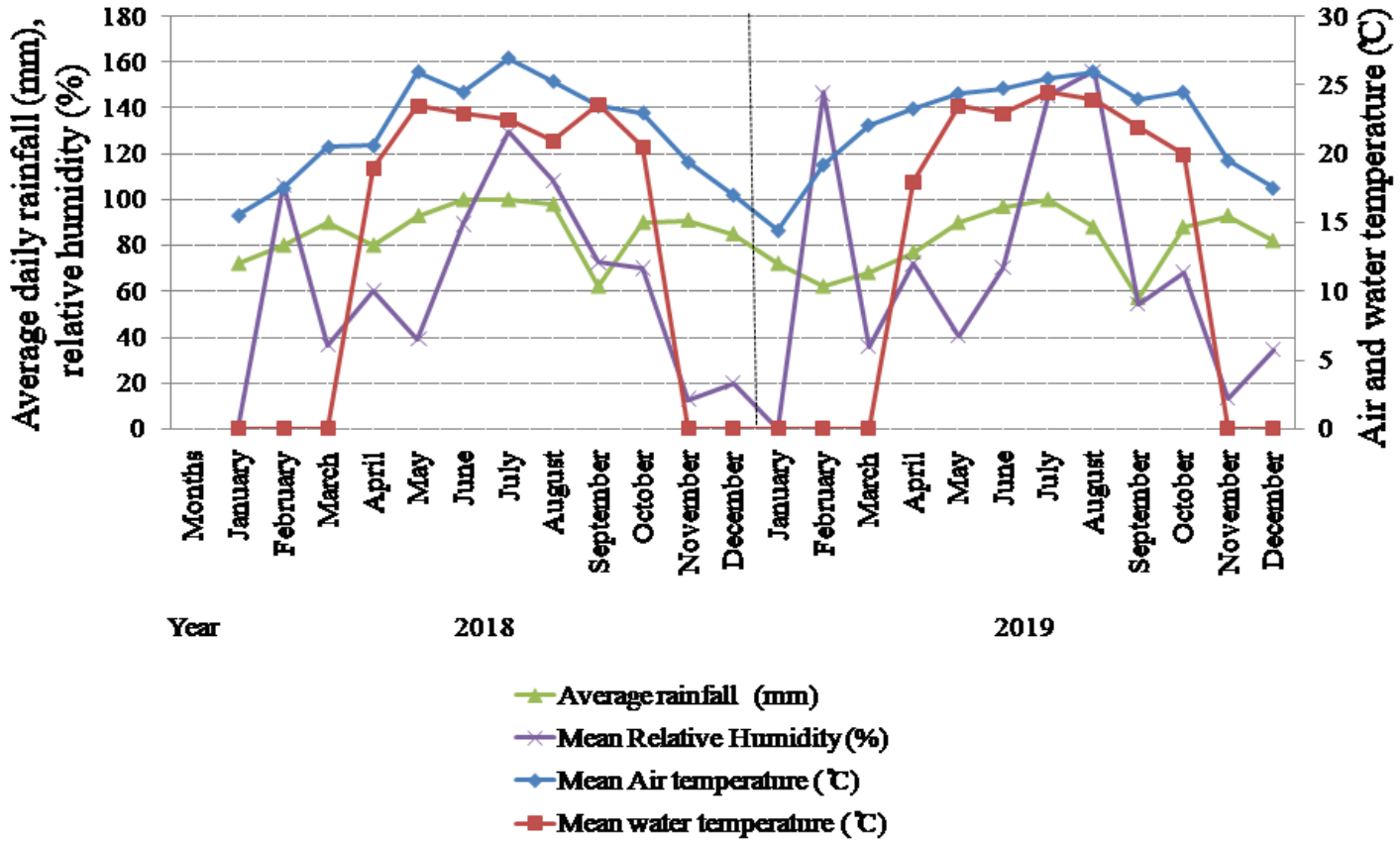

Fig. 10: Variations in the air and water temperatures (℃), relative humidity (%) and average rainfall (mm) of the breeding site at Mawsynram.

Table 1. Diverse reproductive modes of anuran species recorded from a habitat at Mawsynram, Meghalaya, North East India (as per the criteria provided by Haddad and Prado, 2005) (Le: Lentic breeding habitat; Tr: Terrestrial habitat).

Family	Species	Breeding period	Breeding habitat	Oviposition sites	Egg characteristics	Reproductive modes
Rhacophoridae	*Rhacophorus maximus*	March-early May	Tr	Vegetation above water surface and on grasses and stones on the edges of the pond and eggs sometimes floating on pond	Foam nests	**Mode 11:** Foam nest floating on pond, exotrophic tadpoles in ponds **Mode 28:** Foam nest on the humid forest floor; subsequent to flooding, exotrophic tadpoles in ponds **Mode 30:** Foam nest in subterranean constructed nests; subsequent to flooding, exotrophic tadpoles in ponds
	Rhacophorus bipunctatus	April-July	Tr	On leafy vegetation and wooden logs above water and on a humid forest floor	Foam nests	**Mode 33:** Eggs laid on leaves hatching into exotrophic tadpoles that drops in lentic water **Mode 28:** Foam nest on the humid forest floor; subsequent to flooding, exotrophic tadpoles in ponds
	Polypedates himalayensis	March-June	Tr	Damp, moist and also under thick vegetation cover half a meter away from the water	Foam nests	**Mode 28:** Foam nest on the humid forest floor; subsequent to flooding, exotrophic tadpoles in ponds
	Polypedates teraiensis	March-June	Tr	Grasses close to water bodies and sometimes lays its eggs in vegetation cover above water	Foam nests	**Mode 28:** Foam nest on the humid forest floor; subsequent to flooding, exotrophic tadpoles in ponds
	Kurixalus naso	February-March	Tr	Burrows and occasionally in an open soil surface	No foam formation, Eggs scattered as seeds and	**Mode 17:** Eggs and early tadpoles in nests inside soil; subsequent to flooding, exotrophic tadpoles in

					covered with soil	ponds **Mode 18:** Eggs on the ground or rock above water; upon hatching, exotrophic tadpoles move to water
Bufonidae	*Duttaphrynus melanostictus*	February-April	Le	Shallow water with a sandy substratum at the periphery of the pond	Eggs are deposited in the form of strings covered with transparent jelly layer	**Mode 1:** Eggs and exotrophic tadpoles in lentic water
Hylidae	*Hyla annectans*	April-May	Le	Pristine and clear undisturbed puddles on the sides of the breeding habitat	Pigmented eggs covered with thick jelly stick to one another and float as a mass on the air-water interface	**Mode 1:** Eggs and exotrophic tadpoles in lentic water
Dicroglossidae	*Fejervarya nepalensis*	May-July	Le	Vegetation on the sides pond which provides ideal hiding place for the adult frogs	The eggs are pigmented and have a thin layer of jelly cover	**Mode 1:** Eggs and exotrophic tadpoles in lentic water

Table 2. Air temperature, water temperature, relative humidity and rainfall of the study site. (- absence of water at the breeding site and *absence of rain at the breeding site).

Period		Mean Air temperature (℃)		Mean Water temperature (℃)		Mean Relative Humidity (%)		Average daily rainfall (mm)	
	YEAR	2018	2019	2018	2019	2018	2019	2018	2019
Months	January	15.5	14.4	-	-	72	72	*	*
	February	17.5	19.2	-	-	80	62	106.55	146.66
	March	20.5	22.1	-	-	90	68	36.37	36
	April	20.6	23.3	19.0	18.0	80	77	60.09	72.08
	May	26.0	24.4	23.5	23.5	93	90	39.32	40.66
	June	24.5	24.8	23.0	23.0	100	97	89.44	70.33
	July	27.0	25.5	22.5	24.5	100	100	129.46	145.55
	August	25.3	26.0	21.0	24.0	98	88	108.43	156.22
	September	23.5	24.0	23.6	22.0	62	57	72.65	54.44
	October	23.0	24.5	20.5	20.0	90	88	70.10	68.22
	November	19.4	19.5	-	-	91	93	12.85	13.44
	December	17.0	17.5	-	-	85	82	20.00	34.66

ACKNOWLEDGEMENTS

The authors are grateful to the Head of Zoology Department, NEHU, Shillong for providing the essential facilities in the Laboratory for smooth functioning of the work. This study was supported by The Science & Engineering Research Board (SERB), New Delhi [EEQ/2017/000081]for financial assistance to carry out the work. We also extend our gratitude to Dr. Ryan Juvilson Kharbhih (Directorate of Animal Husbandry and Veterinary, Government of Meghalaya) for his constant support and guidance throughout the work. We also thank Mr. Kermiky Symblai and Mr. Marlon Brando Rani for fieldwork assistance.

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Pranikee - Journal of Zoological Society of Orissa, ISSN 0970-4450
Volume XXXII, 2020, pp 16-32

TOXIC AND TERATOGENIC EFFECTS OF VITAMIN A ON ANURAN TADPOLES OF FOUR SUB-TROPICAL SPECIES

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ABSTRACT

Vitamin A is an important micronutrient responsible for appropriate development of vertebrate embryos. Compounds that include all natural and synthetic derivatives of vitamin A are called retinoids and they elicit their biological action in the form of all-trans retinoic acid. Vitamin A and retinoids are teratogenic and affect all organ systems in mammalian embryos. The present study examined the effects of vitamin A palmitate, a retinoid,on tadpoles of four sub-tropical anurans, *Duttaphrynus melanostictus*, *Polypedates maculatus*, *Microhyla ornata* and *Uperodon globulosus*, in laboratory conditions. Vitamin A palmitate proved to be lethal or toxic and mortality of tadpoles was dose dependent. Mortality rate of tadpoles was also species-specific indicating that different species respond differently to vitamin A. Anomalies in body size, tail size, total size, snout, eyes, tooth rows, body pigmentation, branchial chamber, tail shape and morphology and developmental delay in the exposed tadpoles was also dose-dependent and species specific. The study provides reference for toxic concentrations of vitamin A for the tadpoles of the four anuran species and can be helpful for amphibian caretakers and zookeepers undertaking captive breeding programs for anurans.The study emphasizes morphometry as an effective tool to discover deformities of anuran tadpoles in toxicological experiments.

Keywords: Anuran tadpoles, Morphometry, Teratogenic, Toxic, Vitamin A

INTRODUCTION

Vitamin A is an important micronutrient necessary for embryonic development (Clagett-Dame and Knutson, 2011) and retinoids are classes of compounds that include all natural and synthetically derived forms of vitamin A (Clugston and Blaner, 2014). Vitamin A is teratogenic where both deficiency and excess causes similar kinds of defects in eyes, face, ear, dentition, limbs, central nervous system, heart etc.of the developing embryos (Maden, 2000; Dubey et al., 2018). Due to their teratogenicity, Vitamin A and retinoids also pose risks to both captive and natural populations of anurans. Malformations of limbs, nervous system, eye, skin, rostral tentacles and developmental delay due to excess vitamin A and retinoids has been noticed in tadpoles of North American Ranids and *Xenopus laevis* (Weissmann, 1961; Weissmann et al., 1963; Degitz et al., 2000; 2003; Alsop et al., 2004). Conversely, inadequate dietary vitamin A is considered a risk to captive amphibians and altered vitamin A homeostasis to cause population declines of amphibians in the wild (Clugston and Blaner, 2014). In addition, a variety of chemicals, both natural and/or man-made, exist that mimic the activity of natural endogenous retinoids (Zhou et al., 2019). Retinoid activity in effluents from wastewater

treatment plants (Allinson et al., 2011) and sewage treatment plants (Zhou et al., 2019) find their way to aquatic habitats as run-offs or discharges and pose ecotoxicological risk to aquatic fauna (Allinson et al., 2011), including anuran tadpoles.

Vitamin A palmitate is a retinoid found in animal products like eggs, chicken and beef. Commercially available vitamin A palmitate is taken orally or applied topically for treating hypovitaminosis A (Ferrie et al., 2014). After ingestion, it is metabolized to all-trans-retinoic acid, that mediates the biological actions of vitamin A (Conaway et al., 2013). Studies on vitamin A and retinoid exposure of anuran tadpoles is mostly limited to species like *Xenopus* and certain Ranids (Degitz et al., 2000; 2003) and till date, only few studies report vitamin A-induced abnormalities in tadpoles of sub-tropical nurans (Jangir et al., 1994; Das and Mohanty-Hejmadi, 2000). A species wide evaluation of retinoid exposure thus becomes necessary to validate its effects on anurans. Also, there is scanty information on dose-response relationship of vitamin A or retinoid exposure on anuran tadpoles. Present study, thus, investigates the effects of different doses of vitamin A palmitate on tadpoles of four anurans *Duttaphrynus melanostictus* (Family: Bufonidae), *Polypedates maculatus* (Family: Rhacophoridae), *Microhyla ornata* and *Uperodon globulosus* (Family: Microhylidae), in laboratory conditions, to scertain the toxic concentrations of vitamin A palmitate as well as its teratogenic effects at different doses in these four species. The study for the first time uses morphometry to quantify the observed anomalies in body and tail of anuran tadpoles of different species.

MATERIALS AND METHODS

Collection of tadpoles

Egg strings of *Duttaphrynus melanostictus*, foam nests of *Polypedates maculatus*, egg clutches of *Microhyla ornata* and *Uperodon globulosus* were collected from different aquatic habitats during the year 2019 around Baripada, Odisha, India (21°57´27.28´´N, 86°44´22.87´´E) during the monsoons. Care was taken to collect them from areas with minimum human disturbance. They were kept in well-aerated water in the laboratory at room temperature and a 24-hour light-dark cycle was maintained. The average temperature and average humidity during that period varied between 28-30°C and 79-82%, respectively. After hatching, the tadpoles were transferred to glass aquariums (30cmX20cmX25cm) filled with dechlorinated tap water for acclimatization. The tadpoles were fed with boiled egg yolks and *Amaranthus* greens and staged following Gosner (1960). Pre-metamorphic stage tadpoles i.e. Gosner stage 28-30 were selected for vitamin A palmitate treatment. All experiments were performed as per the Institutional Animal Ethical Committee guidelines of North Orissa University.

Vitamin A palmitate treatment and mortality

Toxicity of vitamin A palmitate was assessed by subjecting tadpoles of all four species separately to different concentrations of aqueous vitamin A palmitate (Abbott Health care Pvt Ltd, Mumbai, India). Exposure was continued up to 24 hours as vitamin A palmitate loses its efficacy in aqueous solution within a day (Degitz et al., 2000; Sharow et al., 2012). Briefly, five concentrations of vitamin A palmitate*v iz.*, 0 (control), 5.5, 11, 16.5, and 22µg/ml in five replications were prepared from a saturated stock solution. Feeding was stopped a day prior to the treatment. Twelve tadpoles of each species were exposed to 1 litre of above-mentioned concentrations of vitamin A palmitate solution in

glass bowls (20cm×10 cm). The number of tadpoles that died within 24 hours exposure were scored for mortality. Percentage mortality of tadpoles for all the treated sets and their controls was recorded. The remaining tadpoles that survived the 24-hour treatment were transferred to 1 litre of dechlorinated water and observed up to metamorphosis or death. The duration for which tadpoles survived post treatment and metamorphosed was also recorded.

Morphology and morphometry

A separate set of treatments as described above, comprising 5 replicates of 12 tadpoles (n=60) from each species, was used to score morphology and morphometry. The tadpoles of treated and control sets (n=15) were fixed 15dpe in 10% formalin and measurements such as body length (BL), internarial distance (IND), interorbital distance (IOD), total length (TL), tail muscle height (TMH) and maximum tail height (MTH) were recorded (Fig. 1) as per Altig (2007).

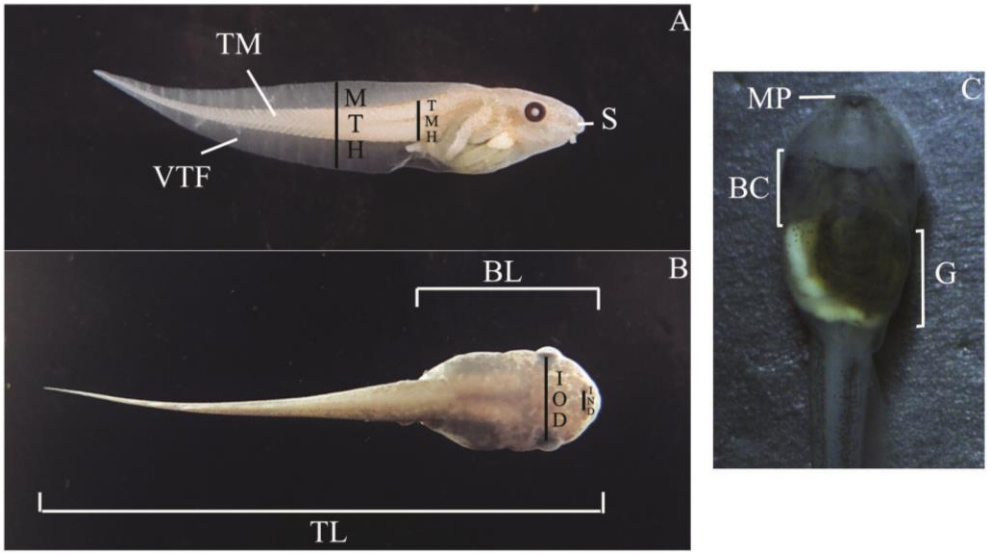

Fig. 1 Morphology and morphometrical parameters of an anuran tadpole.
A: Lateral view; B: Dorsal view; C= Ventral view BC= Branchial chamber; BL= Body Length; G= Gut; IND= Inter-narial distance; IOD= Inter-orbital distance; MP= Mouth parts; MTH=Maximum tail height; TL= Total length; TM= Tail muscle; TMH= Tail muscle height; S= Snout; Photographs not according to scale.

Tadpoles exposed to certain high doses, where only few tadpoles survived 15dpe,were also measured (n=5). High dose treatment sets where tadpoles did not survive beyond 5dpe were excluded from the study. All the measurements were done with an ocular micrometer on a stereo zoom microscope (Hund, Wetzlar). To ascertain the changes in shape of the body, ratios like BL: TL, BL: TAL, TAL:

TL, IOD: BL, IND: BL, IOD: TL, IND: TL, TMH: MTH, TMH: TAL, MTH: TAL,TMH: TL and MTH: TL were evaluated from the morphometric parameters.

Morphological features like the gut, branchial chamber, skin, eyes, snout, mouth parts and tails were also examined for any anomalies on the same day of fixation i.e., 15dpe and photographed. Eosin red was used to stain the mouthparts of tadpoles of *P. maculatus* for proper resolution of characters.

Statistics

Variations in individual morphometrical parameters as well as ratios were tested by Kruskal Wallis (KW) test. The data were statistically analyzed at a significance level of $p < 0.05$ and calculations were performed with SPSS statistical analysis package.

RESULTS

Mortality

Mortality rates in response to vitamin A palmitate, varied in the four anuran species (Table 1) and increased with increase in concentration of vitamin A palmitate.

Tadpoles of all the four species did not survive beyond 15-20dpe, in treatments where more than 15% mortality was recorded. Additionally, tadpoles did not survive beyond 5dpe in treatments that resulted in more than 45% mortality in 24 hours. In the rest of the treatments tadpoles survived upto 30dpe or metamorphosed.

Table 1: Mortality (%) of the tadpoles (n=60) exposed to vitamin A palmitate.

Concentration of Vitamin A palmitate (µg/ml)	*D. melanostictus*	*P. maculatus*	*M. ornata*	*U. globulosus*
0	0.00	0.00	0.00	0.00
5.5	0.00	5.00	1.66	0.00
11.0	0.00	21.66	3.34	40.17
16.5	15.00	56.67	11.67	93.34
22.0	45.00	83.34	25.00	100

Abnormalities in body morphometry and morphology

The effect of vitamin A palmitate was quantified by measuring the morphometrical parameters 15days post exposure (dpe) as it was the minimum duration at which significant differences in morphometry was noticeable between control and treated tadpoles, in all the four species.

Duttaphrynus melanostictus

Variations in individual morphometric parameters like BL, TAL, TL, IND, MTH and TMH and ratios IND:BL, IND:TL, TMH: TAL, TMH:TL and TMH: MTH were dose-dependent (Table 2). Increase in IND: BL and IND:TL in 5.5µg/ml treatment indicated widening of the distance between nares. The snout appeared slightly oblong with the nares positioned more dorsally in contrast to a nearly semi-circular snout and anteriorly positioned nares of the control (Figs. 2A-B). Interestingly, in a treated individual, the right eye was displaced dorsally than its normal lateral position (Fig. 2B). Increase in TMH: TAL, TMH:TL and TMH: MTH ratios indicated expansion of tail muscle along with reduction in thickness of the fins due to treatment. The oral apparatus consisted of jaws with tooth rows (Fig. 2M) as compared to the control which had jaws without tooth rows (Fig. 2L). In 11 and 16.5µg/ml treatments, the morphological anomalies were quite similar like smaller eyes, oblong snout with dorsally placed nares (Figs. 2A and C), jaws with tooth rows, transparent skin and thickened tail muscle. Especially in the 16.5µg/ml treatment, the gut occupied a major portion of the body and the branchial chamber was reduced as compared to the control (Figs. 2D and E). Metamorphosis was delayed in tadpole exposed to lower doses i.e., 5.5 and 11µg/ml, 95% and 90% tadpoles underwent metamorphosis at 23-25dpe and 22-25dpe in 5.5 and 11µg/ml treatments, respectively, while 100% control tadpoles metamorphosed 17-20dpe. Tadpoles exposed to higher doses i.e., 16.5 and 22 µg/ml could not metamorphose and died within 15-20dpe and 5dpe, respectively.

Polypedates maculatus

Variations in individual morphometric parameters and certain ratios were significant in the treated groups (Table 3). In 5.5 µg/ml treatment, there was no noticeable difference except for a reduction in overall size. However, vitamin A treatment resulted in fragmentation of tooth rows and bending of tail. The ventral tail fins were also narrower than the control ones. 70% of tadpoles metamorphosed at 50-60dpe while 100% control tadpoles metamorphosed 30-35dpe. Treatment with 11µg/ml resulted in significant decline in all morphometrical parameters (Table 3) and the tadpoles were diminutive as compared to 5.5 µg/ml and control. The snout of tadpoles appeared semi-circular as compared to a more oblong snout of control ones (Figs. 2F-G). Tooth rows and jaw sheath were fragmented in contrast to the control tadpoles (Figs. 2N and 2O). Such tadpoles could not live to metamorphose and died within 15-20dpe. 16.5 and 22 µg/ml treatment caused high mortality and tadpoles died within 4-5dpe and 2-3dpe, respectively.

Microhyla ornata

The changes observed between individual morphometric parameters and ratios, except BL:TL, TAL:TL and BL:TAL, among control and treated groups was significant and dose dependent (Table 4). Treatment with 5.5µg/ml resulted in overall increase in the size of the tadpoles. Significant increase

in tail ratios and decrease in TMH:MTH ratios indicated increase in the expanse of tail especially of tail fins. Infact, the ventral tail fin was wider in the treated tadpoles. There was decrease in IND: BL and IND:TL ratios and the snout appeared triangular in contrast to semi-circular snout of the control. The skin was more transparent with increased pigmentation on the dorsal surface but the gut and branchial chamber showed no visible anomalies.

Tadpoles of 11µg/ml treatment showed morphological anomalies similar to the 5.5µg/ml treatment in addition to bent tail and shrivelled tail fins. Treatment with 16.5µg/ml caused a significant increase in the BL, TAL, TL, IOD, MTH and TMH in comparison to control but the values were lower and the tadpoles were smaller than 11µg/ml treatment. Besides that, there was substantial increase in IOD:BL and IOD:TL along with decrease in IND: BL and IND: TL and the snout appeared semi-circular (Figs. 2H and I).

In 22µg/ml treated tadpoles, IND: BL andb IND: TL ratios were significantly higher along with IOD: BL and IOD:TL but the snout appeared semi-circular like 16. 5µg/ml treatment. Except IND, other individual morphometric parameters were not significantly different from control and the tadpoles did not survive beyond 15-20dpe. Other anomalies were not very pronounced except the tail where the fins were shrivelled.

The tadpoles of 5.5,11 and 16.5 µg/ml treatments, kept dying intermittently upto 15dpe and regularly after that and did not survive beyond 30dpe. Thus, all the treated tadpoles of *M. ornata* could not metamorphose while all the control tadpoles attained metamorphosis 45-50dpe.

Uperodon globulosus

The differences in the individual morphometric parameters except MTH, IND: BL and IND:TL ratios were dose-dependent (Table 5). Tadpoles treated with 5.5µg/ml showed overall increase in body size. Anterior most part of snout appeared flattened due to increased IND: BL and IND:TL and the snout appeared trapezium shaped. There was also increased pigmentation in both body and tail. Other morphometric ratios did not show any significant difference among control and treated groups without any other remarkable anomalies in the body and tail.

In the 11µg/ml treatment, IND: BL and IND:TL ratios were significantly lower than control. Snout was semi-circular as compared to rectangular snout in control (Figs. 2J and K). Both body and tail showed increased pigmentation as compared to the control.

Treatment with 16.5 and 22µg/ml caused heavy mortality and tadpoles died within 2-3dpe. Tadpoles of 5.5 and 11 µg/ml treatment groups kept dying sporadically up to 15dpe and regularly post 15dpe. The treated tadpoles did not survive beyond 30dpe and could not attain metamorphosis. All the control tadpoles metamorphosed within 60-65dpe.

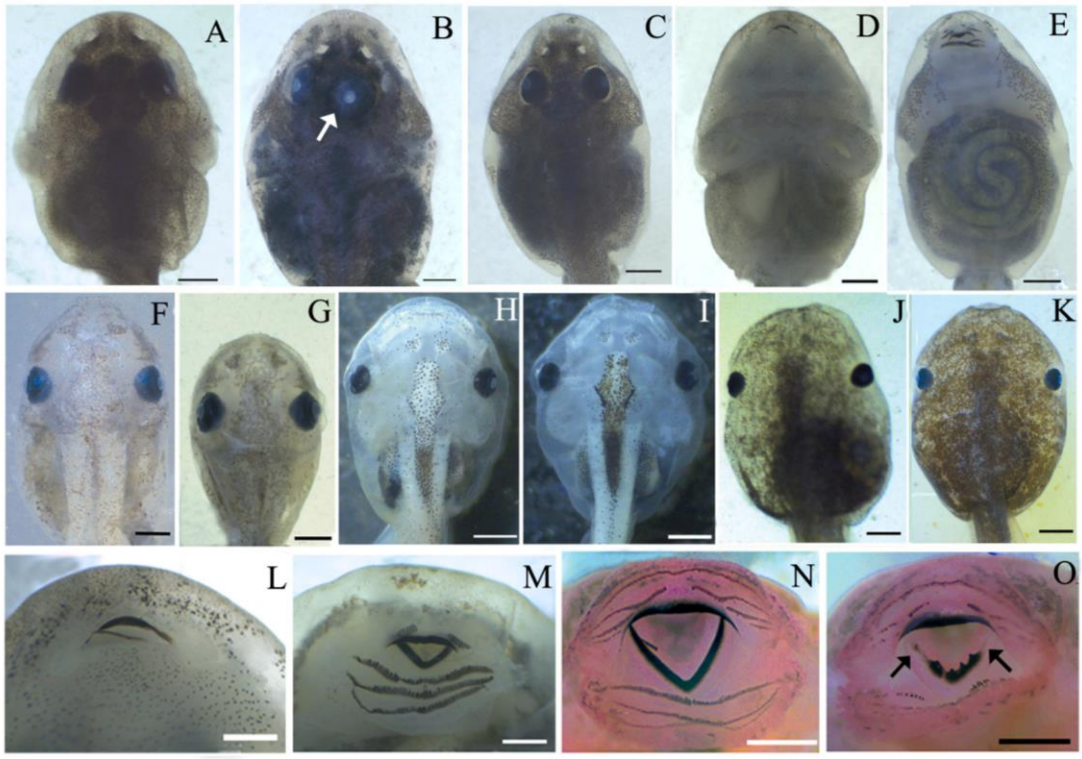

Fig. 2 Morphology of vitamin A treated tadpoles. (A) Dorsal view of body of tadpole of *Duttaphrynus melanostictus* from control group; (B) Dorsal view of 5.5µg/ml treated tadpole of *D. melanostictus*; (C) Dorsal view of 16.5µg/ml treated tadpole of *D. melanostictus*; (D) Ventral view of control tadpole of *D. melanostictus*; (E) Ventral view of 16.5 µg/ml treated tadpole of *D. melanostictus*; (F) Dorsal view of control tadpole of *Polypedates maculatus*. (G) Dorsal view of 11µg/ml treated tadpole of *P. maculatus*; (H) Dorsal view of control tadpole of *Microhyla ornata*; (I) Dorsal view of 11µg/ml treated tadpole of *M. ornata*; (J) Dorsal view of control tadpole of *Uperodon globulosus*; (K) Dorsal view of 11µg/ml treated tadpole of *U. globulosus*; (L) Mouthparts of control tadpoles of *D. melanostictus*; (M) Mouthparts of 5.5µg/ml treated tadpoles of *D. melanostictus*; (N) Mouthparts of control tadpole of *P. maculatus*; (O) Mouthparts of 11µg/ml treated tadpole of *P. maculatus.*

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(White arrow points to the displaced eye; Black arrows indicate broken jaw)

Bars A-K= 1mm; L-O= 0.5mm.

Table 2: Morphometry of vitamin A exposed tadpoles of *Duttaphrynus melanostictus*.

Conc.** (µg/ml)	BL*	TAL*	TL*	IND*	IOD	MTH*	TMH*	BL: TL	TAL: TL	BL: TAL	IND: BL*	IND: TL*	IOD: BL	IOD: TL	TMH: TAL*	TMH: TL*	MTH: TAL	MTH: TL	TMH: MTH*
0	7.93± 0.67	9.04± 0.99	16.97 ± 1.65	1.19 ± 0.15	2.15 ± 0.37	3.13 ± 0.33	1.41 ± 0.21	0.467± 0.041	0.532± 0.007	0.879± 0.025	0.151 ± 0.007	0.070± 0.002	0.269 ± 0.029	0.126± 0.012	0.157± 0.031	0.083± 0.015	0.350± 0.058	0.186± 0.029	0.448 ± 0.04
5.5	9.06± 0.26	10.46± 0.39	19.27 ± 0.44	1.44 ± 0.09	2.39 ± 0.53	3.44 ± 0.33	1.91 ± 0.2	0.469± 0.029	0.530± 0.01	0.883 ± 0.005	0.159 ± 0.008	0.074± 0.003	0.256 ± 0.073	0.131± 0.011	0.190± 0.021	0.100± 0.011	0.326± 0.042	0.176± 0.021	0.575 ± 0.05
11.0	8.21 ± 0.49	9.31± 0.62	17.50± 1.09	1.25 ± 0.08	2.19 ± 0.15	3.16 ± 0.25	1.61 ± 0.15	0.468± 0.023	0.531± 0.006	0.882± 0.021	0.152 ± 0.013	0.071± 0.006	0.266 ± 0.014	0.125± 0.006	0.178± 0.018	0.095± 0.009	0.332± 0.016	0.180± 0.010	0.524 ± 0.04
16.5	7.50 ± 0.35	8.67± 0.22	16.17 ± 0.46	1.27 ± 0.15	2.28 ± 0.26	2.79 ± 0.1	1.76 ± 0.27	0.463± 0.012	0.536± 0.01	0.865 ± 0.039	0.165 ± 0.012	0.075± 0.006	0.285 ± 0.037	0.138± 0.016	0.174± 0.005	0.093± 0.002	0.324± 0.016	0.171± 0.007	0.546 ± 0.03

[All measurements except ratios are represented in mm (Mean ± Standard deviation)] *****: All groups significantly different at $p<0.05$, ** = Concentration

Table 3:Morphometry of vitamin A exposed tadpoles of *Polypedates maculatus*

Conc.** (µg/ml)	BL*	TAL*	TL*	IND*	IOD*	MTH*	TMH*	BL: TL	TAL: TL	BL: TAL	IND: BL*	IND: TL*	IOD: BL*	IOD: TL*	TMH: TAL	TMH: TL	MTH: TAL*	MTH: TL*	TMH: MTH
0	8.38 ± 0.42	13.80± 0.67	22.17± 1.01	1.35 ± 0.11	3.44 ± 0.13	4.02 ± 0.67	2.43 ± 0.35	0.380± 0.008	0.619± 0.008	0.611± 0.020	0.159 ± 0.010	0.060± 0.003	0.406 ± 0.019	0.154± 0.008	0.181± 0.022	0.112± 0.013	0.298± 0.040	0.185± 0.025	0.613 ± 0.082
5.5	7.32 ± 0.22	11.72± 0.76	19.00 ± 0.90	1.7 ± 0.14	3.27 ± 0.32	3.78 ± 0.53	2.11 ± 0.38	0.384± 0.013	0.615± 0.013	0.625 ± 0.034	0.232 ± 0.019	0.089± 0.008	0.447 ± 0.046	0.172± 0.020	0.182± 0.043	0.111± 0.022	0.317± 0.059	0.200± 0.037	0.559 ± 0.064
11.0	6.72 ± 1.	10.3 ± 1.91	17.00 ± 3.21	0.86 ± 0.11	2.92 ± 0.24	2.88 ± 0.81	1.74 ± 0.56	0.394± 0.005	0.605± 0.005	0.651± 0.015	0.133 ± 0.041	0.052± 0.016	0.452 ± 0.122	0.176± 0.040	0.166± 0.042	0.100± 0.025	0.284± 0.039	0.167± 0.022	0.602 ± 0.135

[All measurements except ratios are represented in mm (Mean ± Standard deviation)] *****: All groups significantly different at $p<0.05$, ** = Concentration

Table 4: Morphometry of vitamin A exposed tadpoles of *Microhyla ornate*

Conc.** (µg/ml)	BL*	TAL*	TL*	IND*	IOD*	MTH*	TMH*	BL: TL	TAL: TL	BL: TAL	IND: BL*	IND: TL*	IOD: BL*	IOD: TL*	TMH: TAL*	TMH: TL*	MTH: TAL*	MTH: TL*	TMH: MTH *
0	5.48 ± 0.28	12.49± 0.37	17.97 ± 0.65	0.73 ± 0.3	3.07 ± 0.3	2.04 ± 0.45	1.18 ± 0.07	0.305± 0.005	0.694± 0.005	0.438 ± 0.011	0.135 ± 0.033	0.041± 0.009	0.559 ± 0.057	0.170± 0.016	0.094± 0.008	0.065± 0.006	0.162± 0.031	0.112± 0.021	0.606 ± 0.159
5.5	6.54 ± 0.20	14.81± 0.33	21.40 ± 0.49	0.77 ± 0.11	77 ± 0.44	2.79 ± 0.39	1.36 ± 0.24	0.308± 0.004	0.691± 0.004	0.446 ± 0.008	0.116 ± 0.016	0.036± 0.005	0.570 ± 0.059	0.167± 0.019	0.091± 0.016	0.063± 0.010	0.187± 0.023	0.129± 0.015	0.492 ± 0.095
11.0	7.33 ± 0.43	15.65± 0.75	23.00 ± 1.18	0.78 ± 0.2	4.18± 0.62	3.04± 0.21	1.35 ± 0.1	0.318± 0.002	0.681± 0.002	0.467 ± 0.005	0.105 ± 0.026	0.034± 0.008	0.569 ± 0.120	0.183± 0.037	0.086± 0.01	0.059± 0.007	0.194± 0.018	0.132± 0.013	0.446 ± 0.046
16.5	6.61 ± 0.40	14.18± 0.80	21.00 ± 1.20	0.73± 0.00	4.30 ± 0.27	2.92 ± 0.7	1.50± 0.23	0.317± 0.002	0.682± 0.002	0.465 ± 0.004	0.110 ± 0.006	0.035± 0.002	0.650 ± 0.075	0.207± 0.023	0.105± 0.013	0.072± 0.008	0.205± 0.045	0.140± 0.031	0.528 ± 0.095
22.0	5.68 ± 0.43	12.44± 0.31	18.12 ± 0.59	0.86 ± 0.55	3.54 ± 0.32	2 ± 0.33	1.06 ± 0.15	0.313± 0.016	0.686± 0.016	0.456± 0.034	0.151± 0.019	0.047± 0.003	0.623± 0.036	0.195± 0.014	0.085± 0.01	0.058± 0.006	0.161± 0.056	[illegible] 0.036	0.578± 0.208

[All measurements except ratios are represented in mm (Mean ± Standard Deviation)] ***** All groups significantly different at $p<0.05$, ** = Concentration

Table 5 Morphometry of vitamin A exposed tadpoles of *Uperodon globulosus*

Conc.** (µg/ml)	BL*	TAL*	TL*	IND*	IOD*	MTH	TMH*	BL: TL	TAL: TL	BL: TAL	IND: BL*	IND: TL*	IOD: BL	IOD: TL	TMH: TAL	TMH: TL	MTH: TAL	MTH: TL	TMH: MTH
0	6.09 ± 0.18	8.89± 0.94	14.98 ± 1.13	0.80± 0.18	3.75 ± 0.18	2.87 ± 0.03	1.11 ± 0.25	0.407± 0.018	0.592± 0.018	0.688± 0.052	0.130 ± 0.026	0.052± 0.008	0.615 ± 0.011	0.250± 0.006	0.123± 0.014	0.073± 0.011	0.324± 0.030	0.191± 0.012	0.385 ± 0.081
5.5	7.11 ± 0.66	10.5± 0.96	17.70 ± 1.58	1.08 ± 0.2	4.31 ± 0.34	3.12 ± 0.33	1.33 ± 0.10	0.402± 0.009	0.597± 0.009	0.674 ± 0.025	0.152± 0.024	0.061± 0.009	0.607 ± 0.047	0.244± 0.014	0.126± 0.013	0.075± 0.007	0.299± 0.049	0.178± 0.029	0.428 ± 0.051
11.0	6.44 ± 0.22	9.42± 0.37	15.9 ± 0.5	0.66 ± 0.05	3.92 ± 0.10	3.21 ± 0.55	1.10 ± 0.11	0.406± 0.009	0.593± 0.009	0.684± 0.024	0.102 ± 0.01	0.041± 0.005	0.608 ± 0.014	0.247± 0.009	0.116± 0.013	0.070± 0.006	0.339± 0.049	0.194± 0.006	0.351± 0.089

[All measurements except ratios are represented in mm (Mean ± Standard Deviation)] *****: All groups significantly different at $p<0.05$, ** = Concentration

DISCUSSION

Anuran larva or tadpoles are sensitive to changes during development and hence are affected by teratogens like vitamin A. They exhibit specific morphological characters suited to their respective microhabitats (Altig and McDiarmid, 1999; Baldo et al., 2014) and alteration of morphology and morphometry have serious implications on their survival. The present study provides evidence for dose-dependent as well as species-specific effects of vitamin A palmitate on the tadpoles of four sub-tropical anuran species *Duttaphrynus melanostictus*, *Polypedates maculatus*, *Microhyla ornata*, and *Uperodon globulosus*.

Vitamin A exposure resulted in an increase in body size at lower concentrations and death at higher concentrations. It also affected eye, tooth and jaw development, shape of snout and tail and body pigmentation. Besides, it delayed metamorphosis in tadpoles that survived the exposure.

Vitamin A was toxic to the tadpoles at higher doses as evident from the mortality rates. A dose-response relationship was indicated in mortality rates where vitamin A palmitate caused progressive increase in mortality with increase in doses. Each species had a species-specific response to vitamin A palmitate. For example, the same concentration of vitamin A was not toxic to all the four species; like 16. 5µg/ml vitamin A caused 93.34% mortality in *U. globulosus* while only 11.67% mortality in *M. ornata*. Lower concentrations like 5.5µg/ml caused death of tadpoles of *M. ornata* and *U. globulosus* within 30dpe but tadpoles of *D. melanostictus* and *P. maculatus* were able to metamorphose, although delayed.

A significant increase in growth (body size, BL; tail size, TAL and total size of tadpole, TL) was observed in treatment groups exposed to lower doses in *D. melanostictus, M. ornata and U. globulosus*. Tadpoles of *D. melanostictus* and *M.ornata* increased in size with increase in concentration of vitamin A which then progressively reduced upon further increase in concentration. Maximum size was attained at concentrations of 5.5µg/ml for *D. melanostictus* and *U. globulosus* and 11µg/ml for *M. ornata*, followed by decline in size with increase in concentration. Such dose-dependent effects have been observed in anuran tadpoles of Booroolong frog fed with a retinoid, β-carotene (Keogh et al., 2018). Dietary vitamin A has also been reported to show significant growth in fishes like, *Epinephelus coioides* (Yang et al., 2017), *Oreochromis niloticus* (Guimaraes et al., 2014) and *Carassius auratus* (Shao et al., 2016). Vitamin A and retinoids help in cell growth and differentiation during embryonic development (Clagett-Dame and Knutson, 2011; See and Clagett-Dame, 2009) and the present study confirms such explanation albeit at low doses. Higher doses of vitamin A lead to little or no growth in the tadpoles of above three species and ultimately resulted in death, while it caused diminutive tadpoles in all treatment groups of *P. maculatus*. The morphometric ratios BL:TL, TAL:TL and BL: TAL showed no significant changes between control and treated groups of all four species indicating that there was no change in body shape upon vitamin A exposure. Though there was growth or diminution in tadpoles, body shape remained intact. The, individual morphometric parameters especially, BL, TAL and TL, showed variations in all the four species and hence can be effective markers for immediate identification of vitamin A exposure.

Inter-narial distance (IND), inter-orbital distance (IOD) and their ratios to body length and total length (IND: BL, IOD: BL, IND: TL and IOD: TL) were taken as a measure to record anomalies in the facial

skeleton and antero-dorsal part of the body. The treated tadpoles greatly varied in the shape of their snout and the ratios also validated it. For example, in *P. maculatus* where normally the snout is oblong, significantly higher IOD: BL and IOD:TL ratios resulted in a semi-circular snout. Similarly, increase in IND: BL and IND:TL ratios resulted in oblong snout in *D. melanostictus* which normally had a semi-circular snout. However, if all four ratios are significantly higher then there was no change in shape as seen in 5.5µg/ml of *P. maculatus* and 22µg/ml treatment of *M. ornata*. Although, vitamin A and retinoid mediated anomalies in facial skeleton has not been described in any anuran tadpoles, Wiley et al. (1983) have reported facial skeletal defects in the progeny of retinoic acid treated gravid hamsters. Such defects were caused by delayed and disorganized patterns of migration as well as degeneration of cranial neural crest cells.

The tail is an indispensable organ for the tadpole life. A tadpole's tail is composed of muscle core and tail fins where tail fins are made of single-layered epidermis and scanty connective tissue. Morphometric ratios MTH:TAL, MTH:TL, TMH: TAL, TMH:TL and TMH: MTH gave an inclusive idea about the shape of the tail. Analysis of those ratios showed that vitamin A caused expansion of tail muscle and tail fins in tadpoles of *D. melanostictus* and *M. ornata* respectively. Although there was fluctuation in individual parameters like TMH and MTH, that could only be attributed to the overall growth or diminution of the tadpoles on exposure. Vitamin A administration has been shown to promote myogenesis and postnatal muscle growth by increasing muscle satellite cell density in neonatal calves (Wang et al., 2018). It also caused metaplasia and stimulation of gap junctions in embryonic and neoplastic epidermis (Elias, 1987). Both the tail muscles and fins function to produce undulating movements for swimming. Tail fins and tip also help to escape from predators (Hoff and Wassersug, 2000). Significant changes in shape of tail, shriveled tail fins, abnormalities like bending in *P. maculatus* and pigmentation in *U. globulosus* is expected to cause difficulties in swimming and escaping predators.

The vitamin A exposed tadpoles of *D. melanostictus* showed anomalies like reduction in eye-size and position and reduction in branchial chamber. Absence and reduction in eye size on exposure to vitamin A has been reported in *X. laevis* (Alsop et al., 2004). Reduction in branchial chamber supposedly prevents efficient respiration and ultimately death of tadpoles. Reduction in size of branchial chamber is perhaps the first report on vitamin A induced anomaly. However, anomalies in eye-size and position and reduction in branchial chamber was not observed in the other three species.

The skin of the vitamin A treated tadpoles of *D. melanostictus* and *M. ornata* appeared to be more transparent than the control probably due to thinning of skin epidermis. Hyperpigmentation of skin was also observed in the tadpoles of *M. ornata* and *U. globulosus*. Vitamin A, on the other hand, is known to cause epidermal thickening (Mukherjee et al., 2006) and also causing hyperpigmentation by inhibiting melanin dispersion (Sarkar et al., 2013). Thus, thinning of skin epidermis can be considered as first report for vitamin A exposure. Hyperpigmentation of skin as well as skin hemorrhage to vitamin A exposure has been documented in *X. laevis* (Weissmann et al., 1963). Although hyperpigmentation was observed in exposed tadpoles, skin haemorrhage was not seen.

Exposure to vitamin A also resulted in precocious development of tooth rows in *D. melanostictus* while it caused disruption in the normal jaw development in *P. maculatus*. However, tadpoles of *B.*

melanostictus (= *D. melanostictus*) reared in 15, 20 and 30 IU/ml vitamin A palmitate (equivalent to 8.25, 11 and 16.5µg/ml vitamin A palmitate) showed complete absence of keratinized epidermal material over the jaws and horny labial teeth (Jangir et al., 1994). The present study, thus, disagrees with this report. Vitamin A deficiency causes anomalies during tooth and jaw development (Sheetal et al., 2013) but excess of vitamin A causing tooth and jaw deformities is a new finding. Thus, vitamin A elicits species specific effects on tooth and jaw development of anuran tadpoles. Jaw sheaths and teeth were absent in microhylids, *M. ornata* and *U. globulosus*, hence were not included in the study.

Metamorphosis was also delayed in certain treatment groups of *D. melanostictus* and *P. maculatus* exposed to lower doses of vitamin A. This demonstrates that vitamin A interferes with thyroid hormone mediated metamorphosis in the above two species. Vitamin A mediated developmental delay has been reported in *Xenopus laevis* (Weismann, 1961) but, recently, β-carotene, another retinoid, was shown to accelerate growth and metamorphosis in Booroolong frogs (Keogh et al., 2018). In the present study, however, in most treatment groups, the tadpoles could not attain metamorphosis especially in *M. ornata* and *U. globulosus* although there was increase in the size of the tadpole. This shows that excess vitamin A is detrimental to the survival of microhylids.

CONCLUSION

The present study provides a detailed assessment of the morphological anomalies elicited by vitamin A in tadpoles of four sub-tropical anurans. Morphometry effectively quantified anomalies and identified variations that were statistically significant and facilitated in establishing dose-response relationships. Ratios of morphological parameters helped determine noteworthy changes in the shape of the body. Morphometry can thus be used to quantify morphological anomalies of anuran tadpoles in toxicological experiments.

Anuran amphibians are difficult groups to maintain in captivity because of their diverse life-history. Vitamin A is an important part of the diet of amphibians with captive amphibians being predisposed to both hypo- and hypervitaminosis (Clugston and Blaner, 2014). Adequate amount of vitamin A in the diets of all life-stages is thus vital for captive amphibians (Hadfield et al., 2006). Since, vitamin A is mostly acquired through food, it is essential to determine its precise amounts for diet supplementation, especially for the larval stages, considering its teratogenicity. Recently, there has been increased focus on conservation breeding programmes for amphibians in India (Gupta et al., 2015), but there is lack of scientific data for devising diet plans for larval stages of captive anurans. Since, the present study describes a dose-response relationship and gives reference values of toxic concentration of vitamin A palmitate on tadpoles, it can help amphibian caretakers in zoos to develop diet plans for larval stages. Further studies with endangered anuran species can be done in future to increase the scope and value of this work.

Retinoids and retinoid mimics in the environment present ecotoxicological risks for aquatic organisms (Zhou et al., 2019). This study substantiates the teratogenicity of vitamin A, a retinoid which can play a bigger role in affecting natural population of anurans if they somehow find their way to the environment. However, vitamin A and retinoids as probable threats to anuran population have not been

adequately verified, for which extensive field studies along with identification of potential retinoids and retinoid mimics are needed.

Besides that, endocrinology of anuran metamorphosis is very similar to the human perinatal period (Buccholz, 2017) and vitamin A was found to delay metamorphosis in *D. melanostictus* and *P. maculatus*. Thus, anuran tadpoles can also present a model to study the role of vitamin A in perinatal endocrinology of humans.

ACKNOWLEDGEMENTS

CM thanks the University Grants Commission, Government of India, for a UGC-BSR Start-up Grant.

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Pranikee-Journal of Zoological Society of Orissa, ISSN 0970-4450
Volume XXXII, 2020, pp 33-42

CHALCIDIDAE OF MEGHALAYA: NEW RECORDS OF THREE GENERA WITH A NOTE ON THEIR DISTRIBUTION

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ABSTRACT

Chalcids or parasitic hymenopterans belonging to the family Chalcididae are unexplored in the north eastern state, Meghalaya of India. This paper describes three genera (*Antrocephalus*, *Haltichella* and *Hockeria*) and one species (*Brachymeria eupleoae*) as new records from Meghalaya.

Key words: Chalcididae, New records, Meghalaya

INTRODUCTION

Globally, about 87 valid genera and approximately 1464 valid species of chalcididae are recorded so far out of which 30 genera and 220 species are reported from India (Noyes, 2016), which represents only 32.22% genera and 14.26 % of species to that of the world. This shows poor description of Indian fauna. Chalcididae are paraphyletic relative to Leucospidae (Gibson, 1996). Morphologically, it can easily be differentiated from other families through distinguishable swollen hind femora. Other distinguishing features are geniculate antenna, presence of prepectus, biarticulate trochanter, fore wings with single vein, without closed cells. Their length range from 1.5 mm to 20 mm which shows that they are the largest groups among Chalcidoidea. They are predominantly primary endoparasitoids of insects of orders Lepidoptera, Diptera, Hymenoptera, Coleoptera and Neuroptera (Gibson, 1996). Majority of the work on chalcididae in India are contributed by Masi (1916-1947); Boucek (1982, 1988); Joseph et al., (1973); Boucek and Narendran (1981); Mani (1989); Mani and Kurian (1953); Mani and Dubey (1972); Mani et al. (1973). Gowri et al. (2016) have documented new records of Chalcididae from the states of Andhra Pradesh, Bihar, Karnataka, Himachal Pradesh, Manipur, Mizoram, Nagaland, and Tamil Nadu and union territories like Andaman and Nicobar Islands and Puducherry. In 1986, Narendran reported some Chalcids from Meghalaya like *Brachymeria shillongensis*, *Epitranus nagriceps*, *Conura xanthostigma* whereas Thakur reported *Brachymeria lasus* in 1990. *Brachymeria jambolana* was reported from Meghalaya by Gupta in 2010. In this paper, we describe new records of genus and species belonging to family Chalcididae from the states of Meghalaya for the first time.

MATERIALS AND METHODS

Specimens were collected using Yellow Pan Trap and swept net. The samples were collected from different forest belts of Nongpoh, Mawsynram and Upper-Shillong (Fig. 1). Methods for proper preparation of collected specimen were card mount following Noyes (1982). All the identified specimens were deposited in the entomology laboratory, North Eastern Hill University, Shillong.

Fig. 1 Map showing study sites in Meghalaya

RESULTS

1. Brachymeria euploeae **(Westwood)**

Species examined:

1 ♀ India, North East, Meghalaya, Ingkyrsa, 1214 m, 25°14'30.28"N and 91°27'40.90"E. 9. X. 2017, (Yellow Pan Trap). Coll. B. Kharbisnop.

1 ♀ India, North East, Meghalaya, Ingkyrsa, 1214 m, 25°14'30.28"N and 91°27'40.90"E. 26. XII. 2017, (Yellow Pan Trap). Coll. B. Kharbisnop.

Distribution: Andaman and Nicobar Islands (Gowri et al., 2016; Sheela et al., 2003), Arunachal (Gowri et al., 2016; Sheela et al., 2003), Bihar (Gowri et al., 2016; Kazmi and Chauhan, 2003), Haryana (Gowri et al., 2016; Ahmad et al., 1997), Jharkhan (Gowri et al., 2016; Kazmi and Chauhan, 2003), Karnataka (Gowri et al., 2016; Sheela et al., 2003), Madhya Pradesh (Gowri et al., 2016; Rawat and Modi, 1970), Manipur (Gowri et al., 2016; Singh, 1997), Meghalaya (Gupta, 2010; Narendran, 1986), Mizoram (Gowri et al., 2016), Nagaland (Gowri et al., 2016), Odisha (Gowri et al., 2016; Kazmi and Chauhan, 2003), Punjab (Gowri et al., 2016; Kazmi and Chauhan, 2003), Tamil Nadu (Gowri et al., 2016; Kazmi and Chauhan, 2003), Tripura (Sheela et al., 2003), Uttar Pradesh (Gowri et al., 2016; Ahmad et al., 1997; Husain and Agarwal 1982a; Narendran, 1986; Waterston, 1922), Uttarakhand

(Gowri et al., 2016; Kazmi and Chauhan, 2003) and West Bengal (Gowri et al., 2016; Sheela et al., 2003; Baltazar, 1966; Chatterjee, 1982). This species is new record for Meghalaya (Fig. 2)

***2. Brachymeria lasus* (Walker)**

Species examined:

1 ♀ India, North East, Meghalaya, Upper-Shillong, 25°32'51.09''N 91°51'11.32"E, 1653 m 30. VI. 2016 (Yellow Pan Trap). Coll. B. Kharbisnop.

Distribution: Andaman and Nicobar Islands (Narendran, 1986), Arunachal Pradesh (Sheela et al., 2003), Assam (Sheela et al., 2003), Bihar (Singh et al., 1995), Delhi (Singh, 1980), Gujarat (Kapadia, 1999; Raghwani et al., 1997; Shevale and Khaire, 1999), Himachal Pradesh (Dharmadhikari et al., 1985), Jammu and Kashmir (Amin Masoodi et al., 1986; Fry, 1989; Husain and Agarwal, 1982a,b; Sheela et al., 2003), Karnataka (Peter and Balasubramaniam, 1984; Revannavar et al., 2003; Thippaiah and Kumar, 1999; Vastrad, 1994), Kerala (Fry, 1989; Gupta and Kalesh, 2012; Joy et al., 1978; Mathew, 1980; Pillai and Nair, 1993; Sudheendrakumar, 1986; Sureshan, 2005), Madhya Pradesh (Sheela et al., 2003), Maharashtra (Gupta et al., 2014; Talgeri and Dalaya, 1971), Meghalaya (Thakur, 1990), Punjab (Varma and Shenhmar, 1988), Tripura (Sheela et al., 2003), Uttar Pradesh (Devi and Singh, 2002; Fasih et al., 1989; Sheela et al., 2003; Singh and Goel, 1991), and West Bengal (Chavan, 1984; Sheela et al., 2003; Walker, 1841).

***3. Antrocephalus* (Walker)**

Species examined:

1 ♀,1 ♂, India, North East, Meghalaya, Ingkyrsa, 784 m, 25°14'28.04"N and 91°27'38.36"E. 9. II. 2017, (Yellow Pan Trap). Coll. B. Kharbisnop.

1 ♀ Nongkhyllem Wildlife Sanctuary, Nongpoh, 639 m, 25°55'24.58''N and 91°49'23.70"E. 27. I. 2017 (Yellow Pan Trap). Coll. B. Kharbisnop.

Distribution: Himachal Pradesh (Mani, 1989), Kerala (Mani, 1989), Maharashtra (Mani, 1989), Nagaland (Tripathi, 1995), Meghalaya (New Record), Tamil Nadu (Mani, 1989), Uttarakhand (Mani, 1989), Utter Pradesh (Singh, 1987). (Fig. 3)

***4. Haltichella* (Spinola)**

Species examined:

1 ♀ Nongkhyllem Wildlife Sanctuary, Nongpoh, 641 m, 25°54'18.25''N and 91°52'22.47"E. 07. III. 2017 (Yellow Pan Trap). Coll. B. Kharbisnop.

1 ♀ India, North East, Meghalaya, Ingkyrsa, 389 m, 25°14'28.17"N and 91°27'37.07"E. 26. XII. 2017, (Yellow Pan Trap). Coll. B. Kharbisnop.

Distribution: Delhi (Narendran,1986; Roy and Farooqi, 1984), Kerala (Narendran, 1986), Meghalaya (New Record) ,Tamil Nadu (Narendran, 1986), Uttar Pradesh (Narendran, 1986; Roy and Farooqi, 1984) (Fig. 4)

***5. Hockeria* (Walker)**

Species examined:

1 ♀ India, North East, Meghalaya, Ingkyrsa, 391 m, 25°14'27.42"N and 91°27'35.89"E.05. V. 2016, (Yellow Pan Trap). Coll. B. Kharbisnop.

1 ♀ India, North East, Meghalaya, Ingkyrsa, 405 m, 25°14'28.66"N and 91°27'37.08"E. 25. III. 2016, (Yellow Pan Trap). Coll. B. Kharbisnop.

Distribution: Andaman and Nicobar Island (Gupta and Joshi, 2013), Andhra Pradesh (Narendran, 1989), Assam (Narendran, 1989), Bihar (Narendran, 1989), Delhi (Narendran, 1989), Gujarat (Patel and Patel, 1968), Himachal Pradesh (Dharmadhikari et al., 1985), Karnataka (Narendran, 1989), Kerala (Iqbal et al., 2013; Narendran, 1989; Sheela et al., 2003; Sureshan, 2005; Gupta and Joshi, 2013), Maharashtra (Narendran, 1989; Walker,1841), Meghalaya (New Record) , Odisha (Narendran, 1989), Tamil Nadu (Narendran, 1989; Mani et al., 1974), Tripura (Sheela et al., 2003), Uttar Pradesh (Narendran, 1986; Roy and Farooqi, 1984; Husain and Agarwal, 1982a; Cameron, 1897), West Bengal (Sheela et al., 2003; Narendran, 1989) (Fig. 5)

Fig. 2 *Brachymeria eupleoea*
(Scale bar = 2mm)

Fig. 3 *Antrocephalus*
(Scale bar = 1.8 mm)

Fig. 4 *Haltichella*
(Scale bar = 0.8 mm)

Fig. 5 *Hockeria*
(Scale bar = 0.5mm)

CONCLUSION

The present study documented 10 individuals belonging to four genera of Chalcididae from Meghalaya**.** Three genera viz., *Antrocephalus*, *Haltichella* and *Hockeria* are reported for the first time from Meghalaya. *Brachymeria eupleoae* represented by two females is also reported for the first time from Meghalaya.

ACKNOWLEDGEMENTS

We would like to thank the head of the Department of Zoology, NEHU for providing all the necessary facilities. B.K thankfully acknowledge NEHU for providing UGC-Non NET fellowship.We gratefully acknowledge the PCCF (Wildlife), Meghalaya for giving collection permits from protected areas.

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Pranikee-Journal of Zoological Society of Orissa, ISSN 0970-4450
Volume XXXII, 2020, pp 43-55

MORPHOLOGICAL, BEHAVIORAL AND BIOCHEMICAL CHANGES IN THE INDIAN MAJOR CARP *CATLA CATLA* EXPOSED TO CADMIUM CHLORIDE AND LEAD NITRATE

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ABSTRACT

The present study was conducted to determine the impact of cadmium chloride ($CdCl_2$) and lead nitrate [$Pb(NO_3)_2$] on biochemical parameters like protein, lipid peroxidation and reduced glutathione in muscle of fresh water fish *Catla catla*. Short term tests of acute toxicity were performed for different durations (24 hour, 48 hour and 72 hour) using $1/10^{th}$ of LC_{50} concentration of $CdCl_2$ and $Pb(NO3)_2$ against control. The animals were also exposed to different concentrations of both $CdCl_2$ and Pb $(NO3)_2$ over a period of 24 hours. Results showed significant decrease in protein level, increase in reduced glutathione and lipid peroxidation level with the increase of exposure time and concentration of both $CdCl_2$ and Pb $(NO3)_2$. Besides, some morphological and behavioral changes were also observed. This depletion and elevation levels of biochemical parameters/ morphological/ behavioral changes were apparently indicative of the organism's response to the chemicals under investigation.

Key words: *Catla catla*, Muscle, Lead Nitrate, Cadmium Chloride.

INTRODUCTION

A wide range of pollutants are continuously introduced into the environment mainly due to the increased industrialization, technological advancements and other anthropogenic activities (Lima et al., 2008). Alteration in the chemical composition of a natural aquatic environment by hazardous substances like heavy metals, pesticides and industrial effluents usually affect the behavioural, biochemical and physiological parameters of aquatic fauna including fishes (Radhaiah et al., 1987). Out of 23 heavy metals, lead and cadmium are well known and occupied 3^{rd} and 7^{th} position in the top 20 most hazardous according to Agency for toxic substances and disease registry (Pandey and Madhuri, 2014). Heavy metals accumulate in tissues of fish and other aquatic organisms and passes through food chain causes bio-magnification of heavy metals at different trophic level. The present study was conducted to determine the impact of cadmium chloride ($CdCl_2$) and lead nitrate [$Pb(NO_3)_2$] on some morphological, behavioral and biochemical parameters of fresh water fish *Catla catla*.

MATERIALS AND METHODS

Animal

Fishes (*Catla catla*) aged 80-90 days, 9-12cm long and 9-12g were procured from the local fish farm, Baisingha, Mayurbhanj (21.9320^0 N and 86.7466^0 E), transported in polythene bag half filled with pond water and oxygen from oxygen cylinder. They were acclimatized for seven days in laboratory in an aerated condition before the experiment. They were fed with fish feed. Normal dark and light

periodicity was maintained. Tetracycline hydrochloride (water soluble powder) was used as a disinfectant.

Experimental set up

Four number of aquaria (50 cm × 40 cm × 50 cm) filled with 20 liter tap water, treated with tetracycline hydrochloride (0.25g/ litre) were prepared. Healthy fishes were segregated in two groups: - (i) Control (C_{Cd}/ C_{Pb}) and (ii) Experimental (Subgroups: $E1_{Cd}$/ $E1_{Pb}$, $E2_{Cd}$/ $E2_{Pb}$ and $E3_{Cd}$/ $E3_{Pb}$).

Table1. **Experimental set up for cadmium chloride and lead nitrate exposure**

Aquarium $CdCl_2$/ $Pb(NO_3)_2$	Quantity $CdCl_2$/ $Pb(NO_3)_2$ in mg/L	Duration of treatment (hours)
C_{Cd}/ C_{Pb}	--	0, 24, 48, 72
$E1_{Cd}$/ $E1_{Pb}$	0.453/ 2.685	24, 48, 72
$E2_{Cd}$/ $E2_{Pb}$	0.566 / 3.356	24
$E3_{Cd}$/ $E3_{Pb}$	0.906 / 5.370	24

Preparation of supernatant

Fish was picked up from the desired aquarium, the muscle (just below the pectoral fin) was collected and weighed in a monopan balance (Shimadzu; ELB 300). A 10% homogenate of muscle was prepared in ice-cold 50mM phosphate buffer (pH 7.4) using pre-chilled porcelain motor and pestle by up and down strokes at 4^0C. The homogenate was centrifuged and the supernatant was used for biochemical assay.

Protein estimation

Protein estimation of the samples was made according to the method of Lowry et al. (1951). Protein content was expressed as mg/g weight of the tissue and aqueous BSA (Bovine serum albumin) was taken as standard.

Lipid peroxidation estimation

Lipid peroxidation assay was determined by thiobarbituric acid test (TBA test) following Ohkawa et al. (1979). The concentration of thiobarbituric acid reactive substance (TBARS) was calculated from extinction coefficient of 156 mM^{-1} cm^{-1} (Wills, 1969) and expressed as n mole equivalent of MDA formed per mg protein.

Reduced glutathione estimation

GSH content of the tissue samples was determined following Ellman (1959) with slight modification. The GSH content of the tissue was expressed as mg/g tissue.

Statistical methods

One-way ANOVA and Post Hoc analysis (SPSS 23.0) were carried out to find out the level of significance between *Catla catla* treated with $CdCl_2$ and $Pb(NO_3)_2$ over a period of 24 hour, 48 hour, 72 hour and in control. The difference was taken as significant when p was less than 0.05.

RESULTS AND DISCUSSION

Morphology and Behaviour of *Catla catla*

The fishes showed normal behaviour such as well coordinated active movement, normal gill movement, free gulping, horizontal hanging in water and natural body colour during acclimatization and before exposed to heavy metals. However, after exposure to heavy metals, number of morphological changes like degradation of fins, haemorrhage on skin, fading of body colour and excess mucus secretion were noted. Fishes frequently come to water surface, trying to jump out of water and restlessness were observed. Slight behavioural variations were observed between fishes exposed to two different heavy metals. During lead nitrate exposure fishes became more restless, showed fin degradation and secreted more mucus in comparison to cadmium chloride exposure. But in case of cadmium chloride exposure, haemorrhages were found in head, near mouth and in the ventral region of the body (Figs. 1-8)

Fig.1 Foam formation due to application of tetracycline in the rearing water

Fig. 2 Heavy metal exposed fishes come frequently to the surface of water

Fig. 3 Unexposed fish

Fig. 4 Appearance of haemorrhage in ventral part of the body after $CdCl_2$ exposure

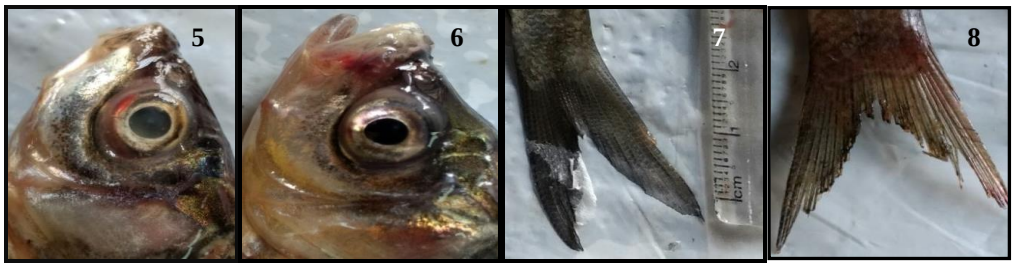

Fig. 5 Head region of normal fish

Fig. 6 Appearance of haemorrhage near mouth after exposed to cadmium

Fig. 7 Fin rays of unexposed fish

Fig. 8 Damage of fin rays after exposed lead

Biochemical investigation at different time intervals

Protein Content

The protein content (mg/g tissue) in muscles of unexposed *Catla catla* was 18.414±1.023mg/g tissue. On treatment with $CdCl_2$ (0.453mg/L), it changed to 16.062±0.663mg/g tissue after 24 hours, 17.298±2.139mg/g tissue after 48 hours and 16.248±1.237mg/g tissue after 72 hours of exposure. The protein content (mg/g tissue) showed fluctuations at different hours of exposure, but always remained below the normal value (Fig. 9). One way ANOVA revealed that the protein content at different time intervals in muscle was significant [$F_{(3, 19)} = 3.101$, $P = 0.056$]. Post Hoc analysis revealed that the protein content at different time intervals when exposed with $CdCl_2$ in muscle was significant at 24 hour, 48 hour and 72 hour ($P>0.05$; LSD).

Protein content (mg/g tissue) in muscle of $Pb(NO_3)_2$ treated *Catla catla* remained 17.292±1.473 after 24 hour, 15.964±1.118 after 48 hour and 10.586±2.789 after 72 hour. The protein content (mg/g tissue) in muscle remained maximum in the control group (18.384±2.101 and gradually decreased with increase in $Pb(NO_3)_2$ exposure (Fig. 9). One way ANOVA and post-hoc analysis revealed that the protein content at different time intervals in muscle is significant [$F_{(3, 19)} = 15.141$, $P = 0.000$] ($P<0.05$; LSD), respectively.

Lipid peroxidation

Lipid peroxidation (n mol of TBARS/mg protein) in muscle of unexposed *Catla catla* was 4.695 ± 0.284 and in exposed with $CdCl_2$ (0.453mg/L) were 4.944 ± 0.576, 4.92 ± 0.361, 5.386 ± 0.309 n mol of TBARS/mg protein at 24 hour, 48 hour and 72 hour respectively. Lipid peroxidation (n mol of TBARS/mg protein) of $CdCl_2$ exposed fishes increased gradually with the increase of time intervals. Lipid peroxidation level was lowest in muscle of unexposed fishes than $CdCl_2$ exposed fishes (Fig. 10). One way ANOVA revealed that the lipid peroxidation at different time intervals in muscle was significant [$F_{(3, 19)} = 2.615$, $P= 0.087$]). Post Hoc analysis revealed that the lipid peroxidation at different time intervals when exposed with $CdCl_2$ in muscle was also significant at 24 hour, 48 hour and 72 hour ($P>0.05$; LSD). Lipid peroxidation (n mol of TBARS/mg protein) contents in muscle of unexposed fishes was 4.816±1.145 and $Pb(NO_3)_2$ exposed was 2.685mg/L. Lipid peroxidation contents

were 4.658±0.871 after 24 hour, 6.28±1.144 after 48 hour and 8.03±2.239 after 72 hour of exposure, respectively. Lipid peroxidation (n mol of TBARS/mg protein) contents in muscle of $Pb(NO_3)_2$ exposed fishes decreased at 24 hour of exposure but gradually increased at 48 hour and 72 hour of exposure in comparison to control unexposed fish. From 24 hour of exposure, lipid peroxidation level increased with the increase of $Pb(NO_3)_2$ exposure time interval (Fig. 10). One way ANOVA revealed that the lipid peroxidation at different time intervals in muscle of was significant [$F_{(3, 19)} = 5.868$, $P = 0.007$]. Post Hoc analysis revealed that the lipid peroxidation at different time intervals when exposed with $Pb(NO_3)_2$ in muscle were significantly different at 24 hour, 48 hour and 72 hour ($P<0.05$; LSD) .

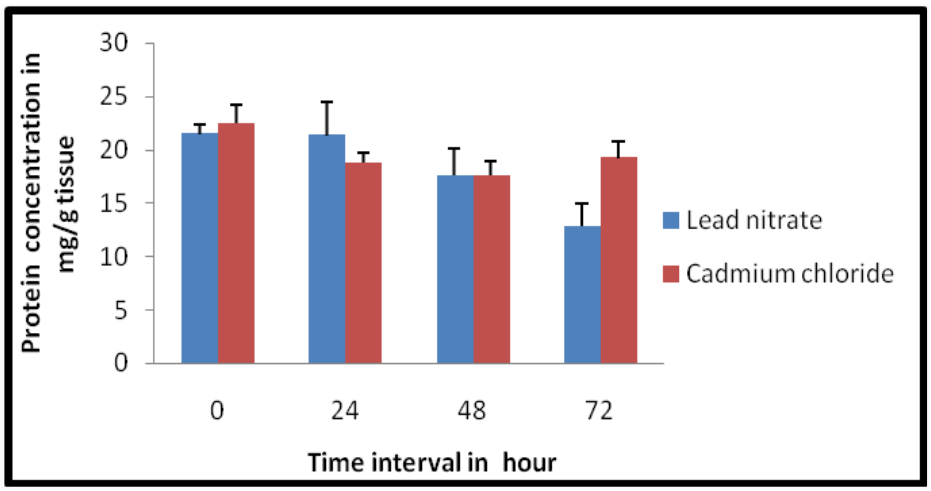

Fig 9. Effect of $Pb(NO_3)_2$ and $CdCl_2$ on protein content of *Catla catla* at different time intervals

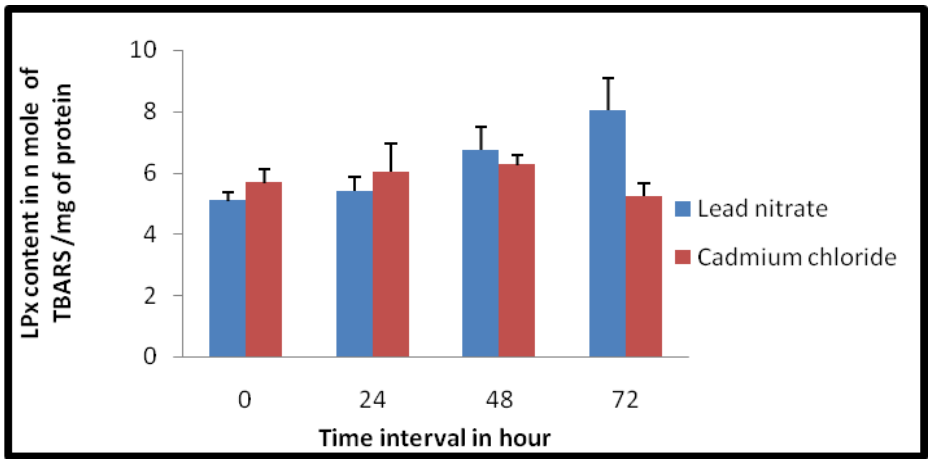

Fig. 10 Effect of $Pb(NO_3)_2$ and $CdCl_2$ on Lipid peroxidation of *Catla catla* at different time intervals

Reduced Glutathione

Effect of $CdCl_2$ (0.453 mg/L) on GSH level of muscle of *Catla catla* in unexposed and after 24 hour, 48 hour and 72 hour of exposure were 0.091±0.001mg/g tissue, 0.107±0.004mg/g tissue, 0.105±0.008mg/g tissue and 0.106±0.007mg/g tissue, respectively. Reduced glutathione (mg/g tissue) content increased at 24 hour of exposure as compared to control. It was decreased in 48 hour of $CdCl_2$ exposure then further increased in 72 hour of exposure.. The reduced glutathione level was the lowest in unexposed and highest at 24 hour of $CdCl_2$ exposure (Fig. 11). One way ANOVA was performed in order to analyze the effect of $CdCl_2$ on the reduced glutathione level at different time intervals. One way ANOVA revealed that the reduced glutathione at different time intervals was significant [$F_{(3, 19)} = 8.215$, $P = 0.002$]. Post Hoc analysis revealed that the reduced glutathione level at different time intervals was significant at 24 hour, 48 hour and 72 hour ($P<0.05$;LSD).

Effect of $Pb(NO_3)_2$ (2.685mg/L) on reduced glutathione level in the muscle of unexposed fishes and after 24 hour, 48 hour and 72 hour of exposure were 0.098±0.003 mg/g tissue, 0.098±0.004mg/g tissue, 0.102±0.006mg/g tissue and 0.092±0.009mg/g, respectively. Reduced glutathione (mg/g tissue) decreased at 24 hour of $Pb(NO_3)_2$ exposed in comparison with unexposed fish but it increased at 48 hour but then decreased at 72 hour of $Pb(NO_3)_2$ exposure. At 72 hour of $Pb(NO_3)_2$ exposure, reduced glutathione level decreased more than unexposed fishes (Fig. 11). One way ANOVA was performed in order to analyze the effect of $Pb(NO_3)_2$ on the reduced glutathione level at different time intervals in muscle of *Catla catla*. One way ANOVA revealed that the reduced glutathione at different time intervals was significant [$F_{(3, 19)} = 2.216$, $P = 0.126$]. Post Hoc analysis revealed that the reduced glutathione at different time intervals was significant at 24 hour, 48 hour and 72 hour($P>0.05$; LSD).

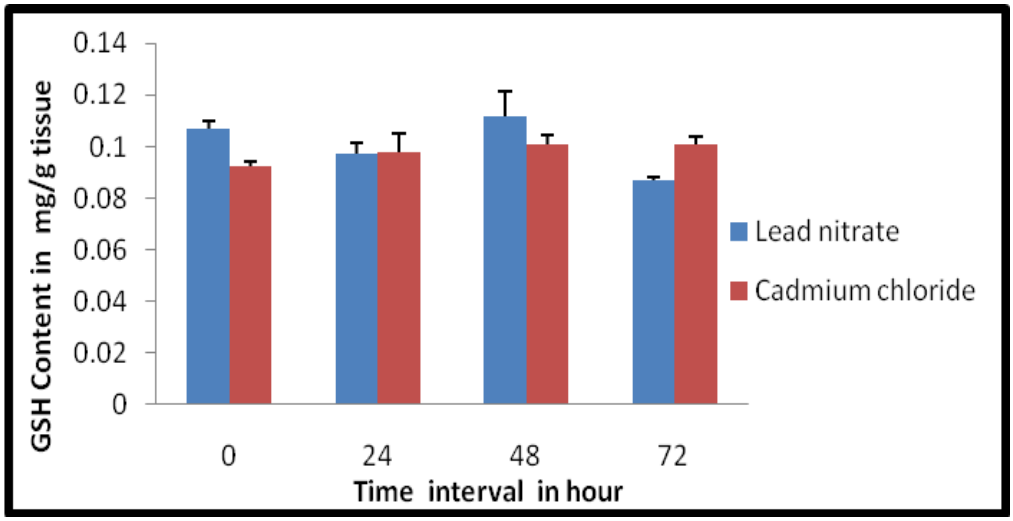

Fig. 11 Effect of $Pb(NO_3)_2$ and $CdCl_2$ on GSH content of *Catla catla* at different time intervals

Biochemical investigation at different Concentration

Protein Content

Protein content (mg/g tissue) in muscle of unexposed *Catla catla* was 18.414±1.023 but 16.062±0.663 at 0.453mg/L, 16.476±1.342 at 0.566mg/L and 16.374±1.653 at 0.906mg/L of 24 hour $CdCl_2$ exposure. Protein content of muscle tissue of $CdCl_2$ exposed fishes was very less at 0.453mg/L $CdCl_2$ concentration than other exposure concentrations. Protein content decreased at different concentrations of $CdCl_2$ exposure than unexposed fishes. Protein content at 0.566mg/L $CdCl_2$ concentration was more than other $CdCl_2$ exposure levels. Protein content of muscle of unexposed fishes was higher than $CdCl_2$ exposed fishes (Fig. 12). One way ANOVA revealed the protein content at 24 hour time interval at different concentrations of $CdCl_2$ exposure was significant [$F_{(3, 19)} = 3.8$, $P = 0.031$]. Post Hoc analysis revealed the protein content of muscle tissue at different concentrations to be significant at 24 hour ($P<0.05$; LSD).

Protein content (mg/g tissue) were 18.384±2.101 in control, 17.292±1.473 at 2.685mg/L, 15.78±3.495 at 3.356mg/L and 17.508±1.543 at 5.370mg/L of $Pb(NO_3)_2$ exposure, respectively. Protein content decreased at any concentration of $Pb(NO_3)_2$ exposure than unexposed fishes (Fig. 13). One way ANOVA revealed that the protein content at 24 hour time interval at different concentrations of $Pb(NO_3)_2$ exposure was significant [$F_{(3, 19)} = 1.106$, $P=0.376$]. Post Hoc analysis revealed that the protein content of muscle tissue at different concentrations was not significant at 24 hour ($P>0.05$; LSD).

Lipid peroxidation

Lipid peroxidation content (n mol of TBARS/mg protein) in muscle of *Catla catla* were 4.695±0.284 in unexposed but 4.945±0.576 at 0.453mg/L, 4.377±0.685 at 0.566mg/L and 5.773±0.819 at 0.906mg/L of $CdCl_2$ exposure during 24 hour. Lipid peroxidation content was more at 0.906mg/L and less at 0.566 mg/L of $CdCl_2$ exposure than other concentrations. But lipid peroxidation content was more at 0.906 mg/L of $CdCl_2$ exposure than other $CdCl_2$ exposure concentration during 24 hour and unexposed fishes (Fig. 14). One way ANOVA revealed that the lipid peroxidation content at 24 hour time interval at different concentrations of $CdCl_2$ exposure was significant [$F_{(3, 19)} = 4.587$, $P = 0.017$]. Post Hoc analysis revealed that the lipid peroxidation content of muscle tissue at different concentrations was significant at 24 hour ($P<0.05$; LSD).

Lipid peroxidation content (n mol of TBARS/mg protein) in muscle of *Catla catla* were 4.186±1.145 in unexposed 4.658±0.871 at 2.685mg/L, 6.022±1.495 at 3.356mg/L and 5.144±0.527 at 5.37mg/L of $Pb(NO_3)_2$ exposure. The content remained much high at 3.356mg/L $Pb(NO_3)_2$ exposure than control and other $Pb(NO_3)_2$ exposure concentrations. One way ANOVA revealed that the lipid peroxidation content at 24 hour time interval at different concentrations of $Pb(NO_3)_2$ exposure was significant [$F_{(3, 19)} = 1.620$, $P = 0.224$] (Fig. 15). Post Hoc analysis revealed that the lipid peroxidation content was not significant at 24 hour ($P>0.05$; LSD) .

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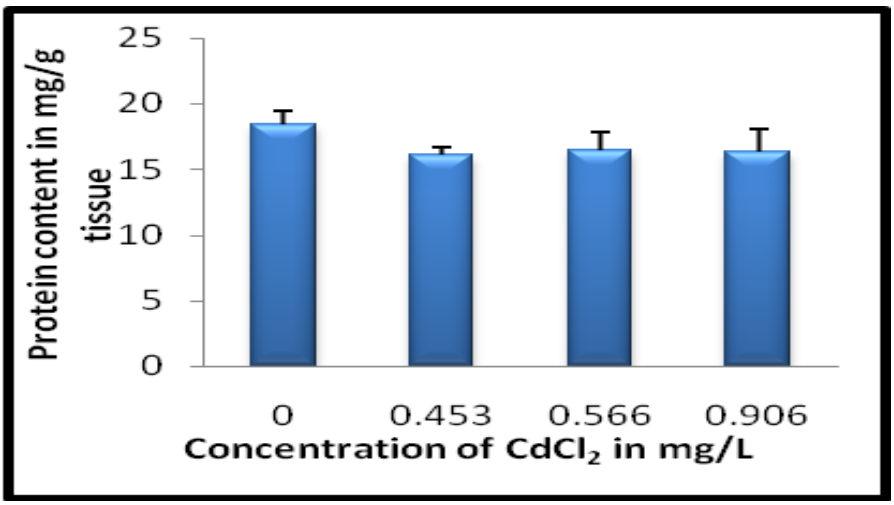

Fig. 12 Protein content in *Catla Catla* exposed to different concentrations of $CdCl_2$ for 24 hours

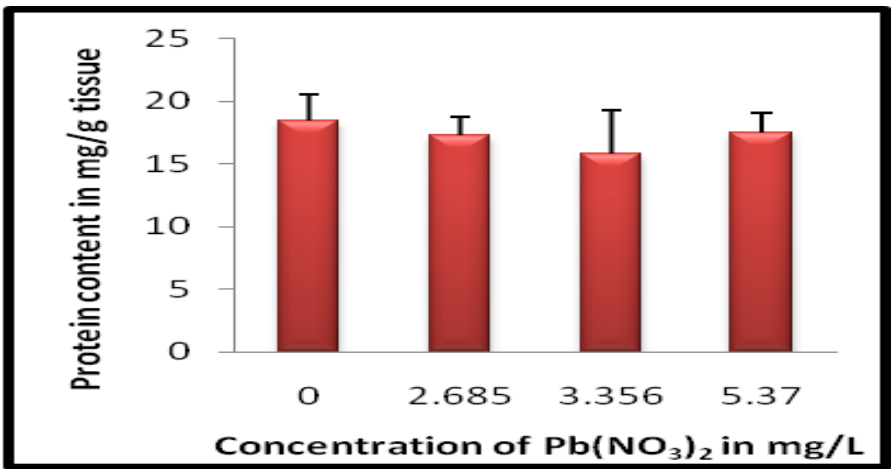

Fig13. Protein content in *Catla catla* exposed to different concentration of $Pb(NO_3)_2$ for 24 hours

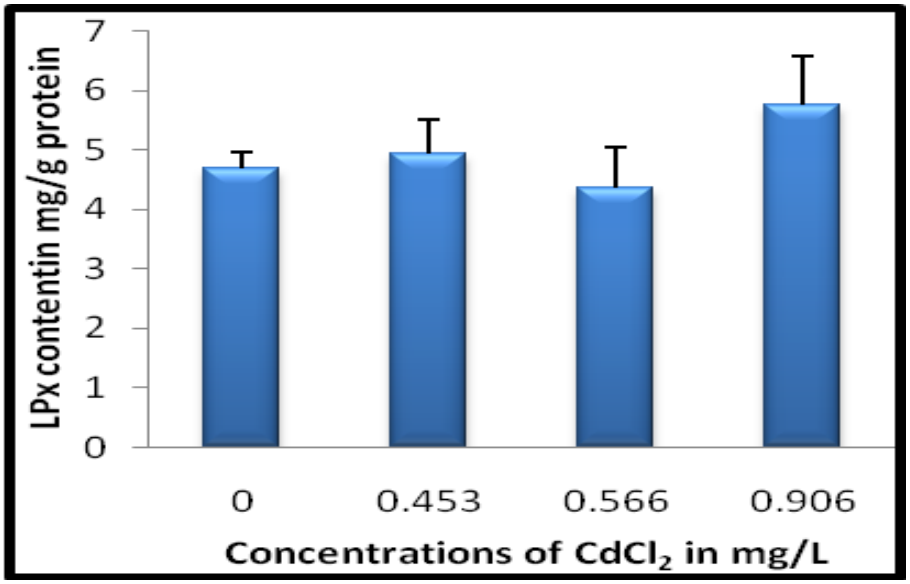

Fig. 14 Lipid peroxidation in *Catla catla* exposed to different concentrations of $CdCl_2$ for 24 hours

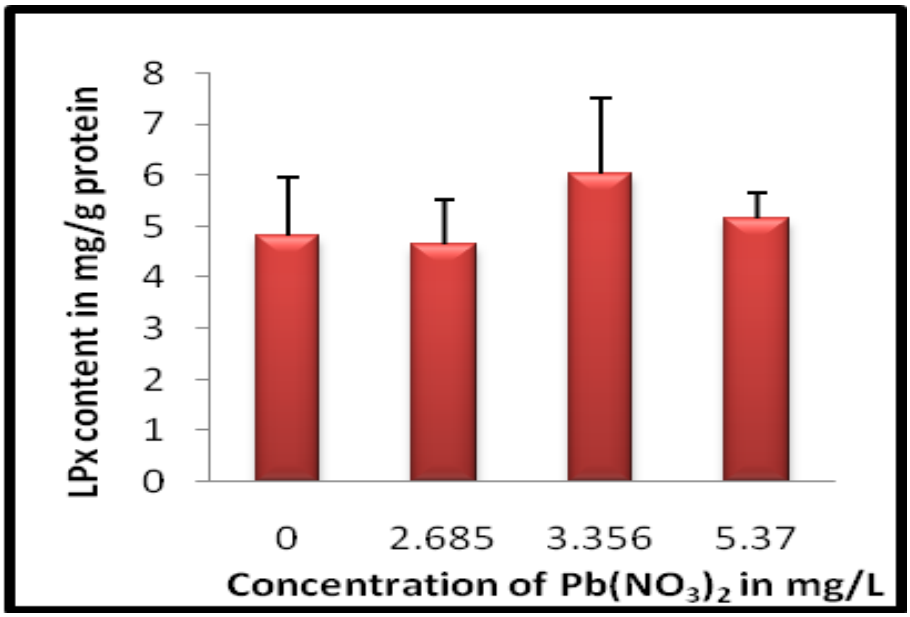

Fig. 15 Lipid peroxidation in *Catla catla* exposed to different concentrations of $Pb(NO_3)_2$ for 24 hours

Reduced glutathione

Reduced glutathione content (mg/g tissue) in muscle of unexposed *Catla catla* were 0.091±0.001 but 0.107±0.004 at 0.453mg/L, 0.084±0.008 at 0.566 mg/L and 0.114±0.004 at 0.906mg/L of $CdCl_2$ exposure during 24 hour. Reduced glutathione content was more at 0.906mg/L and less at 0.566mg/L of $CdCl_2$ exposure than others. At 0.566mg/L $CdCl_2$ concentration the reduced glutathione content is as less as than unexposed control fish. Highest content of reduced glutathione is found in muscle of 0.906mg/L $CdCl_2$ exposed *Catla catla* than other $CdCl_2$ exposed concentration and in unexposed fishes (Fig. 16). One way ANOVA revealed that the reduced glutathione content at 24 hour time interval in muscle tissue at different concentrations of $CdCl_2$ exposure was significant [$F_{(3, 19)} = 35.791$, $P = 0.000$]. Post Hoc analysis revealed that the reduced glutathione content of muscle tissue at different concentrations when exposed with $CdCl_2$ was significant at 24 hour ($P<0.05$; LSD).

Reduced glutathione content (mg/g tissue) in muscle were 0.098±0.003 in unexposed 0.098±0.004 at 2.685mg/L, 0.010±0.005 at 3.356mg/L and 0.101±0.006 at 5.370mg/L of $Pb(NO_3)_2$ exposure. Reduced glutathione level is more at 3.356 mg/L $Pb(NO_3)_2$ concentration than other exposures. At 2.685mg/L of $Pb(NO_3)_2$ concentration of reduced glutathione level decreases from unexposed fishes (Fig. 17). One way ANOVA was performed in order to analyze the effect of different concentrations of $Pb(NO_3)_2$ on the reduced glutathione content at 24 hour time interval in muscle tissue. One way ANOVA revealed that the reduced glutathione content at 24 hour time interval in muscle tissue of *Catla catla* at different concentrations of $Pb(NO_3)_2$ exposure was significant [$F_{(3, 19)} = 0.566$, $P = 0.645$] . Post Hoc analysis revealed that the reduced glutathione content of muscle tissue at different concentrations when exposed with $Pb(NO_3)_2$ in *Catla catla* was not significant at 24 hour ($P>0.05$; LSD) .

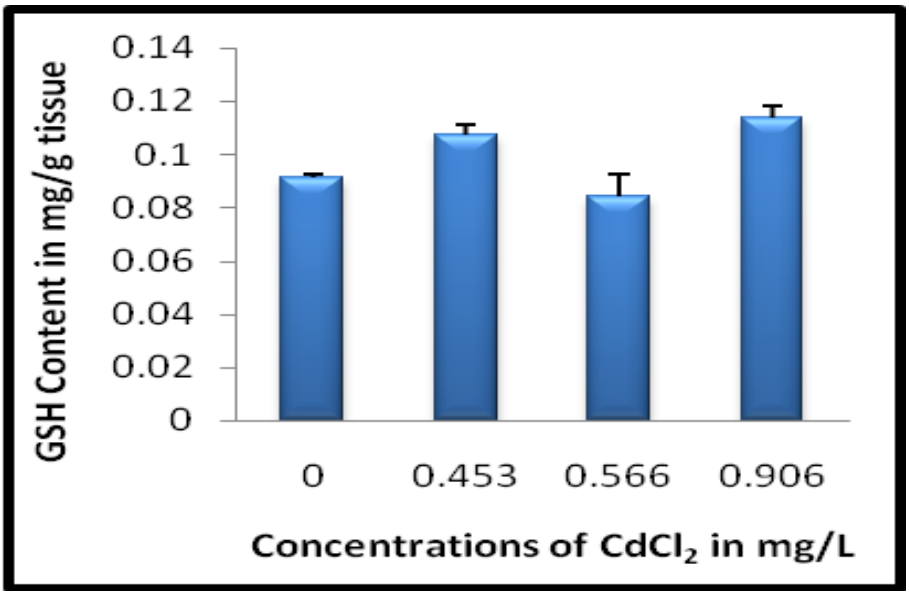

Fig. 16 Level of GSH in *Catla Catla* exposed to different concentrations of $CdCl_2$ for 24 hours

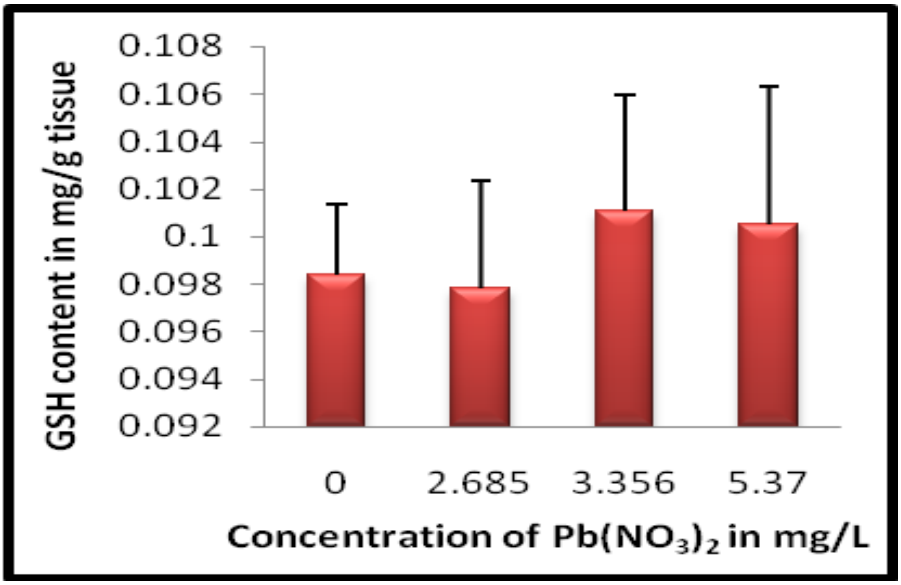

Fig. 17 Level of GSH in *Catla Catla* exposed to different concentrations of $Pb(NO_3)_2$ for 24 hours

Fish consumption is increasing across the globe because of its health and nutritional (essential source of protein, vitamins, minerals, and unsaturated fatty acids) value (Medeiros et al., 2012). However, all aquatic organisms particularly fishes are directly and indirectly influenced by the physical characteristics of aquatic environment, especially the physico-chemical parameters of water (Gillis et al., 2008). Mbabazi and Wasswa (2010) noted that industrial, agricultural, and domestic waste discharge have increased the levels of heavy metals in the lake, putting aquatic organisms and human consumers at risk. Contaminants, such as toxic metals, hydrocarbons, trace organic contaminants, pesticides, nanoparticles, microplastics, and other emerging contaminants, are a threat to human health, ecological services, and sustainable socioeconomic development (Li, 2020; Li and Wu, 2019).

According to Zhang et al. (2011), heavy metals caused histological alterations mainly via generations of reactive oxygen species. Maurya et al. (2019) studied bioaccumulation and human health risk due to Zn, Pb, Cu, Cd, and Cr pollution in *Cirrhinus mrigala*, *Cirrhinus reba*, *Catla catla*, *Labeo rohita*, *Crossocheilus latius*, *Clupisoma garua*, and *Mystus tengara* collected from Ganga river basin.

CONCLUSION

The present study revealed that $CdCl_2$ and $Pb(NO_3)_2$ were responsible for oxidative stress in muscle tissues of *Catla catla* as evident by increase in level of lipid peroxidation and reduced glutathione. Also there was decrease in the level of protein with increase in exposure period as well as concentrations of both $CdCl_2$ and $Pb(NO_3)_2$. As muscle tissue is the most commonly consumed edible portion of fish by human, there is a chance that heavy metal can accumulate in amplified manner in tissues of aquatic and terrestrial animals through food chain and cause physiological disorders in higher animals. However, as the importance of aquaculture continues to expand, and almost one third of the fish used for human consumption are

produced through aquaculture (FAO, 2000), it has become all the more important to critically monitor various chemicals that are being used in aquaculture to avoid human health hazards.

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Pranikee-Journal of Zoological Society of Orissa, ISSN 0970-4450
Volume XXXII, 2020, pp 56-64

EFFECTS OF INTRAPERITONEAL EXPOSURE OF *ESCHERICHIA COLI* ON THE HISTOPATHOLOGY, SPLENIC LYMPHOCYTE LOAD AND SERUM SGPT AND SGOT LEVELS IN IMMUNIZED EXPERIMENTAL MICE

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ABSTRACT

Intraperitoneal injection of heat-killed *Escherichia coli* into male Swiss albino mice resulted in several histopathological changes in organs such as lungs and liver and also in selected secondary lymphoid organs like spleen, mesenteric lymph nodes and Peyer's patches of the intestine. The first indication of innate immune response against this antigen exposure was evident in the form of neutrophil infiltration in the lung and liver histology, respectively. Moreover, lesions and formation of edema was also observed in the immunized lung sections, indicating site-specific tissue injury due to inflammatory immune response. Presence of immune cell aggregates in the intestinal Peyer's patches as well as formation of germinal centers in mesenteric lymph nodes clearly suggest effective interaction of the antigen with the resident immune cells in these lymphoid structures. Similar histological alterations, such as increased growth in white pulp and constriction of the red pulp in the spleen confirm the role of the injected antigen in activating the adaptive immune responses. This is, further confirmed by increased lymphocyte load in this organ. The elevated serum levels of the liver enzymes such as serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT) also provide a clear evidence of the onset of infection in the immunized mice and thereby validate the histology and splenic lymphocyte load results.

Keywords: Antigen, Secondary lymphoid organs, Histopathology, Splenic Lymphocyte load; Liver enzymes

INTRODUCTION

The immune system in higher vertebrates is equipped with launching both non-specific and specific immune responses, characteristic of the innate and the adaptive immune systems, respectively. Exposure to antigen acts as the first signal in activating the adaptive immune system in the individual, while the second signal is provided by the non-specific interactions of the antigen with the innate immune barriers. The immune system is functionally compartmentalized into primary lymphoid organs responsible for the generation and differentiation of naive T and B cells and into secondary lymphoid organs where immune responses are initiated. Only after activation, do T and B cells migrate from secondary lymphoid organs to seek antigen in the peripheral lymphoid organs. Secondary lymphoid organs include the spleen, lymph node, and organized lymphoid tissues associated with mucosal membranes such as the tonsils, the appendix, and the Peyer's patches. These highly organized secondary lymphoid structures provide the cellular scaffold where antigen is efficiently retained and presented and where ordered cellular interactions between antigen presenting cells, T cells, and B cells take place to initiate and promote efficient immune responses. These interactions eventually lead to prominent morphological and histological alterations in the organs concerned. Although it has been postulated that T or B cells get energized during possible initial peripheral encounter with antigen,

there is good experimental evidence indicating that antigen encounter by naive T cells does not occur, in general, outside of organized lymphoid tissues and as such, does not lead to activation (Karrer et al., 1997). Increased serum concentration of liver enzymes such as serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT) also act as important markers to diagnose liver function and its integrity, including antigen exposure or the onset of infection (Davachi et al., 2009).

This study aims to investigate the cellular immune responses in various secondary lymphoid organs in antigen exposed Swiss Albino mice leading to diagnostic histological changes in such organs, together with estimation of splenic lymphocyte load and serum concentration of liver enzymes to further substantiate the findings.

MATERIALS AND METHODS

Experimental Animals

Eight-weeks old male Swiss Albino mice, each weighing 25 ± 0.5g, were collected from animal house facility of The Institute of Advanced Study in Science and Technology (IASST) (IASST/2019-20/751) and given access to food and water *ad libitum* during the experiment.The mice were divided into two groups, the PBS-injected control group and the *Escherichia coli* injected immunized group. All animal procedures and protocols were approved by the Institutional Animal Ethics Committee, IASST and all efforts were made to minimize animal discomfort and suffering.

Preparation of *E. coli* for Immunization

E. coli was cultured in the laboratory following standard protocol (Dietert et al., 2017). *E. coli* cells were inoculated in nutrient broth for 24 hrs at 37°C and then washed with PBS by centrifugation at 3000 rpm for 5 min at 4°C. They were then re-suspended to appropriate density of $1x10^7$ CFU/ml in PBS, followed by heat killing at 60°C for 60 minutes (Ali et al., 2018).

Immunization

Mice from both the control and immunized groups were injected intraperitoneally for this experiment. The control group was injected with 250µl PBS whereas the immunized group was injected with 250µl PBS containing 10^7 CFU/ml of heat-killed *E.coli* (Ali et al., 2018).

Histopathology

Lungs, liver, spleen, mesenteric lymph nodes and intestine with Peyer's patches were collected from both control and immunized groups for histopathological studies after 3days of injection as per standard protocol (Ali et al., 2018). These tissues were then embedded in paraffin, sectioned at 2µm thickness using microtome (Unilab GE-70) and stained with Hematoxylin-Eosin for observation under the microscope (Labomed LX-200) identifying distinct morphological alterations in the said tissues (lungs, liver, spleen, mesenteric lymph nodes and Payer's patch) as a result of antigen exposure (Sordi et al., 2013).

Lymphocyte isolation and spleenic lymphocyte load

The spleens from both groups were collected after 3 days of injection, placed in cold BSA-PBS medium and cut into small pieces. The tissues were homogenized using a 1 ml syringe plunger and the

homogenate was passed through a 100 µm fine cell strainer mesh to obtain a cell suspension, the concentration of which was adjusted to 2×10^7 nucleated cells/ml of medium. The cell suspension was then layered over HiSep gradient (LSM 1077, HiMedia Laboratories) in a 15ml centrifuge tube and centrifuged for 20 minutes at 1000-1500g at room temperature (Boyum, 1968; Lim et al., 2016). A white fuzzy layer was observed at the interface comprising mainly of lymphocytes which was then carefully removed using a Pasteur pipette and transferred to a new tube. This was further diluted with the medium and centrifuged at 800g for 10 minutes to pellet the lymphocytes. The supernatant was discarded and the lymphocytes were washed with medium and then centrifuged again. The procedure was repeated thrice, after which the total lymphocyte count was calculated using Neubauer's improved hemocytometer (Louis and Siegel., 2011). Statistical analysis of the data, i.e. one-way analysis of variance (ANOVA) of the data was performed using the statistical software OriginPro 8.

Liver enzyme assay

Serum concentrations of Serum Glutamic-Pyruvic Transaminase (SGPT) and Serum Glutamic-Oxaloacetic Transaminase (SGOT) in both control and immunized group were estimated using enzyme kit assay (Erba SGPT/SGOT kit, UV-kinetic method recommended by IFCC) after 3 days of injectionin. For this experiment, mice from each group were terminally anaesthetized for collection of blood through cardiac puncture. The blood was collected in glass tubes and then carefully allowed to coagulate at room temperature for efficient pooling of serum without hemolysis. For each reading, 0.1ml of the serum was mixed with 1.0 ml of the working solution provided with the kit, mixed thoroughly and then absorbance was recorded immediately at 60 seconds, and subsequent readings were recorded at 30 seconds interval at 340nm in a UV-Spectrophotometer (Systronics-2202).

RESULTS

Histopathology studies

Histological sections of lungs from immunized mice showed indications of early immune response characterized by mild infiltrations of neutrophils, specifically in the interstitial spaces, and their distinct aggregation in and around the blood vessels, also termed as arterial congestion. Areas of cellular fragmentation, decay and loss of cellular details such as tissue lesions, light-pink alveolar edema and hemorrhage were also observed as compared to the normal tissue (Figs.1A and 1B). Similar patterns of neutrophil infiltration as well as tissue lesions were also identified in the liver sections obtained from immunized mice as compared to the control sections (Figs. 2A-2C). The lung and liver sections of the control group exhibit normal tissue histology, devoid of any immune-related alterations. Immunized spleen sections showed prominent constriction of the red pulp and well-defined expanded white pulp or core region as compared to normal spleen pathology of the control group (Figs. 3A and 3B), revealing the extensive proliferation of the immune cells upon activation by the antigen. Intestinal sections of Peyer's patches obtained from the immunized group contained distinct aggregates of immune cells, clearly not evident in the control group (Figs. 4A and 4B). Moreover, the mesenteric lymph nodes from the immunized group showed clear, spherical germinal centers indicative of antigen exposure and interaction with the resident immune cells in these lymphoid tissues while no such germinal centers were observed in the lymph node sections obtained from the control group (Figs. 5A and 5B).

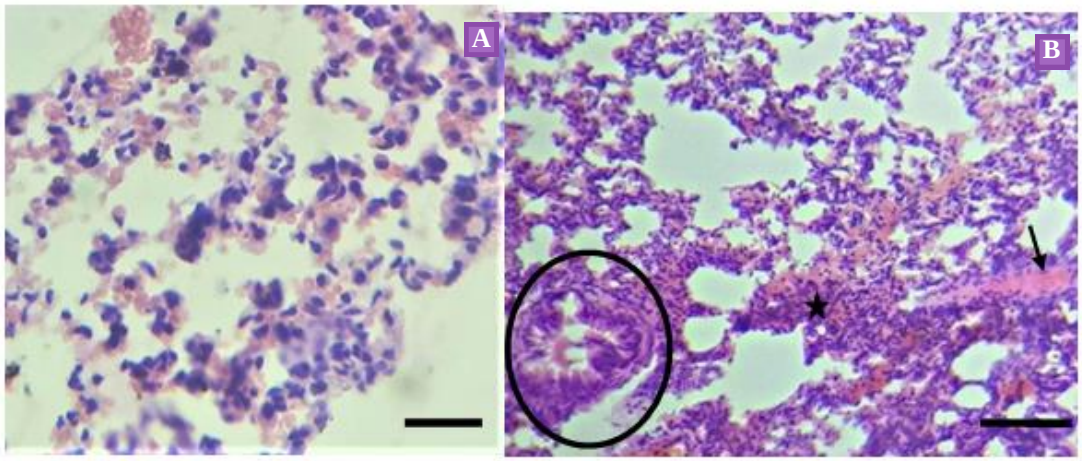

Fig. 1 (A) Regular alveolar morphology with few resident immune cells in the lungs of control group; (B) distinct edema (arrow), loss of cellular organization (*) and heavy neutrophil infiltration around the artery (encircled) in the immunized group.
Scale bars (A and B = 20 μm)

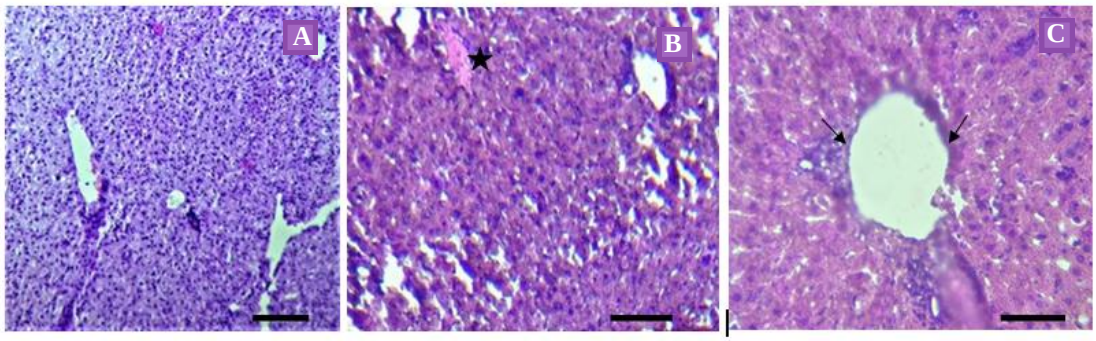

Fig. 2 (A) Liver tissue organization in normal tissue; (B) Initiation of tissue fragmentation with distinct edema (*) and (C) neutrophil infiltration around the artery (arrows) in immunized rat liver tissue.
Scale bars (A = 20μm; B and C = 200μm)

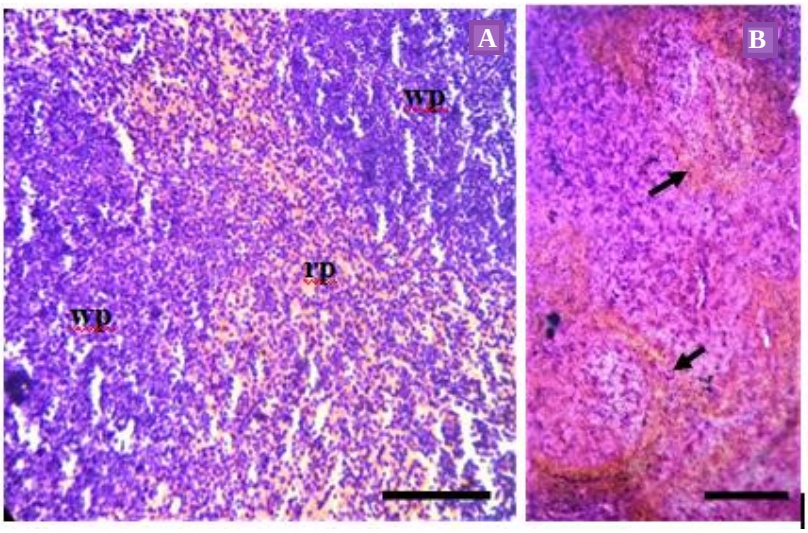

Fig. 3 (A) Well demarcated white pulp (wp) and red pulp (rp) region in the spleen of control group; (B) Constricted red core region (arrows) and the white pulp regions making contact with one another due to their expansion as a result of cell proliferation in immunized group (Scale bars A and B = 50μm).

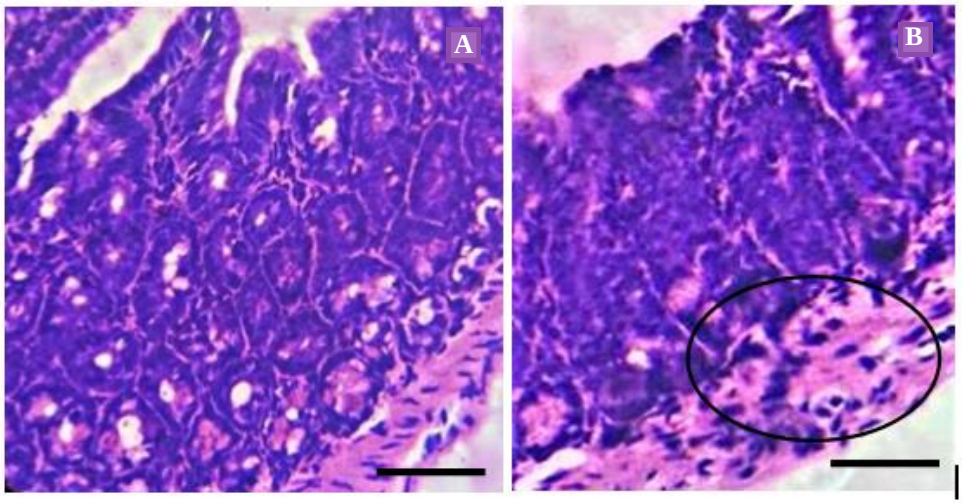

Fig. 4: (A) Normal villi organization and mild aggregation of immune cells in the subcutaneous layer, without any organized Peyer's patch in the intestinal section of control group; (B) Increased number of immune cells forming structured aggregates of lymphoid tissue in the subcutaneous region together with flattened villi, indicating infection in the immunized group (Scale Bars A and B = 50μm).

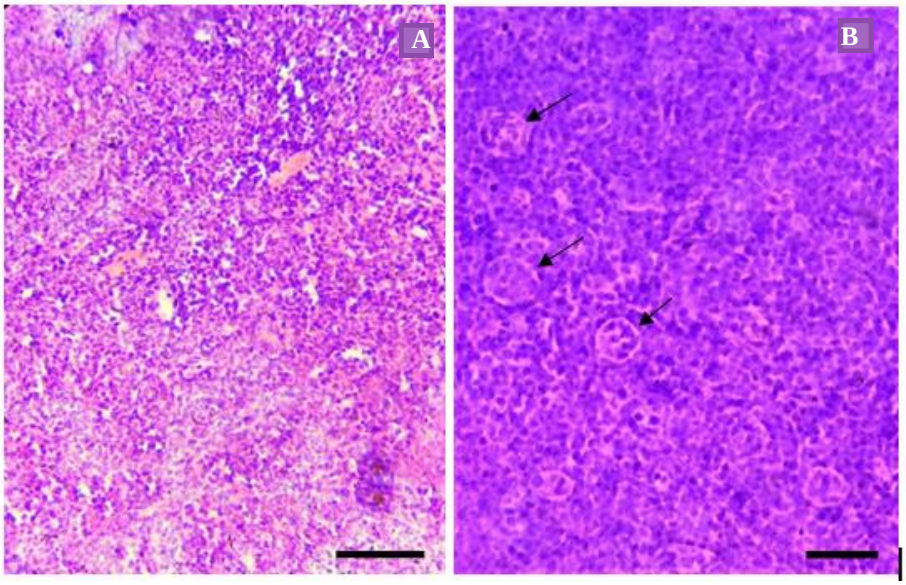

Fig. 5(A) Normal tissue organization in the mesenteric lymph node section of control; (B)Number of spherical germinal centers (arrows) indicating active interaction of the antigen with resident immune cells in the lymph node for effective initiation of the adaptive immune responses in the immunized group. (Scale bars A and B = 200μm).

Spleen Lymphocyte load

The lymphocyte load from the immunized spleen were significantly higher as compared to that of the control group (Fig. 6). This was in confirmation with the histological alterations observed in the immunized spleen, where the white pulp was observed to be greatly expanded while the red pulp regions were constricted from all sides.

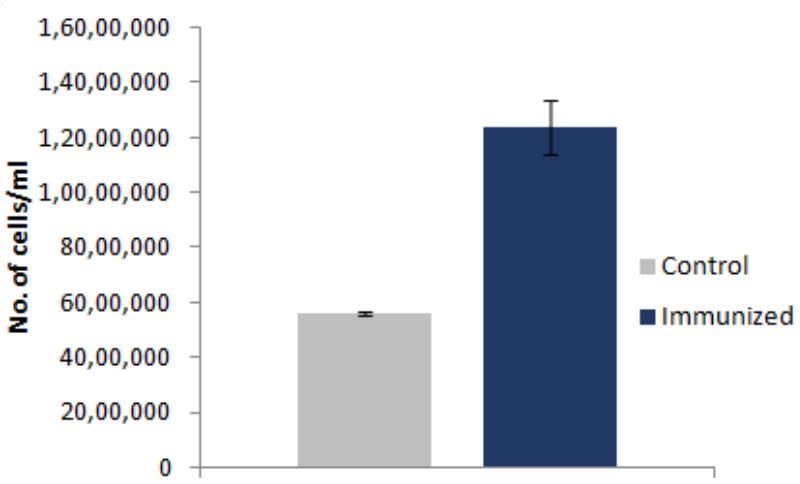

Fig. 6 Comparison of total splenic lymphocyte in both control and immunized mice.

Liver enzyme assay

The serum concentrations of both SGPT (control group: 45 U/L, immunized group: 82 U/L) and SGOT (control group: 44 U/L, Immunized group: 76 U/L) were observed to be significantly higher in the immunized group than corresponding control groups (Figs. 7A and B).

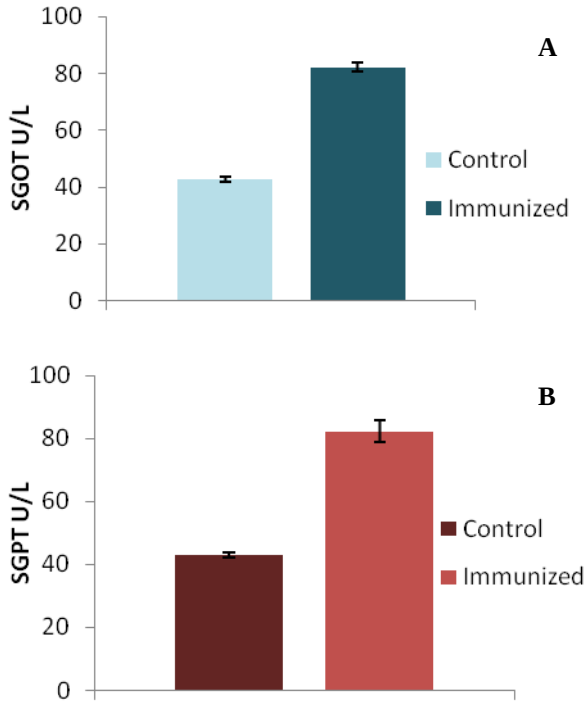

Fig. 7 (A) SGOT and (B) SGPT levels (U/L) in control and immunized mice.

DISCUSSION

Immunization is the process by which an individual's immune system becomes fortified against a foreign agent, referred to as immunogen. When this system is exposed to foreign molecules, called non-self, it orchestrates an immune response, and will also develop the ability to quickly respond to a subsequent encounter because of immunological memory (Nicholson, 2016). This study aims at documenting the preliminary histological changes in selected organs upon antigen exposure, using *E. coli* as the antigen. Since, secondary lymphoid organs or tissues are the first zones of interaction between antigen and the naïve immune cells, spleen, lymph nodes and intestinal Peyer's patches were selected for histological study in addition to lungs and liver. Histopathological investigation in administrated mice in this study revealed that *E. coli*, as an antigen, had a marked effect on different tissues characterized by the infiltration of inflammatory cells such as neutrophils, lymphocytes and macrophages, accompanied by loss of cellular organization, edema, hemorrhage and tissue necrosis, depending on the extent, route and duration of the exposure. The present observations were in conformation with several authors utilizing different micro-organisms as well as bacterial surface antigens such as lipopolysaccharides (Kafa et al., 2010; Dietert et al., 2017; Ali et al., 2018).

An important indication of any infection in the body is the increased numbers of circulating lymphocytes in the blood (Minemura et al., 2014). However, this study investigated the variation in resident lymphocytes within the spleen in response to antigen exposure (Boyum, 1968) and was found to be much higher as compared to that of the control. This increased lymphocyte numbers were inaccordance to those observed in experimental mice model injected with *Mycoplasma pneumonia* and in sepsis related studies (Hayakawa et al., 2002; Szabo et al; 2002). The liver is often involved in systemic infections, resulting in various types of abnormal liver function tests (Minemura et al., 2014), the cause of which has been attributed to vasodilation and increased microvascular permeability due to bacterial products and cytokines (Szabo et al., 2002; Kanai et al., 2008). This eventually leads to increased serum concentrations of the liver enzymes which act as critical markers of infection. Therefore, comparison of serum levels of important liver enzymes in both control and immunized group was imperative in this study, so as to validate activation of immune response against the antigen, the intraperitoneally injected *E. coli.*

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Pranikee-Journal of Zoological Society of Orissa, ISSN 0970-4450
Volume XXXII,2020, pp 65-78

EFFECTS OF LONG TERM DIETARY REPLACEMENT OF FISH MEAL WITH *ARTHROSPIRA PLATENSIS* AND SOYBEAN MEAL ON GROWTH PERFORMANCE, HEMATOLOGICAL AND ANTIOXIDANT PROFILES IN *LABEO ROHITA* FINGERLINGS

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ABSTRACT

Present study has been made to assess the appropriate level of replacement of fish meal (FM) with alternative protein sources such as *Spirulina* (*Arthrospira platensis;* SP) and soya meal (SM) in the feed to evaluate the growth performance of *Labeo rohita* fingerlings. The 60-day feeding trial was conducted in an indoor rearing system with seven isoproteinous (~30% crude protein) dietary regimes, namely, Group-I: fed with FM as protein source in the diet, Group-II and III: diet replaced by 5% and 10% (w/w) SP; Group-IV & V: diet replaced by 5% and 10% (w/w) SM; Group-VI: diet replaced by 5% each of SP and SM, Group-VI: diet replaced by 10% each of SP and SM. Feed with 10% SP replacement showed the highest significant growth performance evidenced by elevated feed conversion efficiency, specific growth rate, percentage body weight gain rate, proportionate gain of muscle protein and protein efficiency ratio. SP at 10% concentration neither affected blood parameters (the content of RBC, WBC, and Hb) nor induced oxidative stress as shown by the level of lipid peroxidation and antioxidant enzyme activities while 10% SM augmented gill lipid peroxidation. The results suggest that 10% SP supplementation is good for fish health and growth in aquaculture practices.

Keywords: *Labeo rohita*, *Arthrospira platensis*, *Spirulina*, soybean meal, antioxidant

INTRODUCTION

Nutrients are the substances which nourish the body, increases growth and helps to maintain and repair the whole body parts (Srivastava and Srivastava, 2008). Fish require sufficient nutrition in order to grow and survive. The quantity and quality of feed consumed have a prominent effect on growth rate. A complete diet supplies with all the essential ingredients such as proteins, carbohydrates, fats, vitamins, and minerals those crucial for the optimal growth and health of the fish. Protein is the costliest component in the formulated fish feeds. It provides the essential and non-essential amino acids necessary for muscle formation and enzymatic functions and it provides energy for survival of the fish (Yang et al., 2002). Soybean meal (SM) has been widely used as the most cost-effective alternative for high-quality fishmeal in diets for many aquaculture animals (Storebakken et al., 2000; Hernández et al., 2007; Li et al., 2012). Alternatively, a high level of soybean protein concentrate in a compound diet may cause reduced feed intake, poor nutrient utilization (Kader et al., 2010), and digestibility (Lech and Reigh, 2012). Furthermore, SM is reported to induce enteritis in both carnivorous and omnivorous

fishes (Booman et al., 2018; Garcia-Ortega et al., 2016; Gu and Wu, 2016; Uran et al., 2008; Wang et al., 2017, 2018) but has rarely been studied in herbivorous fish (Wang et al., 2015; Wu et al., 2018). On the other hand, the biomass of the *Cyanobacterium* i.e., *Arthrospira platensis*, the micro algae commonly known as Spirulina, is a potential protein source. Reports on various bioactive constituents of Spirulina like phycocyanin, β-carotene, γ linolenic acid, and phenolic compounds that show antioxidant, antimicrobial, immuno stimulants and disease resistance properties are available **(**Watanuki et al., 2006; Mala et al., 2009; Chu et al., 2010; Tayag et al., 2010; Lin et al., 2010; Arts et al., 2011; Hetta et al., 2014; Kumar and Sibi, 2020). Spirulina also used as palatability enhancers in order to maintain feed attractiveness and induce adequate feed consumption rate by fish (Kissil, 2000;Papatryphon and Soares et al., 2000).

Fish is an important dietary animal protein source in human nutrition containing all the essential amino acids and minerals viz., iodine, phosphorus, potassium, iron, copper and vitamin A and D in desirable concentrations while low in carbohydrate and unsaturated fat contents. *Labeo rohita*, an Indian major carp, is one of the most important species among Indian major craps, which contributes more than 60% of the total carp production in India due to its palatability, high market value and rapid growth (Mohanta et al., 2008). *Labeo rohita* is primarily herbivorous but turns to be omnivorous in adult stage and prefers to feed on plant materials (Talwar and Jhingran,1992). There is an increasing demand for the development of a cost effective aqua feed that could maintain the growth and health status of this species. With this background, in this study, an attempt has been made to assess the effect of micro algae S*pirulina* and soyabean meal in different concentrations replacing the traditional protein source (fish meal in fish diet) on growth, haematological parameters and oxidative stress responses in *Labeo rohita* fingerlings.

MATERIALS AND METHODS

Chemicals

Unless and otherwise mentioned all chemicals used were of analytical grade and prepared in MiliQ water.

Preparation of experimental diet

The experimental diets were formulated which contains crude protein (Table 1). Dough was prepared with the addition of required amount of lukewarm water. Oil was added to the dough and mixed for uniform distribution. It was allowed for proper conditioning by keeping at room temperature for 1 hour and then steam cooked in pressure cooker for 10 minutes. All the ingredients except vitamin, mineral mixture and vitamin C (Emix^{TM} Plus, India) were mixed in a big plastic bowl to get a homogeneous ingredient mixture after cooling. The dough mixed with all components had been pressed through hand pelletizer to obtain uniform sized pellets, air dried for 4 hours, followed by oven dry at 50°C till complete dryness. Thereafter, the prepared pellet had been kept in properly labeled air tight sealed polythene packs at 4°C until further use.

Collection and acclimatization of experimental animals

Labeo rohita fingerlings (average weight 4.8±1.1g and 5-7cm length) were collected from the Kailash Hatchery Mayurbhanj, Odisha, India and were transported in to the laboratory by a large aerated polythene bag kept within carrier container. At the laboratory, they were treated with 0.1% of

potassium permanganate solution for 5 minutes to get rid of any possible fungal infection and then allowed for acclimatization to laboratory conditions for 3 weeks in dechlorinated tap water with continuous aeration at constant (12h:12h) light:dark cycle in the glass aquaria tanks (Tank capacity: 65 L size) kept at 27± 1.1°C. During acclimatization the fish were fed 3% of their body weight with control diets in the morning at 8 am and evening at 5 pm. About 20% of the water has been changed daily at 9 a.m. to maintain the water quality to avoid deposition of excess fecal matters.

The hydrobiological parameters such as water temperature and pH were maintained within the range of 28°C to 30°C, 6.8 to 7.2, respectively. Similarly, the level of dissolved oxygen and total hardness range of water was maintained at 6.4 to 8.2 mg L^{-1} and 101 to 104 ppm of $CaCO_3$, respectively. The water quality parameters were maintained within the normal range recommended for fish production indicating that the fish were not under stress condition (Banerjee, 1967). Dissolve oxygen (6.9 to 7.1 mg/L), pH (6.8 to7.3) and ammonia concentrations (0.03 to 0.07mg/L) were also standardised (Wu et al., 2014).

Experimental design

The fish were randomly divided into seven experimental groups (N=20 in each group) and in duplicate in separate aquaria. The experimental diets were prepared on the basis of Spirulina (SP) and Soybean meal (SM) such as Group-I with fish meal only (contro l0; Group-II: replaced with SP 5%,; Group-III: replaced with SP 10%; Group-IV: replaced with SM 5%; Group-V: replaced with SM 10%; Group-VI: replaced with SP 5% and SM 5%; Group-VII: replaced with SP 10% and SM 10%. The experimental was conducted for 60 days. Feed was given at the rate of 5% of body weight twice daily at 7 am and 6pm. The unconsumed feed was collected and dried in sunlight after one hour of feed provision (James et al., 2009). During the study period the hydro-biological parameters were measured and maintained.

Growth performance

During initiation and termination of the feeding trials, 5 fish from each tank (10 per group) were randomly selected and batch weighed for calculating the growth parameters like initial body weight (IBW), final body weight (FBW), weight gain rate (WGR %) = [100× (final body weight − initial body weight)/initial body weight], specific growth rate (SGR %) = [(ln (final body weight) – ln (initial body weight)/ experiment period (60days)] × 100, feed conversion ratio (FCR) = total diet fed (dry weight)/ total body weight gain (wet weight), food conversion efficiency (FCE) = Biomass (g)/ Feed intake (g) and protein efficiency ratio (PER) = total body weight gain (wet weight)/ total dietary protein intake, where W_t and W_0 were final and initial weight of fish taken, respectively, and t is the duration of feeding (in days), and survival = $(N_0/N_f) \times 100$, where N_0 is the initial number of fish and N_f is the final number of fish.

Sample collection

At the end of the feeding period, fish were starved for 24 hours, and then anesthetized with 2-phenoxyethanol 500µl/L (~1:10,000 v/v) (Varkey and Sajeevan, 2014). After determination of the body weight, blood from lateral line was collected following which muscle, liver and gill tissues were collected and stored in -20°C until analysis.

Hematological parameters

After anaesthetization 0.5ml of blood was collected from caudal vein in 1 ml insulin syringe. Red blood cells (RBCs) and white blood cells (WBCs) were counted using Neubauer`s cell counting chamber under a light microscope. Hemoglobin levels were immediately assessed using cyanomethaemoglobin method (Ochei and Kolhaktar, 2000).

Tissue processing

A 20% (w/v) homogenate of muscle, gill and liver were prepared in 50 mM potassium phosphate buffer (pH 7.4 containing 0.25 M sucrose) with the help of a pre-cooled porcelain mortar and pestle (Mohanty and Samanta, 2018). The homogenates were centrifuged at 200×g for 10 minutes at 4°C in a cooling centrifuge (REMI, CPR 30) to remove tissue debris. The obtained supernatant (crude homogenate) was kept for estimation of proteins and lipid peroxidation (LPx) assay. Remaining crude homogenates were centrifuged at 600×g for 10 minutes at 4°C to sediment nuclei. The supernatant thus obtained were centrifuged at 10,000×g for 20 minutes at 4°C for catalase (CAT) assay while it was passed through Sephadex G-25 column to get rid of small molecules and used for superoxide dismutase (SOD) assay. Protein estimation was done (Lowry et al., 1951) through standard curve prepared from Bovine serum albumin (BSA).

Measurement of oxidative stress parameters and antioxidant defense

In the present study, LPx was used as marker of oxidative stress and measured in terms of thiobarbituric acid reactive substances (TBARS) while principal antioxidant enzymes like SOD and CAT were assayed as antioxidant defense marker (Mohanty and Samanta, 2018).

Lipid peroxidation (LPx) assay

Lipid peroxidation (LPx) in the crude homogenate was estimated as thiobarbituric acid reactive substances (TBARS) using thiobarbituric acid (TBA) reagent. Results were calculated from the molar extinction co-efficient of TBARS 156 mM^{-1} cm^{-1} and were expressed as nmoles equivalent of malondialdehyde (MDA) formed mg^{-1} protein and expressed as nmoles TBARS formed/mg protein**.**

Catalase activity assay

For catalase activity (Aebi, 1984), sample were suitably diluted with potassium phosphate buffer (pH 7.0, 50 mM) and taken in micro centrifuge tubes properly mixed after addition of 1% absolute ethanol. This was incubated in ice for 30 minutes. Following incubation, 0.1%, triton-X-100 was added to it. The post mitochondrial supernatant fraction (PMF) sample (0.1 ml) was appropriately diluted with freshly prepared 12mM H_2O_2 in buffer (2.9 ml) so as to contain 25-50µg protein. The decrease in absorbance was recorded at 240 nm in a UV-VIS spectrophotometer at every 15 second intervals upto 3 min. Enzyme activity was calculated taking 43.6 $M^{-}1$ $cm^{-}1$ as molar extinction coefficient of H_2O_2 and expressed as nkatal/mg protein.

Superoxide dismutase activity assay

Superoxide dismutasr (SOD) protects cells by dismutating $O_2^{\cdot -}$ to H_2O and H_2O_2 and O_2. This assay is based on indirect method involving scavenging of $O_2^{\cdot -}$ by SOD. Photo reduction of riboflavin

generates $O_2^{\cdot -}$, which is allowed to react with hydroxylamine hydrochloride to produce nitrite. The nitrite reacts with sulfanilic acid to produce a diazonium compound that subsequently reacts with naphthylamine forming a red-azo compound with absorbance at 543nm. SOD present in sample scavenges $O_2^{\cdot -}$, hence nitrite formation is inversely proportional to the amount of SOD. Enzyme activity was expressed in unit (U) mg^{-1} protein where one unit of enzyme activity is defined as the amount of SOD capable of inhibiting 50% nitrite formation under standard assay conditions.

Statistics

All statistical analyses was done using SPSS version 20 software. Data (mean ± SD) on each parameter studied were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene test). As the data didn't show normal distribution and homogeneity of variance, a non-parametric test (Kruskal-wallis ANOVA) was carried out. Differences among the means were considered significant at $p<0.05$ level.

RESULTS

Growth performance and nutrient utilization

The ingredient and proximate composition of diets used in seven experimental groups is presented in Table 1. The crude protein (CP) content was maintained around 30% (ranged from 32.22 to 34.36%).

Table 1. Composition of experimental diet and their proximate analysis**.**

Compositions (%)	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
Fish Meal	30	25	20	25	20	20	10
Spirulina	00	05	10	00	00	05	10
Soya meal	00	00	00	05	10	05	10
Wheat meal	32	32	32	32	32	32	32
Deoiled Rice Bran	10	10	10	10	10	10	10
Broken Rice	10	10	10	10	10	10	10
Corn meal	10	10	10	10	10	10	10
Oil	05	05	05	05	05	05	05
Vitamin (Premix)	02	02	02	02	02	02	02
Mineral (Premix)	01	01	01	01	01	01	01
Proximate analysis							
Crude protein	32.22	33.52	34.21	32.89	34.26	33.64	34.36
Crude fat	4	4	4	4	4	4	4
Moisture	19	9.8	8	9.5	8.2	9	8
Fiber	5	4.8	4.2	5	4.6	4.1	3.8

The values of initial weight, final weight, net weight gain (NWG), feed conversion ratio (FCR), specific growth rate (SGR), and survival rate are presented in Table 2. SP 10% was able to positively impact growth performance when given alone or in combination with soyabean meal as evidenced by augmented BWG, SGR, FCE and PER along with a decline in FCR. Similarly, 10% SP supplementation enhanced the protein content of gill and liver including the edible part muscle suggesting nutritional augmentation (Fig. 1).

Table 2. Effect of dietary replacement of fish meal with *Arthrospira platensis* (Spirulina) and soybean meal on growth performance of *Labeo rohita* fingerlings.

Parameters	GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V	GROUP VI	GROUP VII
IBW(g)	4.10 ± 0.09	4.90 ±0.25	4.70 ± 0.43	4.80 ± 0.50	4.70 ± 0.50	5.00 ± 0.47	5.20 ± 0.29
FBW(g)	16.6 ±0.90	18.2 ±1.95	21.9 ±1.55	17.8 ±0.70	17.8 ±1.37	20.8 ±1.24	21.6 ±0.82
BWG (%)	304.8 ±1.07	271.4 ±1.38	365.95 ±1.43*	270.83 ±1.03	278.72 ±1.77	316.00 ±1.58*	315.38 ±2.36*
SGR (%/day)	2.33 ±0.01	2.18 ±0.01	2.56 ±0.04*	2.18 ±0.02	2.11 ±0.07	2.37 ±0.02	2.31 ±0.06*
FCR	1.42 ±0.01	1.42 ±0.01	1.12 ±0.01*	1.44 ±0.01	1.44 ±0.01	1.19 ±0.01*	1.21 ±0.01*
FCE	0.70 ±0.00	0.70 ±0.001	0.89 ±0.02*	0.69 ±0.02	0.69 ±0.01	0.84 ±0.02*	0.83 ±0.01*
PER	9.46 ±0.06	8.10 ±0.08	10.71 ±0.03*	8.23 ±0.01	8.13 ±0.02	9.39 ±0.03	9.18 ±0.02

* indicates values significantly different at $p<0.05$

IBW= initial body weight, FBW= final body weight, SGR= specific growth rate, FCR= feed conversion ratio, FCE= food conversion efficiency, PER= protein efficiency ratio

Hematological parameters

The hematological parameters of *Labeo rohita* fingerlings with different feeding regimes are presented in Table 3. The blood parameters of fingerlings after 60 days of feeding trial were assessed for change in RBC, WBC, and hemoglobin among the groups. None of the dietary regimes bring about any significant change in the hematological parameters studied.

Oxidative stress parameters

Except for gill tissue, none of the dietary regimes was able to induce Lpx. However, 10% SM alone or in combination significantly increased Lpx in the gill. Similarly, both 5 and 10% double supplementation (SP + SM) was able to induce SOD activity in the gill only. On the other hand CAT activity was augmented in all supplemented diet categories in comparison to control (Table 4). None of the supplemented diet was able to alter any of these oxidative stress/antioxidant responses in the edible part of the fish.

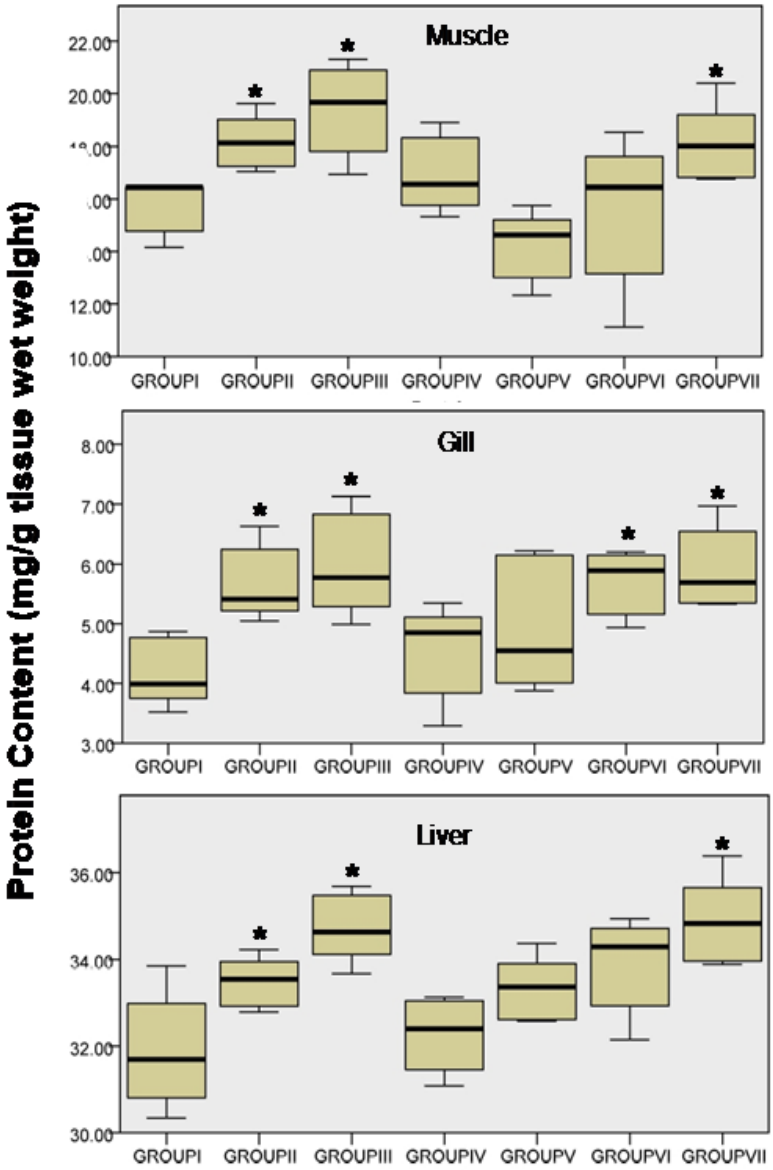

Fig. 1. Box plot showing effect of dietary replacement of fish meal with *Arthrospira platensis* (Spirulina) and soybean meal on protein content in tissues of *Labeo rohita* fingerlings.*$p<0.05$ with respect to control (Group-I).

Table 3. Effect of dietary replacement of fish meal with *Arthrospira platensis* (Spirulina) and soybean meal on hematological parameters of *Labeo rohita* fingerlings.

Hematological parameters	GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V	GROUP VI	GROUP VII
RBC ($10^6 \mu l^{-1}$)	1.38 ± 0.05	1.42 ± 0.06	1.57 ± 0.12	1.39 ± 0.15	1.42 ± 0.18	1.51 ± 0.16	1.45 ± 0.198
WBC ($10^3 \mu l^{-1}$)	6.10 ± 0.30	6.37 ± 0.16	6.89 ± 0.36	6.25 ± 0.18	6.34 ± 0.07	6.55 ± 0.21	6.59 ± 0.190
Hb(g dL^{-1})	8.0 ± 0.24	9.0 ± 0.04	9.0 ± 0.08	8.0 ± 0.12	8.20 ± 0.12	8.4 ± 0.12	9.0 ± 0.16

Table 4. Effect of dietary replacement of fish meal with *Arthospira platensis* (Spirulina) and soybean meal on oxidative stress parameters of *Labeo rohita* fingerlings.

Parameters	Tissues	GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V	GROUP VI	GROUP VII
LPx	**Muscle**	1.15 ±0.40	0.82 ±0.07	1.26 ±0.43	0.48 ±0.03	0.89 ±0.42	0.89 ±0.26	0.73 ±0.14
	Gill	0.42 ±0.21	0.59 ±0.01	0.36 ±0.04	0.47 ±0.20	0.90 ±0.20*	0.48 ±0.18	0.64 ±0.09*
	Liver	4.52 ±2.00	4.93 ±1.29	2.30 ±0.723	2.63 ±0.34	3.43 ±0.46	3.34 ±0.462	3.41 ±1.86
Catalase	**Muscle**	57.07 ±13.17	67.73 ±17.48	80.89 ±9.11	78.82 ±5.86	70.81 ±13.51	78.64 ±4.44	80.10 ±7.26
	Gill	8.79 ±4.63	12.81 ±4.41	11.93 ±3.54	11.71 ±4.29	9.09 ±3.56	11.66 ±4.38	13.57 ±4.80
	Liver	749.9 ±68.50	1109.07 ±76.14*	1323.92 ±69.62*	969.22 ±94.44*	1106.37 ±82.09*	1146.00 ±46.80*	1226.00 ±37.62*
SOD	**Muscle**	6.43 ±0.02	6.46 ±0.08	6.80 ±0.28	6.45 ±0.21	6.57 ±0.44	6.56 ±0.20	6.69 ±0.25
	Gill	5.02 ±0.16	5.14 ±0.29	5.65 ±0.72	5.54 ±0.20	5.55 ±0.16	6.02 ±0.24*	6.16 ±0.19*
	Liver	8.49 ±0.24	8.21 ±0.48	8.65 ±0.26	8.52 ±0.28	8.61 ±0.28	8.74 ±0.79	8.88 ±0.55

* indicates values significantly different at $p<0.05$

DISCUSSION

About 40–50% of the total production cost in intensive fish culture systems is fish feed (Alceste and Jory, 2000) which is not only responsible for growth of the fish, but also is pivotal in maintenance of the quality of eggs (Kjørsvik et al., 1990). In developing countries like India, majority of fish farmers primarily depend on locally made fish feeds or commercially available alternatives. The principal source of protein in fish feed comes from fishmeal component of the commercially available feed, as it augments the feed efficiency and growth through better food palatability, and enhancement of nutrient uptake, digestion, and absorption. However, upon long term storage, depending on storage time and condition, it may get fermented and in presence of C*lostridium* spp produce histamine and ammonia, the primary toxic substances in fish meal (Yuningsih, 2002). Bioconcentrate monomethyl mercury in protein matrices, and organohalogen pollutants are observed especially in farmed fish that are fed with feed having fish meal and can be passed on in the fat components of derived foods to humans (Dorea, 2006). Therefore, over the years different supplements have been tried to improve fish production. In this investigation, we used two important feed supplements for fish meal (SP and SM) against fish meal for the study of the growth in the major Indian carp *Labeo rohita* fingerlings.

SP at both 5 and 10% concentrations didn't show any toxic effect as revealed by survival rate, hematological parameters and induction of oxidative stress measured as peroxidation of membrane lipids. Though 5% SP supplementation failed to achieve substantial augmentation of the growth parameters, 10% SP showed significant increase in all the growth parameters studied along with food conversion efficiency and protein efficiency ratio suggesting positive modulation of the fish physiology by *Sprirulina*. This was further corroborated by increase in total protein content of the fish tissues particularly the economically important muscle tissue. *Spirulina*, the blue-green algae is not only a rich source of protein (60-70%), vitamins, essential amino acids, minerals, and essential fatty acids such as palmitic acid, linolenic acid and linoleic acid (Radhakrishnan et al., 2014; Lupatini et al., 2016), but also have abundant pigment contents such as β-carotene, xanthophylls, zeaxanthin, echinenone, cryptoxanthin, and phycocyanin resulting in antioxidant and anti-inflammatory activities (Abdel-Daim, 2014; Liu et al., 2019). Therefore, it is often recommended as feed additives for animal species, particularly birds and fishes (Lee et al.1998; Lorenz 1999; Hoseini et al. 2013; Kulshreshtha et al. 2008; Ibrahim and Abdel-Daim, 2015; Abdel-Diam, 2014). In fact, both the doses of *Spirulina* were able to increase the activity of CAT in the liver and SOD activity of the gill, two principal metabolic and detoxification organs of the fish. On the other hand, none of the SM supplemented diets could affect the survival rate or hematological parameters. They also were unable to elicit any augmentation in any of the growth parameters studied; rather 10% SM increased lipid peroxidation in the gill tissue when treated alone or in combination with 10% SP. Most often, SM is used as a protein supplement in fish feed because of its low cost and easy access. In fact, Sales (2009) have shown through meta-analysis that SM supplementation didn't show any significant enhancement in growth of fishes, rather is reported to play a pivotal role in the onset of soyabean-induced enteritis in Atlantic salmon (*Salmo salar* L.) (Knudsen et al., 2008; Krogdahl et al., 2015). Further positive changes observed in fishes treated with combination of 10% each of SM and SP may be attributed to *Spirulina.*

Based on the present studies, it may be envisaged that replacement of *Spirulina* as a source of protein in place of fish meal in fish diet may be a better option than soya based products as it not only serves as

neutraceutical but also an economically viable one as this can be grown in presence of sunlight in the culture pond itself.

ACKNOWLEDGEMENTS

Nalinibala Behera (Rajiv Gandhi National Fellow) gratefully acknowledges the University Grants Commission (UGC), Government of India for the financial support as JRF and SRF [F.16-1825(SC)/2010(SA-III)] for this work. LS, DS also acknowledge the DST-FIST (2014-17), Government of India and the Department of Higher Education, Government of Odisha under OHEPEE (HE-PTC-WB-02017) for financial assistance to the Department of Zoology for Centre of Excellence in Environment and Public Health under the World Bank.

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Pranikee-Journal of Zoological Society of Orissa, ISSN 0970-4450
Volume XXXII, 2020, pp 79-91

THE ORAL APPARATUS IN *DUTTAPHRYNUS MELANOSTICTUS*, *EUPHLYCTIS CYANOPHLYCTIS*, *FEJERVARYA ORISSAENSIS* AND *POLYPEDATES MACULATUS* TADPOLES OF ODISHA

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ABSTRACT

The microanatomy of the oral discs of the tadpoles of four anuran tadpoles namely *Duttaphrynus melanostictus*, *Euphlyctis cyanophlyctis*, *Fejervarya orissaensis* and *Polypedates maculatus* were described. Tadpoles of *D. melanotictus* and *F. orissaensis* have two labial tooth rows on the upper labium with a medial gap in second row proximal to the mouth and three uninterrupted rows on the lower labium while that of *E. cyanophlyctis* have a single tooth row on the upper labium and two in the lower labium. Tadpoles of *P. maculatus* have four labial tooth rows on the upper labium with a medial gap in three rows proximal to the mouth and three rows on the lower labium with a medial gap in the row proximal to the mouth. Labial tooth row formula and order of the length of denticle rows are A2 (1)/P3 and A1>P1>P2>P3>A2, A1/P2 and P1+P2>A1, A2 (1)/P3 and A1>P1>P2>A2>P3 and 4(3)/P3 (1) and A2>P1>P2>A1>P3>A3>A4 for *D. melanostictus*, *E. cyanophlyctis*, *F. orissaensis* and *P. maculatus*, respectively.We also quantified the deformities in the marginal papillae, tooth rows, and jaw sheaths of tadpoles of Gosner stages 32-37 using a stereo microscope. Four oral deformities were recognized such as the lack of keratinization only in upper and/or lower jaw sheaths, lack of keratinization only in upper or lower tooth rows, deformities both in the jaw sheaths and tooth rows, and deformities in the structure and arrangement of marginal papillae. Tooth row deformities comprised 69% of all oral deformities, and jaw sheaths and marginal papillae comprised 18 and 13%, respectively. Tooth rows in the tadpoles of *D. melanostictus* had recorded the highest oral deformities. *E. cyanophlyctis* tadpoles had recorded the highest anomaly in jaw sheaths whereas marginal papillae of the tadpoles of *P. maculatus* were observed with maximum anomaly. Analysis of the samples revealed that tooth rows A1 and P1 had the most tooth row deformities, and P2 had the minimum deformities. Studies examining the etiology of oral deformities and the resulting impacts on the survival and success of post-metamorphic life stages will be important for allowing us to gain a better understanding of oral deformities and their implications in tadpoles.

Keywords: Amphibia, tadpoles, oral disc, labial tooth row formula, anomaly.

INTRODUCTION

Amphibians are distinct among animals physiologically, morphologically, and medically. Despite their relatively uniform body plan, anuran tadpoles exhibit an amazing morphological diversity, such as the position of spiracle opening, the eyes and the mouthparts. This morphological variation reflects the ecological and phylogenetic diversification that anurans have undergone (Duellman and Trueb, 1986). Some remarkable adaptations to the environment are found especially in the arrangement of mouthparts such as variations in the size and shape of the oral disc, the papillae at the margins of the oral disc, the shape of the jaws, the numbers of teeth rows and any gap(s) in those rows are some of the habitat-specific remarkable adaptations shown by anuran tadpoles. Such features are important both in the identification of tadpoles of different speciesand for inferring ecological adaptions (Duellman and

Trueb, 1986). The oral apparatus in anuran tadpolesis unique, complex and composed of soft upper and lower labia with transverse tooth ridges, marginal and sub marginal papillae, keratinized jaw sheaths, and labial teeth that are positioned on crests of the tooth ridges. The component of oral apparatus is arranged in such a manner that they help the tadpoles to adapt to its natural habitat.The oral apparatus seem to reflect lineage and habitats even among closely related taxa (Grandison, 1981; Duellman and Trueb, 1986; Channing, 2001).

Dental formula of a tadpole depicts the number and arrangement of tooth rows on its oral disc. The number which is written on the left of "/" refer to the anterior labium, while that written on the right is for posterior labium. Open numbers indicate the total number of tooth rows on each labium, number(s) in parenthesis is the number of interrupted rows in order of arrangement on labium. A dental formula of 2(2)/3(1-2) indicates two rows on anterior labium where the second one is interrupted with a median gap, while that of the posterior labium has three rows where the first and second one only are interrupted (Channing, 2001). The keratinized teeth are derived from cells present at the base of the tooth ridge (Gosner, 1959). The function of teeth in frogs is primarily to grasp food/prey or to position it for swallowing. The oral apparatus of different anuran tadpoles is different which reflects different feeding habits. The ontogeny of the labial teeth row structure of anuran tadpoles inhabiting temperate regions has been studied by several workers (Bonacci et al., 2008; Tolledo et al., 2009; Erik et al., 2010; Luna et al., 2012).

Deformities in dental architecture are of concern to ecologists because tadpoles with deformities may experience lower survival rates (Johnson et al., 2001). Obvious external deformities have been documented in amphibians since early 1700s (Vallisneri, 1706; Ouellet, 2000). The majority of these reports dealt with missing digits or portions of a limb and supernumerary limbs and deformity in oral structure with possible causes being increased ultraviolet (UV) radiation, environmental chemical contaminants, parasitic infections (Blaustein and Johnson, 2003), and eutrophication (Johnson and Chase, 2004).The developing larvae may remain longer in ponds with longer hydroperiods and thus have a greater incidence of developing oral deformities (Snodgrass et al., 2000). The variability of oral deformities as pigmentation in keratinized cells in tooth rows and jaw sheaths may also be related to either seasonal changes in temperature (Rachowicz, 2002) or infection by *Batrachochytrium dendrobatidis* (Vieira et al., 2013). Deformities in the oral discs have been reported for animals either exposed to the inorganic pesticides like DDT or to coal combustion residues (Rowe et al., 1996, 1998a, 1998b; Peterson et al., 2008). However, oral deformities have an effect on the feeding ability and growth performances.

Oral deformities may be used as biomonitoring tools, both for detecting contamination and for determining the efficacy of occurrence of deformities both in non-contaminated and in contaminated water bodies (Cooke, 1981). This paper deals with the detailed oral structure of the tadpoles of *Duttaphrynus melanostictus*, *Euphlyctis cyanophlyctis*, *Fejervarya orissaensis* and *Polypedates maculatus* including the incidence and form of oral deformities. Most reports involving oral deformities of tadpole have been in association with the tooth rows rather than the entire oral apparatus (Bresler, 1954; Bresler and Bragg, 1954; Grillitsch and Grillitsch,1989). We focused on oral deformities as irregularities in mouthparts can lead to erroneous identifications (Johnston and Altig, 1986). Besides, variations in oral morphology are of interest from ecological, developmental, and evolutionary

perspectives; morphological plasticity in trophic features in some species of tadpoles (Pfennig and Murphy, 2000; Vences et al., 2002; Relyea and Auld, 2005) can translate into differential survival for individuals of different phenotypes (Pfennig, 1992).

MATERIALS AND METHODS

Tadpoles of *D. melanostictus*, *E. cyanophlyctis*, *F. orissaensis* and *P. maculatu*s were collected from different water bodies of the Jagatsinghpur district (20º 19' 76"N and 86º 33' 77"E) of Odisha. The sites sampled were known to support successful amphibian breeding and metamorphosis, but no data were available on the oral apparatus and oral deformities of tadpoles. A total of 2156 tadpoles were collected by dip netting during May to September 2019. The live tadpoles were transported to the laboratory, maintained in separate aquaria with pond water under room temperature (25 - 30ºC), and fed with boiled spinach and algae collected from the sampling locations. Water was changed normally in every 3 days or as per requirement and the aquaria were checked to remove the dead individuals, if any. The developmental stages 32-37 (Gosner, 1960) were selected for circumoral studies, since at these stages the tadpoles have typical morphology and the oral disc is fully developed and functional. Besides, Thibaudeau and Altig (1988) have opined that the oral deformities in these stages to be neither due to developmental nor due to metamorphic responses. A minimum of 100 tadpoles for each species were euthanized using 1:10000 MS-222 in distilled water and were examined using a dissecting microscope for the oral architecture.

RESULTS

The four species of anuran tadpoles selected for the present study were collected from diverse habitats. *D. melanostictus* and *E. cyanophlyctis* occurred in wide range of habitats, *P. maculatus* were common in temporary rain pools and ponds, whereas *F. limnocharis* was generally found in rice fields.

Family: Bufonidae (Gray, 1825)

Genus: *Duttaphrynus* (Frost et al., 2006); **Species: *melanostictus*** (Schneider, 1799)

All soft and keratinized structures surrounding the mouth comprised the oral apparatus or mouth parts. The oral disc had equal-sized upper (anterior) and lower (posterior) labia with transverse tooth ridges.The oral disc was antero-ventral and emarginated in position. Single row of marginal papillae spread on the lateral corners of the oral disc and none seen on both the labia; two to three submarginal papillae seen at the lateral corners. The labial tooth row formula was A2 (1)/P3. Order of the length of denticle row was A1>P1>P2>P3>A2. Moderately keratinized jaw sheaths were very feeble and completely serrated with uniform sized small serrations with a wide base and a rounded head. Supra-rostrodont was convex, longer, the median being slightly broad and protruding posteriorly with keratinization and tapering to a long thin lateral process. The infra-rostrodont was V-shaped with a concave median. Denticles were closely packed and strongly curved towards the mouth at the apex. The oral angle was slightly obtuse; the sheath was narrow and the body was slightly broader. The tip of the head was broad with 10-12 long and moderately rounded cusps present on each denticle. Oral sucker was absent.

Family: Dicroglossidae (Anderson, 1871)

Genus: *Euphlyctis* (Fitzinger, 1843); **Species: *cyanophlyctis*** (Schneider, 1799)

The oral disc was near ventral in location, emarginated; not visible dorsally, single row of marginal papillae were mostly concentrated on the lateral corners; gaps in the distribution of marginal papillae were seen medially on both the equal-sized labia. Submarginal papillae were absent. The labial tooth row formula was A1/P2. Order of the length of denticle rows was P1+P2>A1. Both the jaw sheath were well developed, massively keratinized, and completely serrated with uniform sized serrations havinga short base and a triangular head. Supra-rostrodont was longer than wide and convex with the median protruding towards the posterior while the infra-rostrodont was U-shaped, convex laterally and concave medially. The non-cusped denticles were widely spaced and strongly curved towards the apex.The oral angle was straight except at the apex where it was slightly curved. The sheath was the broadest part of the denticle followed by the body; the body of the denticle was long and the tip of the head was deeply curved.

Genus: *Fejervarya* (Bolkay, 1915); **Species: *orissaensis*** (Dutta, 1997)

The oral disc was anteroventral in location, emarginated; not visible with a single row of marginal papillae spread on lateral corners of both the equal-sized labia. The marginal papillae gaps were observed at anterior and posterior regions of the oral disc with four to five submarginal papillae at the lateral corners. The labial tooth row formula was A2 (1)/P3. Order of the length of denticle rows was A1>P1>P2>A2>P3. The jaw sheaths were well developed, moderately keratinized and completely serrated with uniform sized serration with a wide base and a pointed head.The supra-rostrodont was longer than wide and broadly convex with the lateral process extending at the tip. The U-shaped infra-rostrodont was convex laterally and concave medially. The widely spaced denticles were strongly curved towards the apex. The oral angle was straight except at the apex where it was slightly curved. The sheath was the broadest part of the denticle followed by the body.The body of the non-cusped denticle was long and the tip of the head was deeply curved.

Family Rhacophoridae (Hoffman, 1932)

Genus: *Polypedates* (Tschudi, 1838), **Species: *maculatus*** (Gray, 1830)

The oral disc was anteroventral in location, emarginated, not visible dorsally with a single row of marginal papillae spread on both the equal-sized labia and the lateral corners.Two to three submarginal papillae were present at the lateral corners. The labial tooth row formula was A4 (3)/ P3 (1). Order of the length of denticle rows was A2>P1>P2>A1>P3>A4. The moderately keratinized jaw sheaths were well developed, completely serrated with uniform sized serrations and a wide base and short triangular pointed head. The supra-rostrodont was arch shaped with thin lateral process while the infra-rostrodont was U-shaped, convex laterally and concave medially.

Oral deformity

Tooth row deformities comprised 69% of all the oral deformities while that of jaw sheaths and marginal papillae comprised of 18% and 13%, respectively (Table 1). Deformities in the tooth rows accounted for 87.8 % of the oral deformities in *D. melanostictus* tadpoles, with 8.6 and 5.1% recorded in the jaw sheaths and marginal papillae, respectively (Table 1, Fig.1). The tooth rows of the tadpoles

of other three species studied had 60.4%, 54.6%, 73.6% of the deformities, with jaw sheaths and marginal papillae having 27.2%, 12%, 20.6% and 5.5%, 17.3%, 8.6% of the deformities, respectively (Table 1, Fig. 1). Tooth rows in the tadpoles of *D. melanostictus* had recorded the highest oral deformities. *E. cyanophlyctis* tadpoles had the highest anomaly in jaw sheaths whereas marginal papillae of the tadpoles of *P. maculatus* were observed with maximum anomaly. Tooth rows A1 and P1 had the maximum deformities, and P2 had the minimal deformities (Table 1). The most common defects were the missing teeth with disrupted supporting tissue. Upper and lower jaw sheaths without keratinisation were the most common jaw sheath defects followed by erosion of the cutting edges. Missing a portion was the most common defect in the lower and ventral area of marginal papillae. Extraneous projections, including teeth on the marginal papillae, were also a part of the marginal papillae deformities.

DISCUSSION

The oral disc of the tadpoles of *D. melanostictus, E. cyanophlyctis, F. orissaensis and P. maculatus h*ad the same general organization of keratinized jaw sheaths with keratinized tooth rows, the most common feature of the oral discs of most of the tadpoles (Thibaudeau and Altig, 1988; Altig and Johnston, 1989). However, the oral discs of these species differed specifically in orientation. It was anteroventral in *D. melanostictus and P. maculatus* and near ventral in *E. cyanophlyctis* and *F. orissaensis*. The anteroventral disposition of oral disc of tadpoles of *D. melanostictus* and *P. maculatus* indicated them primarily to be detritus feeders. Besides, they graze on algal vegetation and also filter feeds the planktonic bloom of the pond (Khan and Mufti, 1994a, 1994b). Nevertheless, some differences in the arrangement and morphology of the mouthparts among the tadpoles studied was observed. First, the arrangement of the upper and lower jaw sheaths varied among these species. The differences among the shape and size of the oral discs could be related to the nature of the food particles ingested by both species. In fact, *Dicroglossidae and Rhacophoridae* tadpoles feed probably by taking large bites of macrophytes and algae attached on submerged substrates, while *Duttaphrynus* tadpoles ingest smaller particles of detritus and algae through rasping (Savage, 2002; Kinne et al., 2004).

The structure, length and arrangement of the marginal and submarginal papillae also varied among the species investigated. The distribution pattern of the marginal papillae of *Dicroglossidae and Rhacophoridae* was the most common pattern taxonomically and ecologically (McDiarmid and Altig, 1999). On the other hand, a dorsal gap among the marginal papillae seen among *Duttaphrynus* tadpoles matches with the configuration that occurred commonly in most bufonids (McDiarmid and Altig, 1999). In fact, Van Dijk (1981) assumed that the presence of a ventral gap in the marginal papillae row of bufonidae tadpoles could play a role as a "weir-like flow-controlling structure"which acts as a barrier against water flow. The marginal papillae have tactile and chemosensory functions and help to control the water flow conveying food particles towards the mouth (McDiarmid and Altig, 1999), but the functional significance of the differences of this papillary pattern is still not understood. In *B. variegata*, it was found that the marginal papillae surrounded the entire oral disc (Altig and Johnston, 1989). They confirmed that this configuration was found among the stream inhabitant larval types of several families. Dicroglossid and Rhacophorid tadpoles lack a dorsal gap probably due to their carnivorous nature. Haas (2003) was of the opinion that the tadpoles use their complete papillary row as a filter for water flow and for a better adhesion to the irregularities of substrate surfaces.

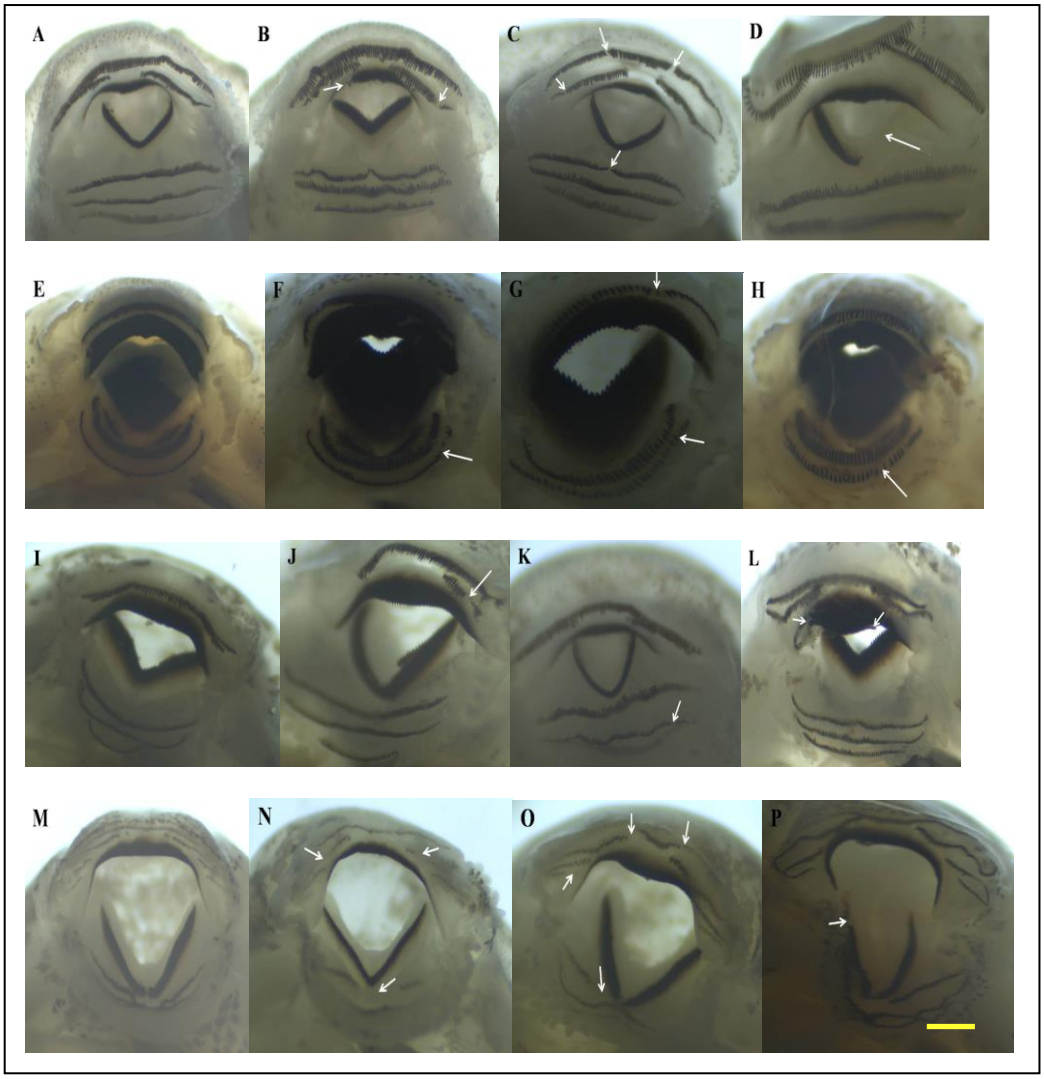

Fig. 1 Oral deformities among anuran tadpoles. *D. melanostictus*: (A) normal oral apparatus; (B) dekeratinized upper jaw sheath and missing teeth in A2; (C) missing teeth in A1, A2 and P1; (D) dekeratinized lower jaw sheath; *E. cyanophlyctis*: (E) normal oral apparatus; (F) missing labial teeth in P2 (G) missing labial teeth in A1 and P2 and (H) missing labial teeth in P2; *F. orissaensis*: (I) normal oral apparatus; (J) absence of teeth and stunted teeth in A2; (K) missing teeth in P2 and overlapping of P3 over P2; (L) dekeratinized upper jaw sheath; *P. maculatus*: (M) normal oral apparatus; (N) missing labial teeth in A2; (O) missing labial teeth in A1, A2, A4 and P1 and (P) dekeratinized lower jaw sheath. (Scale bar = 0.3mm)

Table 1 Percentage of oral deformities observed per tadpole sample per morphological region (U: upper, L: lower, V: ventral, A1: First row in anterior labium, A2: Second row in anterior labium, A3: Third row in anterior labium, P1: First row in posterior labium, P2: Second row in posterior labium, P3: Third row in posterior labium) (n = 100).

Species	Percentage deformity	Marginal Papillae				Anterior tooth rows			Posterior tooth rows				Jaw sheaths		
		U	L	V	Total	A1	A2	Total	P1	P2	P3	Total	U	L	Total
D. melanostictus	58	0	3.4	1.7	5.1	12	8.6	20.6	29.3	15.5	22.4	67.2	0	8.6	8.6
E. cyanophlyctis	33	3	6	3	12	36.3	0	36.3	15.1	9	0	24.1	6	21.2	27.2
F. orissaensis	53	3.7	1.8	0	5.5	9.4	28.3	37.7	11.3	7.5	16.9	35.7	5.6	15	20.6
P. maculatus	46	0	4.3	4.3	8.6	28.2	23.9	52.1	8.6	8.6	4.3	21.5	4.3	13	17.3

The labial tooth row formula (LTRF) was 2/3 in *D. melanostictus* and *F. orissaensis*, 1/2 in *E. cyanophlyctis*, and 4(3)/3(1) in *P. maculatus*. This pattern is in accordance with the observation of McDiarmid and Altig (1999) for Bombinatroids and that of Grillitsch and Grillitsch (1989) and Tubbs et al., (1993) for *Duttaphrynus* tadpoles. Our observation supports the uniform morphology of the oral apparatus in the tadpoles of species considered for the present study as proposed by McDiarmid and Altig (1999). Variations, if any, could be related to the pond dimensions. It is known that tadpoles in temporary ponds have few labial tooth rows because the progress of development and the metamorphosis are induced before the full development of the oral structures (Vences et al., 2002). They also assumed that the first tooth row on the upper labium is always longer than the lower tooth rows. The lower tooth rows have a similar length in *D. melanostictus*, *E. cyanophlyctis* and *P. maculatus* which decreases from the proximal to the distal row in *F. orissaensis*. It seems that the tooth row lengths are correlated with the tadpole microhabitats. Species occupying standing water may have a shorter distal lower tooth row than the proximal one; whereas tooth rows are typically long in species inhabiting running water.

Deformities in the oral apparatus of tadpoles are common in the species and sites sampled during this study. The total number of deformities and their patterns of distribution within the oral apparatus varied among populations and were mostly explained by taxonomic affinity (family). The location of deformities within the oral apparatus was not random, with the majority of deformities found in the tooth rows. The incidence of deformities in one area of the oral apparatus was independent of their occurrence in another region. Extra keratinized structures in the marginal and submarginal papillae in the tadpoles of *D. melanotictus* were observed. The present results coincide with similar observations those aid in stuffing large pieces of food into oro-pharyngeal passage of the tadpole (Atlig and Johnson, 1989; Khan and Mufti, 1994a; Hopkins et al., 2000; Drake et al., 2007). In addition, oral anomalies such as eroded jaw sheaths and gaps in tooth rows were also reported in the natural ponds (Altig, 2007). Drake et al. (2007) have reported oral deformities (teeth in the marginal papillae, tooth rows and jaw sheaths) of tadpoles from nine anuran species (*Rana sphenocephala, Rana sevosa, Rana clamitans, Hyla versicolor, Hyla chrysoscelis, Hyla squirilla, Bufo nebulifer, Bufo fowleri and Bufo woodhousii*). The frequency of oral deformities can be high in natural populations due to the presence of *Batrachochytrium dendrobatidis* infection, which exerts a strong influence on the occurrence and type of oral deformities in tadpoles. The chytrid fungus *B. dendrobatidis* was found to cause oral abnormalities in *Rana muscosa* (Fellers et al., 2001) and in *Rhinella quechua* (Barrionuevo et al., 2008). *B. dendrobatidis* also induces oral deformities in *Bufo fowleri* and *Hyla chrysoscelis* tadpoles. On the other hand, the incidence of oral deformities increased in the American Bullfrog (*Lithobates catesbeianus*) tadpoles due to their exposure to coal combustion residues (Rowe et al., 1996, 1998a and 1998b; Peterson et al., 2008). Generally, the jaw sheaths exhibit significantly more deformations than labial teeth (Venesky et al., 2010).

The results of our study are informative when one considers differential larval development durations and other ecological and developmental features across taxa. North American *Rana* tadpoles have prolonged larval development compared to *Hyla* and *Bufo*, and some *Rana* species (*R. catesbeiana*) take up to two years to metamorphose (Duellman and Trueb, 1994). *Rana* also typically inhabit more permanent water bodies (Dundee and Rossman, 1989) and are more benthic in their feeding habit. In

contrast, *Hyla* tadpoles metamorphose in a comparatively shorter period, live in more ephemeral water bodies (Dundee and Rossman, 1989), and are nektonic in their feeding. Such general differences at the family level may result in differential exposure to environmental components which may play a role in the development of oral deformities. Rowe et al. (1996) suggested that "deformities would be frequent in tadpoles only after a longer period of exposure to pollutants" and that "physiological, developmental or other differences between the species affect the response to conditions in polluted sites".

It is unclear why the deformities are mostly anterior in *E. cyanophlyctis, F. orissaensis* and *P. maculates* tadpoles but posterior in *Duttaphrynus*. Often it seems that structures such as tooth rows (A1 and P1) appearing earlier in oral ontogeny (Thibaudeau and Altig, 1988) are less susceptible to disruption than those (P2, the ventral marginal papillae) appear later. Grillitsch and Grillitsch (1989) examined taxa with more than two anterior and three posterior (2/3) tooth rows and reported that often tooth rows appearing later and further from the mouth (2/4 or 2/5 tooth row formulae) commonly possess deformities. Tooth rows farther from the mouth are under less stringent developmental control and more susceptible to insult, or are of lesser importance in feeding. A similar trend also occurred in the ventral marginal papillae, which appear later or does not appear at all (*D. melanostictus*) and have more deformities than the lateral papillae.

Further studies are required to verify the present observations on malformations. All life stages (including the larvae) should be examined to determine the overall health of amphibian populations and the potential role that deformities play in their decline. Our data provide another step towards achieving this goal by quantifying the frequency and types of oral deformities that can occur in larvae. More intensive examinations of tadpoles at family, species, developmental stage, site, cohort levels, year of development, and over multiple years will refine our understanding of the patterns of deformities and their implications. The common occurrence of oral deformities in the major components of the oral disc reinforces and assertion that the entire oral disc should be examined more closely when evaluating tadpoles for oral deformities and emphasizes the need to explore the etiology of the deformities and their potential impacts on future life stages.

ACKNOWLEDGEMENTS

Jasmin Rout is thankful to the CSIR, Government of India for funding through Junior Research Fellowship (File No. 09/173(0134)/2020-EMR-I) and to the Head, Department of Zoology, Utkal University for extending necessary facilities to carry out the work.

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Pranikee-Journal of Zoological Society of Orissa, ISSN 0970-4450
Volume XXXII,2020, pp 92-117

HAEMATOLOGICAL PROFILE OF THE DEVELOPING TADPOLES OF *DUTTAPHRYNUS MELANOSTICTUS*

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ABSTRACT

The present study concerns with the haematological changes in the Asian toad, *Duttaphrynus melanostictus* (Schneider, 1799): Bufonidae, during the development of the tadpole. The limb bud stage tadpoles were collected from natural pools of Kendrapara (20.50°N 86.42E), Odisha, India. Study of red blood cells (RBCs) revealed presence of comma, spindle, round and oval shaped cells. Several dividing cells, apoptotic cells and degenerating cells were also found. A negative correlation was observed between the developmental stages with respect to length (r = -0.529), aspect ratio (r = -0.504), area (r = -0.244) of RBCs. Similar negative correlation were also observed in length (r = -0.466), breadth (r = -0.529), area (r = -0.516) of nuclei of RBCs. The leukocyte profile during developmental stages was also investigated. A negative correlation existed between the developing stage and percentage of leucocytes whereas positive correlation was observed in percentage of neutrophils, basophils and monocytes.

Keywords: *Duttaphrynus melanostictus*, Leucocyte profile, Erythrocyte

INTRODUCTION

A characteristic of metamorphosis, in the animal kingdom in general, is that the polymorphic animals express itself in such a way that one form replaces another during the normal life of the individual (Paik and Cohen, 1960). Amphibians posses a unique metamorphosis which include transition of aquatic tadpole to terrestrial adult. Amphibians have been a subject of investigations in several directions due to their exclusive ontogeny and mode of life (Wojtaszek and Adamowicz, 2003). As a part of metamorphosis, blood cell populations are renewed (Rosennkilde et al., 1994) and number of leukocyte changes (Davis, 2009). Metamorphosis thus provides physiologists an opportunity to understand the process involves in hematopoiesis. Blood is also considered as an indicator of physiological condition of an animal (Zutshi, et al., 2010). Amphibian Blood is composed of plasma in which nucleated erythrocytes, leukocytes and thrombocytes are suspended (Jordan, 1938). The concentration of each cell type is consequently maintained in the blood within well defined limits, unless the balance between production and elimination is disturbed.

The phenomenon of tissue growth and lysis during metamorphosis has an effect on the relative distribution of white blood cells in circulation (i.e., leukocyte profile) and it has been considered as an important biological phenomenon since early part of twentieth century (Davis, 2008). Studying the

changes in the parameters in blood, which dynamically react to the external stressors, makes it possible to use the data in the system of biomonitoring (Cabagna et al., 2005; Lajmanovich et al., 2012 and Zhelev, 2012). Hematological indicators are very specific and they fluctuate within narrow parameters (Gül et al., 2011; Mahapatra et al., 2012), which allow their use as markers in different physiological and pathological process taking place at the levels of the organism and the ecosystem (Peskova, 2001; Zhelev et al., 2006 and Lajmanovich et al., 2008). Moreover, interest in counts of leukocytes in amphibian for environmental monitoring emphasizes the need to understand how the blood cells naturally vary throughout larval life (Jordan and Speidel, 1922, 1924; Kiesecker 2002; Barni et al., 2007; Davis, 2008).

Most vertebrates have five types of White Blood cells (WBCs): lymphocytes, neutrophils, eosinophils, basophils and monocytes. The morphology of each cell type is conserved across taxa, except in the case of neutrophil, which is replaced with the heterophil (Davis et al., 2008) in birds, reptiles with the same immunological function (Hawkey and Dennett, 1989; Jain, 1993) and occasionally in amphibians (Cabagna et al., 2005; Forbes et al., 2006). However, in case of amphibian heterophil appears more similar to a neutrophil (Thrall, 2004). Neutrophils/heterophils and lymphocyte make up the majority (i.e., nearly 80% combined) of WBCs of mammals (Jain, 1993) birds (Rupley, 1997), amphibians (Bennette et al., 1972; Cathers et al., 1997; Thrall, 2004) and reptiles (Eliman,1997; Fisse et al., 2004; Werner, 2007) are considered as the primary phagocytic leukocyte which proliferate in circulation in response to infections, inflammations and stress (Jain,1993; Campbell, 1995; Rupley, 1997; Harmon, 1998; Thrall, 2004). Lymphocytes are involved in a variety of immunologic function such as immunoglobulin production and modulation of immune defense (Campbell, 1996). The remaining 20% of the leukocyte represent eosinophil, basophil and monocytes. Eosinophils are pleiotropic, multifunctional leukocytes involved in host protection against parasites (Rothenberg et al., 2006). The eosinophils acquire their unique contribution in initiating inflammatory and adaptive responses due to their bidirectional interaction with the dendritic cell and T cell as well as their large pannel of secretory cytokines and soluble meditors (Blanchard and Rothenberg, 2009). Monocytes are long lived phagocytic cells associated with defense against infections and bacteria (Campbell, 1995; Davis et al., 2004). Basophils are the last type of amphibian white blood cell (WBC) which function is not clearly understood (Rupley, 1997); but thought to involve in inflammation (Campbell, 1995).The leukocyte profiles are increasingly incorporated into research goals as they are altered by stress and can be directly related to stress hormone levels and the change brought on by stress leads to increase in number of neutrophil and decrease in lymphocyte.

During metamorphosis, renew of erythrocyte involves a switch shift to a new terrestrial type of hemoglobin with lower oxygen affinity. In all vertebrates, erythrocytes are responsible for sorting and transporting oxygen throughout the body and are composed of little else but a nucleus (except in mammals) and hemoglobin in cytoplasm (Davis et al., 2009). There is considerable variation in cell morphology among species, especially within amphibians (Vernberg, 1955; Kuramoto, 1981) and this variation is thought to stem from variation in metabolism (Smith, 1925; Vernberg, 1955).

Research on amphibian larval Red Blood cells (RBCs) reveals the lifespan of the same to be 100 days rather than 120 days as found in mammals (Forman and Just, 1975). Amphibian RBC size and number

in different species (*Ambystoma jeffersonianum*, *Rana lesonae*, *Rana esculenta*) have been reported by Smith (1925), Fankhauser (1945), Davison (1959), Uzzell (1964), Uzzell and Berger (1978), Gunther (1977), Uzzelle and Hotz (1979), Kuramoto (1981), Ravindra and Soushag (1988), Berger and Ogielska (1994), and Davidson and Mortimer (2003). The number of red blood cells in amphibian circulating blood show a wide individual variation and considerable interspecies difference (Jordan and Speidel, 1923; Roufe, 1961; Hutchison and Szarski 1965; Hollyfield, 1966; Szarski and Czopek,1966; Maniatis and Ingram, 1971; Kaloustian and Dulac, 1982; Sinha, 1983; Wakahara and Yamaguchi, 2001) depending on body mass, age, sex (Arvy, 1947; Goniakowska,1973; Sinha, 1983; Choubey et al., 1986; Banerjee, 1988), habitat conditions (Ruiz et al., 1983, 1989) and season (Zhukova and Kubantsev, 1979; Sinha, 1983; Samantaray, 1985; Wojtaszek et al., 1997). The erythrocyte size has also been described to be used in ploidy determination (Atatür et al., 1999; Cianciarullo et al., 2000). Hematological studies have been carried out in different species of anurans. Blood parameters of *Rana temporaria* (Alder and Huber, 1923), *Rana pipiens* (Rouf, 1969); *Bufo vulgaris* (Fankhauser, 1945); *Rana esculenta* (Sinha, 1983); *Rana tigrina* (Mishra and Banerjee, 1983); *Hyla septentrionallis*, *Rana catesbeiana* (Carmena et al., 1980), *Bombina bombina* (Atatür et al., 1999; Wojtaszek and Adamowicz, 2003), *Rana ridimunda*, *Bufo viridis*, *Bufo bufo*, *Pelobates syriacus*, *Bombina bombina* and *Hyla arborea* (Atatür et al., 1999), *Polypedates maculatus* (Mahapatra et al., 2012) and *Microhyla ornata* (Hota et al., 2013) have been studied. However, report on larval hematology is very scarce except for erythrocyte morphology in larval mole salamander *Ambystoma talpoideum* (Davis and Maerz, 2008), Leukocyte profile in *Rana catesbeiana* (Davis, 2009), blood cell profiles of Indian tree frog *Polypedates teraiensis* (Das and Mahapatra, 2012) and blood cell study of ornate frog *Microhyla ornata* (Hota et al., 2013). Erythropoiesis has been reported in *Rana catesbeiana* (Benbassat, 1970; Maniatis and Ingram, 1971).

The common Asian toad, *Duttaphrynus melanostictus* is a species of the family Bufonidae of order Anura. These toads are nocturnal, terrestrial, mid to large sized and characterized by a modestly sized head, short limbs and stout body. The important physical feature showed by the toad is covering of the back with a series of warts. Asian toads are insectivorous and known to feed on many insect pests known to humans (Mogali et al., 2011).

Although in India, blood parameters of adult *Bufo melanostictus* (New name, *Duttaphrynus melanostictus*; Banerjee and Banarjee, 1966; Banerjee and Sinha, 1978; Banerjee et al., 1980) have been reported, hematological studies of larva remain unexplored in this species. So, the present piece of study was to examine metamorphosis related changes in shape and sizes of erythrocytes and also leukocyte profile in different larval stages. Tadpoles of limb bud stage were collected from a natural pool of Kendrapara (20.50°N 86.42°E), Odisha, India. A correlation between the leukocyte related changes during successive developmental stages, size and area occupied by RBCs and their nuclei, along with the ratio of length and breadth of RBCs and their nuclei have been investigated in the present study.

MATERIALS AND METHODS

Limb bud stage tadpoles of the Asian toad, *Duttaphrynus melanostictus* were collected during the month of July 2018 from their natural habitat from Kendrapara (20.50°N 86.42°E), Odisha, India. The

tadpoles were reared following standardized procedure (Mohanty-Hejmadi, 1977). The tadpoles were fed with boiled Amaranthus leaves *ad libitum*.

Tadpoles Investigated

In the present study tadpoles from Gosner stages (Gosner, 1960) 28 to 46 were taken into account (Figs. 1-3). A pool of three tadpoles from each stage was selected for investigation. The tadpoles were divided into three groups i.e., premetamorphic (stages 28 to 32); pro-metamorphic (stages 33 to 41) and metamorphic (stages 42 to 46) (McDiacmid and Altig, 2000).

Preparation of Blood smears

Prior to amputation, the tadpoles were anesthetized with 0.1% Tricaine methane sulphonate solution. The blood of tadpoles from stage 28 to 44 was obtained from tail by amputation through the middle of the tail by maintaining the tadpole laterally on a pre-sterilized porcelain plate. In case of stages 45 and 46, the blood was collected from heart by a fine syringe of gauge 26. Blood smears were prepared by using push slide technique. The slides were stained with Giemsa's stain, which was prepared by mixing 20ml of concentrated Giemsa stain with 20ml of distilled water and 60ml of phosphate buffer solution of pH 7. Following staining slides were examined under light microscope.

Identification and Counting of Blood Smears

The blood smears were observed under a standard light microscope. The different shapes of RBCs were identified as oval, elliptical, comma and spindle shape. The white blood cells were identified as lymphocytes, monocytes, neutrophils, eosinophils and basophils following Hadji-Azimi et al. (1987) and Thrall (2004). Slides were viewed in zig-zag pattern, covering all parts of the blood smear and all leukocytes were counted in each field of view until 100 cells were viewed.

The size of RBCs and nuclei were measured by inbuilt software in microscope eyepiece digital camera (model Catcam130:1.3MP). The area of RBCs which were elliptical was calculated as given below.

Area= short (minor) axis × long (major) axis × 0.7854

Photographs of erythrocytes and leukocytes were taken with the help of microscope eyepiece digital camera, (model Catcam130:1.3MP).

Statistical Analysis

The relationship between developmental stages and morphometric parameters of erythrocytes and percentages of leukocytes were assessed by drawing scatter plots. Pearson's correlation coefficient was calculated for each parameter with the developmental stages. The graphs were done in Microsoft office Excel-2007.

RESULTS

Blood cell profiles of tadpoles of *D. melanostictus* observed in the study are represented in Table 1 and 2.

Morphology of Erythrocytes

Oval shaped erythrocytes were the most dominant cell types observed in tadpoles throughout development (Fig. 4A). Round erythrocytes were also found in tadpoles of stages 42 to 46 (Fig. 4B).

Besides, other forms of RBCs such as comma shaped (stages 37- 41) (Fig. 4C) and spindle shaped (stages 34-42) (Fig. 4D) were also observed. Dividing and degenerating cells were found in the tadpoles throughout development (Figs. 4E, G). Poikilocytosis i.e., increased variation in shape of RBCs was found in stages 28-39 (Fig. 4F). Apoptotic cells and large number of degenerating cells were evident in later stages (37- 42) of development (Fig. 4G). Rouleaux formation was found in stages 39- 44 (Fig. 4H).

Morphology of Leukocytes

The leukocytes observed in the present study were of five categories i.e., lymphocyte (large and small), monocyte, eosinophil, neutrophil and basophil. The first two were agranulocytes while the rest were granulocytes. Both types of lymphocytes observed had rounded morphology differing only in size. The deep violet nuclei of the lymphocytes covered the entire cells, leaving only a thin rim of cytoplasm towards the periphery (Figs. 5A, B). Monocytes were either irregular or rounded with kidney shaped nuclei (Figs. 5C, D). The neutrophils observed in the present study were either band shaped (Figs. 5E and F), bilobed or trilobed (Figs. 5G and H). However, all eosinophils were rounded cells with bilobed nuclei (Fig. 5I). Basophils were identified as large rounded cells having undifferentiated cytoplasm and nuclei (Fig. 5J).

Profile of Erythrocytes

The length (L), breadth (B), aspect ratio (L/B), area (A) of erythrocytes and length (L'), breadth (B'), aspect ratio (L'/B'), area (A') of nuclei of erythrocytes were investigated in the present study. The length, breadth and aspect ratio of erythrocytes ranged from 16.95±1.72µm (Gosner stage 45) to 22.89±2.29 (Gosner stage 29), 10.19±1.75 (Gosner stage 37) to 14.36±1.69 (Gosner stage 34) and 1.36±0.14 (Gosner stage 45) to 1.92±0.3 (Gosner stage 38), respectively (Table 1). Similarly, the length, breadth, aspect ratio of nuclei of erythrocytes ranged from 7.93±1.44 (Gosner stage 42) to 11.79±1.92 (Gosner stage 33), 5.01±0.80 (Gosner stage 38) to 8.20±1.64 and 1.24±0.15 (Gosner stage 34) to 1.74±0.32 (Gosner stage 38), respectively. Moreover, area occupied by the RBCs ranged between 145.03±36.55 (Gosner stage 37) to 226.81±48.7 (Gosner stage 29) while that of nuclei ranged from 33.98±7.88 (Gosner stage 38) to 62.44±12.22 (Gosner stage 46) and the area of nuclei of RBCs to area of RBCs ratio ranged between 0.16±0.002 (Gosner stage 41) to 0.40±0.10 (Gosner stage 30).

Profile of Leukocytes

Lymphocytes were the dominating leukocytes observed during the developmental stages of the tadpoles of *D. melanostictus* with a maximum number of 95% in tadpoles of stage 26 (Table 2). A decline in lymphocyte number was evident towards the climax stage during metamorphosis with a minimum of 60% in stages 41 and 45. The mean lymphocyte count estimated was 74.789±10. Monocytes were found to be the second most abundant leukocytes having mean 10.368±6.22. The number of these cells ranged from 0 (Gosner stage 29) to 24 (Gosner stage 45).The average percentage of neutrophil was found to be 7.684±6.17. The neutrophil percentage ranged from 0 (Gosner stages 28, 29, 30, 32) to16 (Gosner stage 38). The eosinophil number increased significantly during metamorphic climax as compared to pre and pro metamorphic stages. The average percentage of eosinophils was found to be 4.473±3.061. The eosinophil percentage ranged from 0 (Gosner stages 28, 31, 35, 38) to 10 (42, 41) (Table 2). Basophils were the least abundant leukocytes during the development of tadpoles

with a mean 1.842±2.242 (Table 2). The percentage of basophils ranged from 0 (Gosner stages 30, 32, 33, 34, 35, 37, 38, 42 and 43) to 8 (Gosner stage 40).

Statistical Analysis

The correlation between various parameters of erythrocytes and different developmental stages of tadpoles were investigated by calculating the Pearson's correlation coefficient. A negative correlation was observed between different developmental stages and length of erythrocyte (r= -0.529) (Fig. 6A; Table 1). The correlation observed between different developmental stages and breadth of erythrocyte was positive (r= +0.088) (Fig. 6B; Table 1). The correlation between length: breadth (L/B) ratio of erythrocytes and the area of erythrocytes with respect to developmental stages was negative with a correlation coefficient 'r'= -0.504 and 'r'= -0.244, respectively (Figs. 7A and B; Table 1). The correlation between length (L') and breadth (B') of nulei of erythrocytes with respect to different developmental stages were negative with correlation coefficient 'r'= -0.466 and -0.529, respectively (Figs. 8A, B; Table 1). While a positive correlation was observed between different developmental stages with respect to aspect ratio of nulei of erythrocytes (r= 0.069) (Fig. 9A; Table 1). A negative correlation was observed between different developmental stages with respect to area of nuclei of erythrocytes and A': A ratio (r= -0.516 and r= -0.388) (Fig. 9B and Table 1).

A negative correlation between lymphocytes percentage and developmental stages was observed (r= -0.843) (Fig. 10 and Table 2). The correlation coefficient 'r' was observed to be 0.445 for monocytes percentage with respect to different developmental stages (Fig. 11A; Table 2). The correlation observed between the eosinophils number and developmental stages was found to be positive having the correlation coefficient value, 'r'= 0.480 (Fig.11B; Table 2). The correlation between neutrophils percentage and developmental stages was found to be positive (r= 0.852) (Fig. 12A and Table 2). Similarly, a positive correlation was found between percentage of basophils and developmental stages (r= 0.237) (Fig. 12B and Table 2).

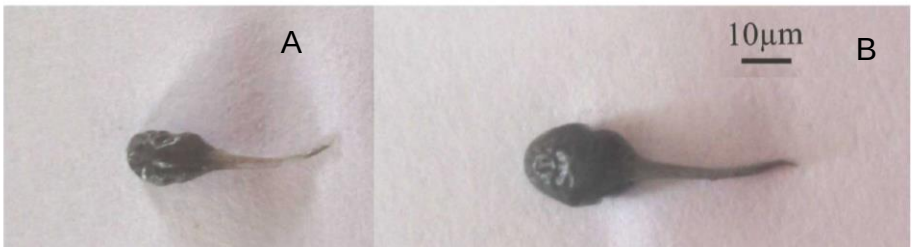

Fig. 1 Pre metamorphic stages of tadpoles of *D. melanostictus* (A) Tadpole of stage 30; (B) Tadpole of stage 32. (Scale bar = 10µm)

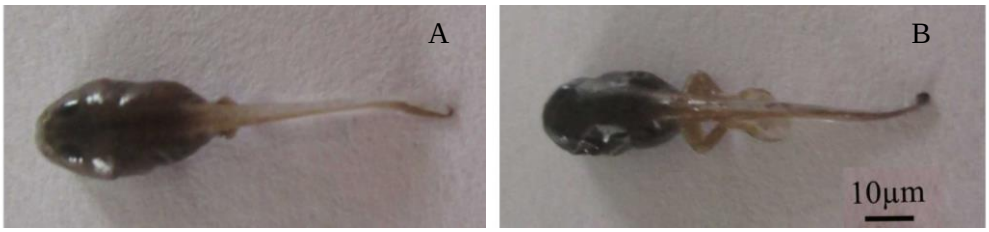

Fig. 2 Pro metamorphic stages of tadpoles of *D. melanostictus* (A) Tadpole of stage 36; (B) Tadpole of stage 38. (Scale bar = 10µm)

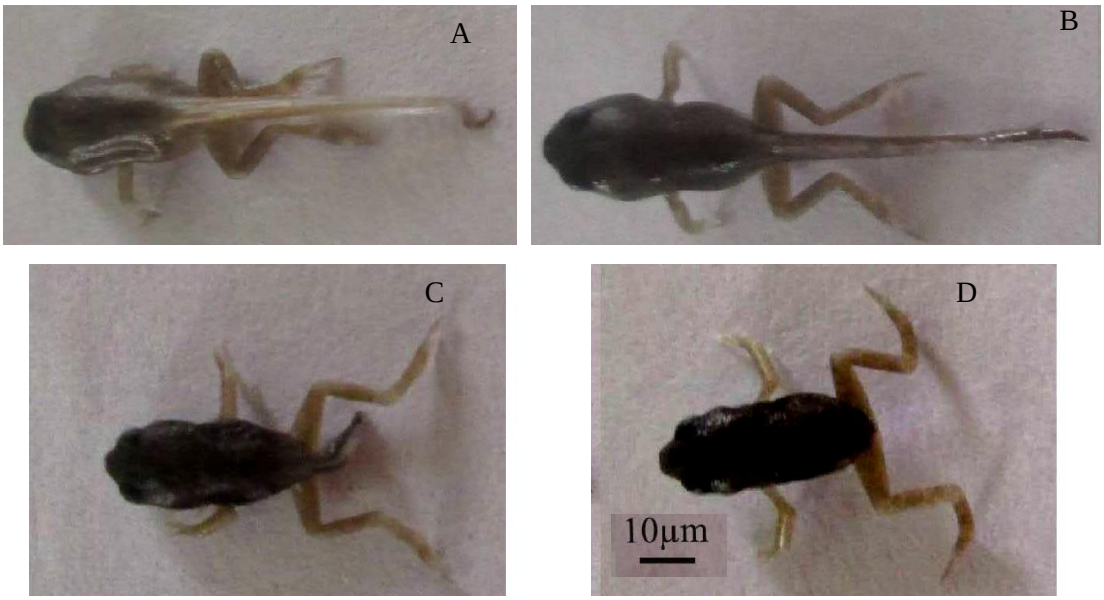

Fig. 3 Metamorphic stages of tadpoles of *D. melanostictus* (A) Tadpole of stage 42; (B) Tadpole of stage 44; (C) Tadpole of stage 45; (D) Tadpole stage 46. (Scale bar =10µm)

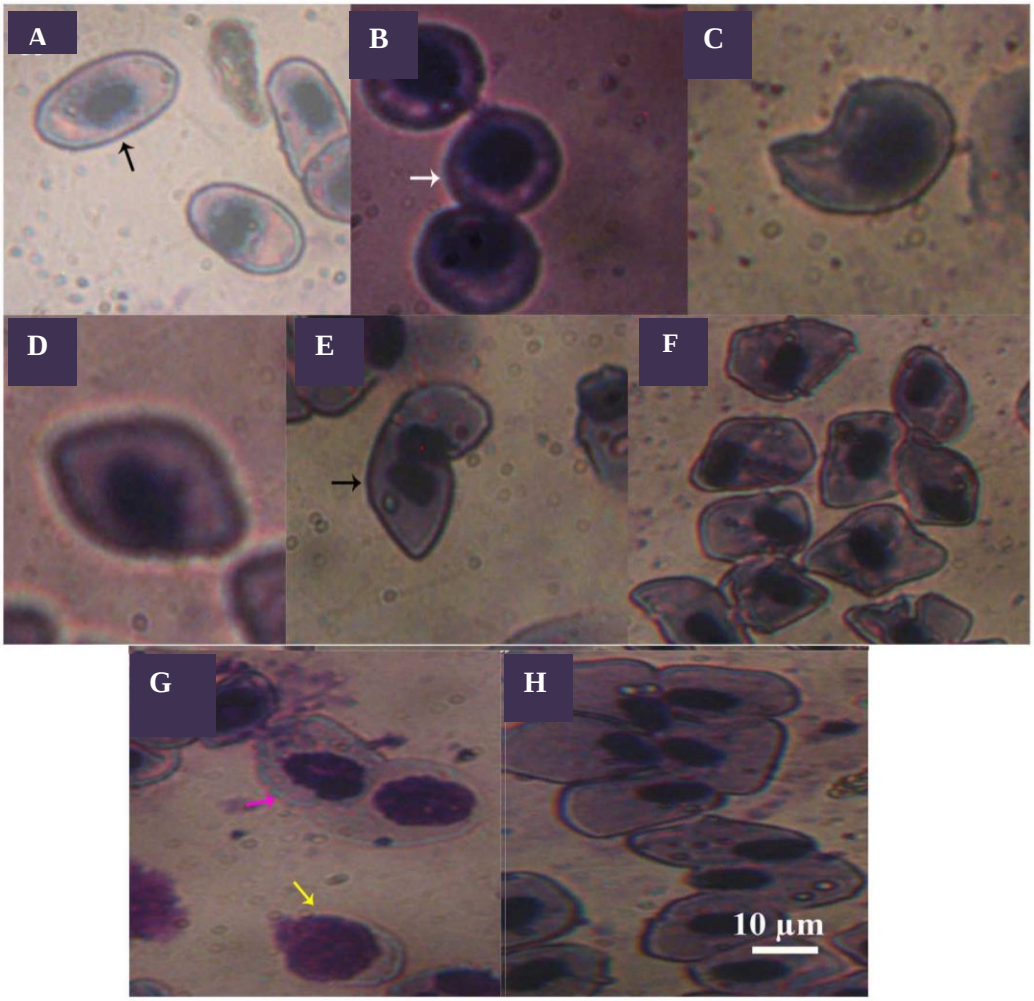

Fig. 4: Different types of RBCs observed in different developmental stages of the tadpoles of *D. melanostictus*. (A) Oval RBC; (B) Round RBC; (C) Comma shaped RBC; (D) Spindle shaped RBC; (E) Dividing RBC; (F) Poikilocytosis of RBCs; (G) Apoptotic RBC and Degenerating RBC; (H) Rouleaux formation of RBC (Scale bar =10µm).

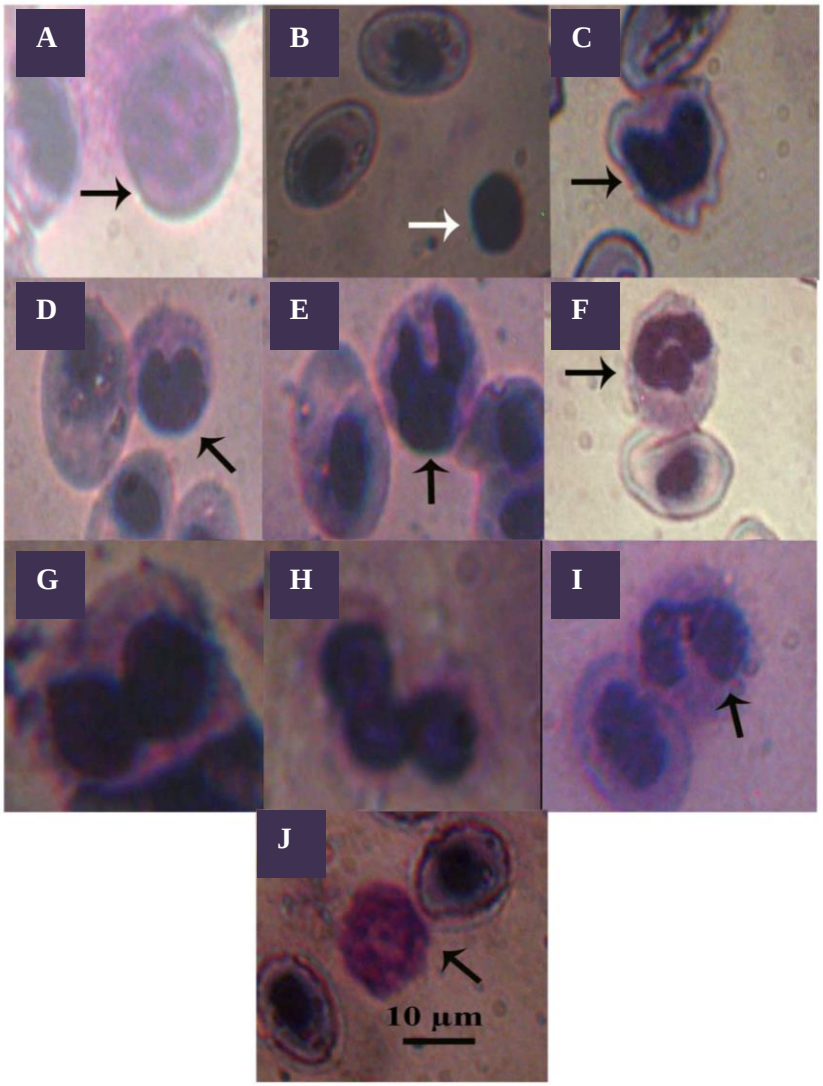

Fig. 5 Different types of WBCs observed in different developmental stages of the tadpoles of *D. melanostictus*; (A) Large lymphocyte; (B) Small lymphocyte; (C) Irregular shaped monocytes; (D) Round shaped monocyte; (E) and (F) Band shaped neutrophil; (G) Bilobed neurtophil; (H) Trilobed neutrophil; (I) Eosinophil; (J) Basophil (Scale bar = 10µm).

Table 1: Erythrometry of different developmental stages of tadpoles of *Duttaphrynus melanostictus*

Gosner stages	Red Blood Cell				Nuclei				A'/A (µm²)
	L (µm)	B (µm)	L/B	A (µm²)	L' (µm)	B' (µm)	L'/B'	A' (µm²)	
28	19.45 ±2.62	11.9 ±2.97	1.71 ±0.42	182.66 ±57.37	10.65 ±1.41	6.6 ±0.7	1.62 ±0.22	55.52± 10.69	0.31 ±0.06
29	22.89 ±2.29	12.5 ±1.50	1.83 ±0.13	226.81 ±48.7	10.52 ±1.31	7.78 ±0.88	1.37 ±0.21	64.17 ±10.09	0.29 ±0.06
30	20.2 ±3.53	11.39 ±1.39	1.77 ±0.26	182.79 ±45.09	11.27 ±2.34	8.19 ±2.55	1.44 ±0.36	73.17 ±27.26	0.40 ±0.10
31	22.56 ±2.03	12.38 ±1.56	1.84 ±0.24	220.06 ±39.23	8.31 ±1.06	6.44 ±2.52	1.39 ±0.34	41.70 ±14.86	0.19 ±0.08
32	19.85 ±1.78	11.83 ±1.45	1.71 ±0.34	183.48 ±18.36	10.98 ±1.82	6.9 ±1.26	1.63 ±0.39	59.64 ±14.73	0.32 ±0.07
33	19.41 ±1.80	12.06 ±1.18	1.55 ±0.2	192.41 ±27.24	11.79 ±1.92	8.20 ±1.64	1.47 ±0.31	76.62 ±22.2	0.39 ±0.1
34	19.91 ±3.21	14.36 ±1.69	1.41 ±0.32	223.35 ±35.7	8.77 ±1.22	7.07 ±0.98	1.24 ±0.15	49.25 ±11.56	0.22 ±0.04
35	20.09 ±2.81	12.26 ±1.57	1.64 ±0.19	195.52 ±48.40	9.64 ±0.98	6.56 ±0.64	1.48 ±0.22	49.76 ±7.59	0.26 ±0.06
36	20.86 ±1.79	13.64 ±3.17	1.60 ±0.36	223.33 ±52.92	10.50 ±1.35	6.26 ±0.40	1.68 ±0.2	51.73 ±7.64	0.24 ±0.05
37	18.01 ±2.74	10.19 ±1.75	1.80 ±0.35	145.03 ±36.55	9.84 ±1.08	6.92 ±1	1.43 ±0.18	53.99 ±12.38	0.38 ±0.1
38	20.25 ±2.19	10.62 ±0.86	1.92 ±0.3	168.36 ±18.13	8.58 ±1.20	5.01 ±0.80	1.74 ±0.32	33.98 ±7.88	0.20 ±0.04
39	19.22 ±2.35	12.49 ±1.56	1.55 ±0.26	188.73 ±33.61	9.73 ±1.52	6.37 ±1.16	1.55 ±0.26	49.45 ±14.68	0.27 ±0.10
40	19.57 ±1.49	12.39 ±0.76	1.58 ±0.18	190.18 ±13.63	8.92 ±1.76	7.14 ±2.08	1.30 ±0.35	50.99 ±19.91	0.27 ±0.10
41	20.65 ±1.32	13.43 ±1.24	1.55 ±0.2	217.62 ±22.19	8.43 ±0.88	5.43 ±0.52	1.56 ±0.23	35.98 ±5.01	0.16 ±0.02
42	17.92 ±2.46	11.57 ±1.02	1.41 ±0.22	181.85 ±28.31	7.93 ±1.44	5.78 ±0.90	1.30 ±0.24	40.80 ±12.66	020 ±0.04
43	19.51 ±0.97	13.89 ±0.89	1.4 ±.09	213.12 ±19.77	8.64 ±0.35	5.19 ±0.76	1.70 ±0.30	35.31 ±5.83	0.16 ±0.03
44	19.88 ±2.13	11.16 ±0.69	1.78 ±0.22	174.49 ±22.54	9.53 ±1.26	6.27 ±1.1	1.55 ±0.33	47.03 ±10.37	0.27 ±0.09
45	16.95 ±1.72	12.5 ±1.08	1.36 ±0.14	167.06 ±26.91	8 ±0.46	5.96 ±0.64	1.35 ±0.15	37.52 ±4.83	0.23 ±0.05
46	19.58 ±1.35	12.61 ±1.11	1.56 ±0.23	193.3 ±11.58	11.05 ±1.27	7.16 ±1.03	1.56 ±0.28	62.44 ±12.22	0.32 ±0.06
'r'	-0.529	+0.088	-0.504	-0.244	-0.466	-0.529	+0.069	-0.516	-0.388

SD: standard deviation 'r': correlation coefficient

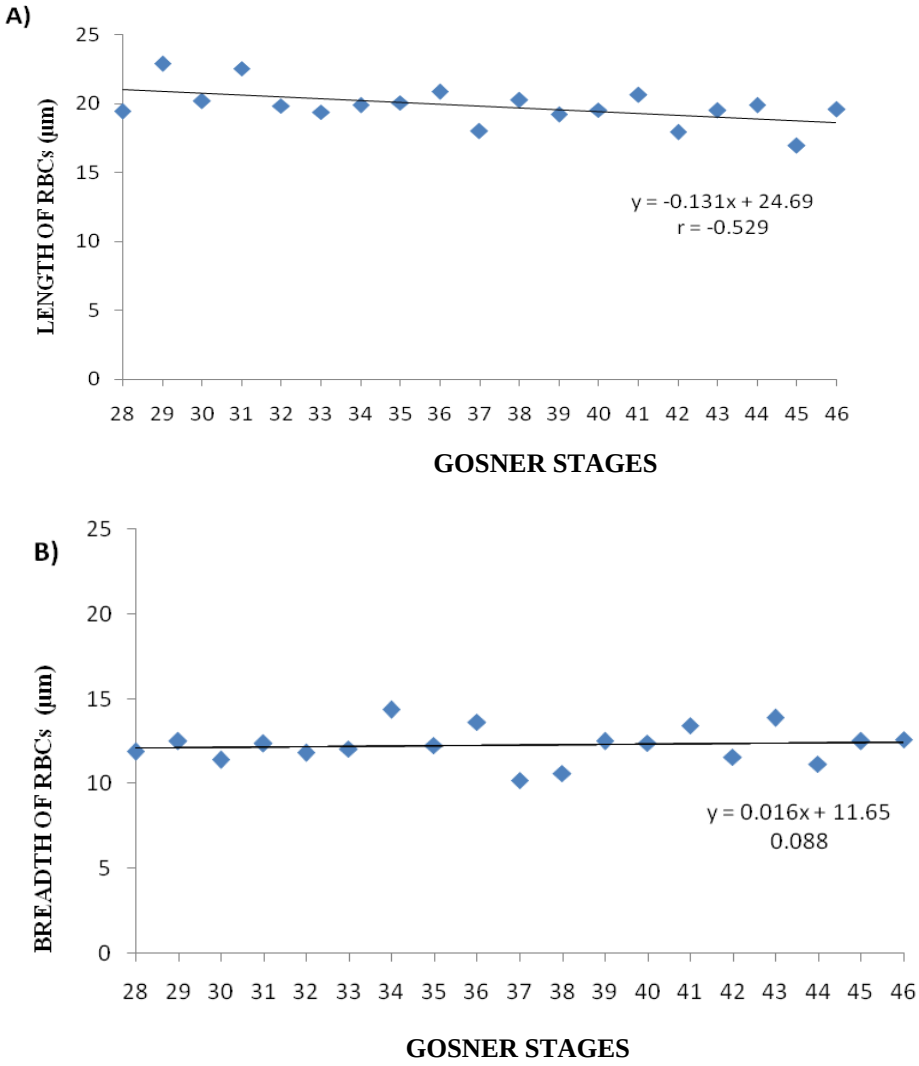

Fig. 6 (A) Correlation between different developmental stages and length of RBCs and (B) Correlation between different developmental stages and breadth of RBCs in the tadpoles of *D. melanostictus.*

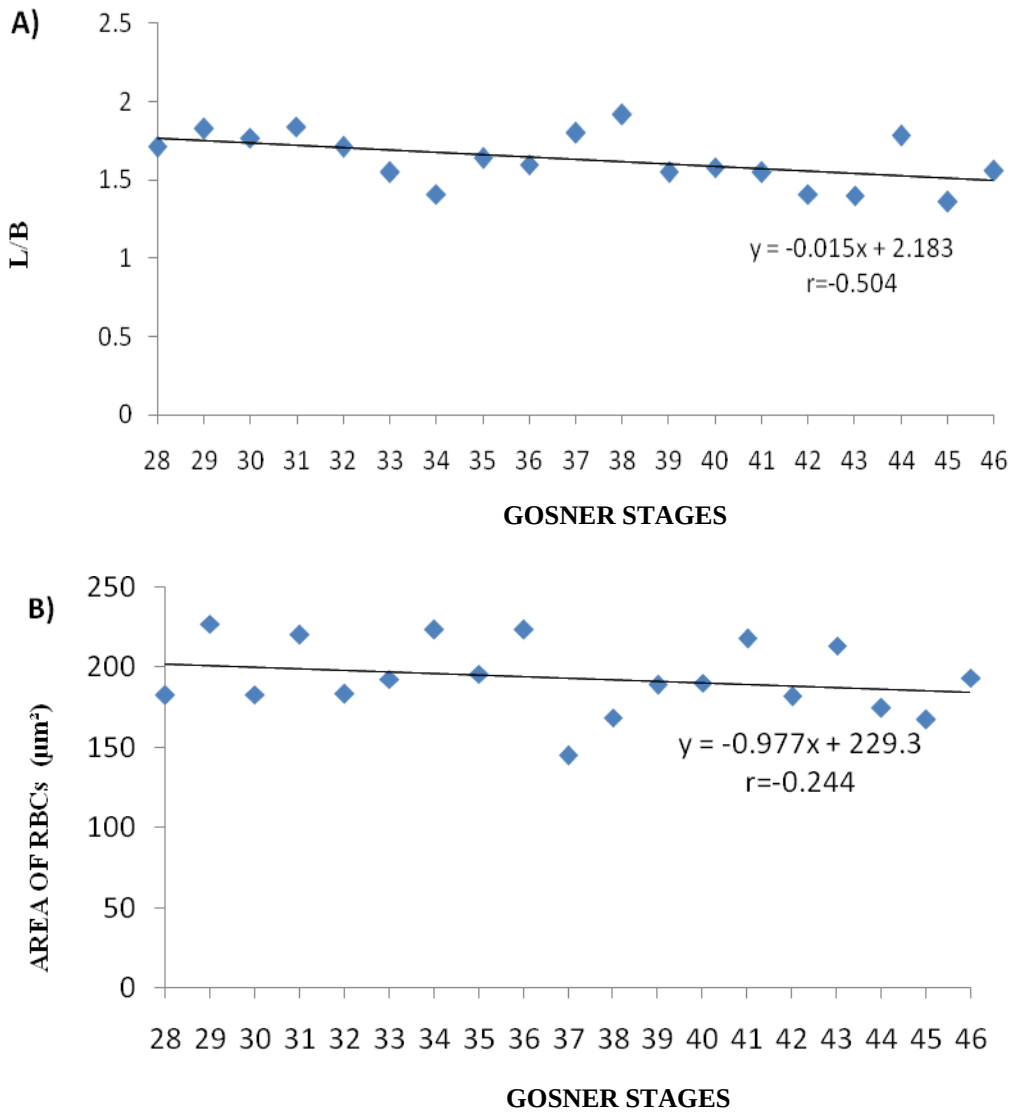

Fig. 7 (A) Correlation between different developmental stages and length: breadth of RBCs; (B) Correlation between different developmental stages and area of RBCs in the tadpoles of *D. melanostictus*.

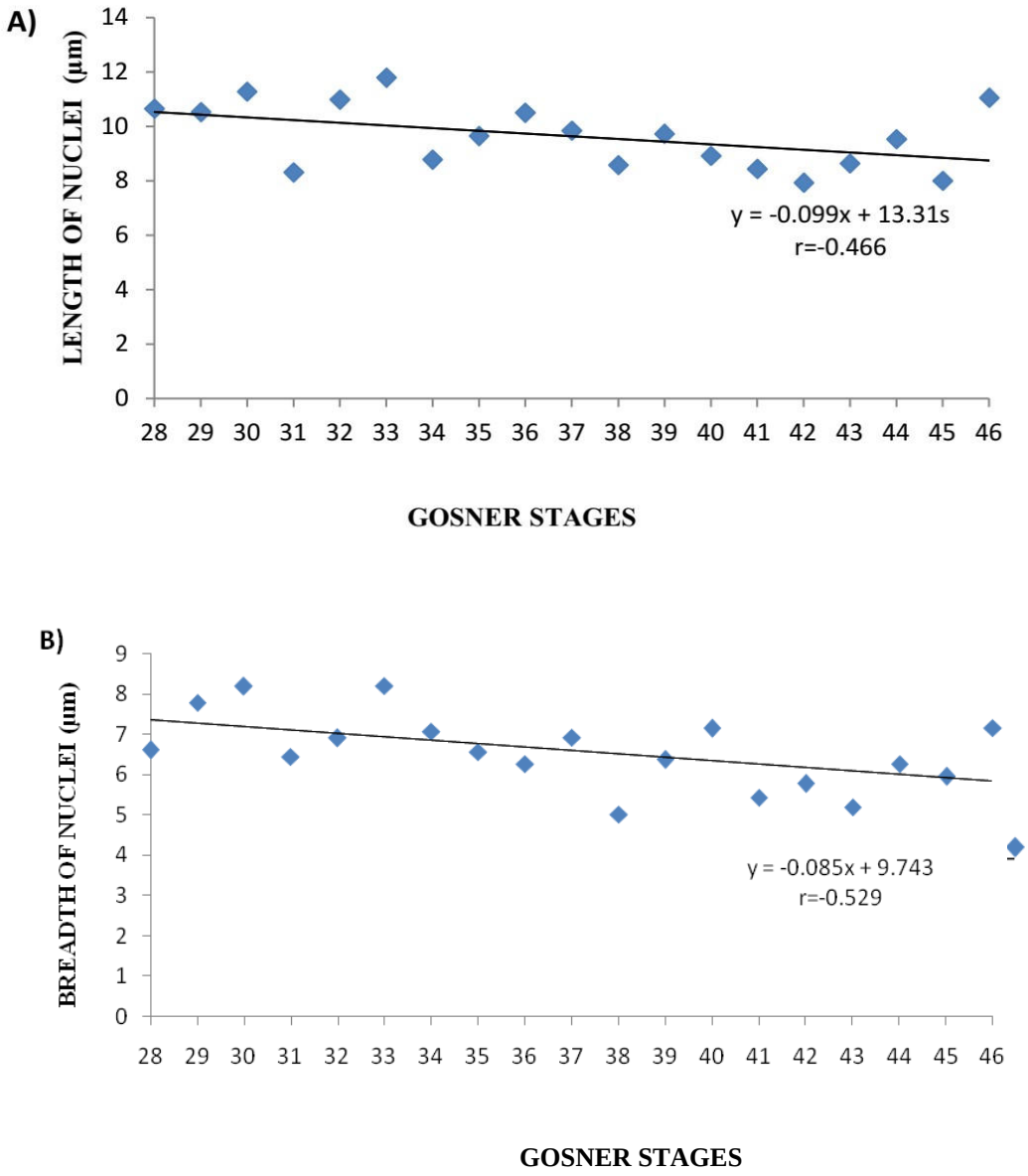

Fig. 8 (A) Correlation between different developmental stages and length of nuclei of RBCs; (B) Correlation between different developmental stages and breadth of nuclei of RBCs in the tadpoles of *D. melanostictus*.

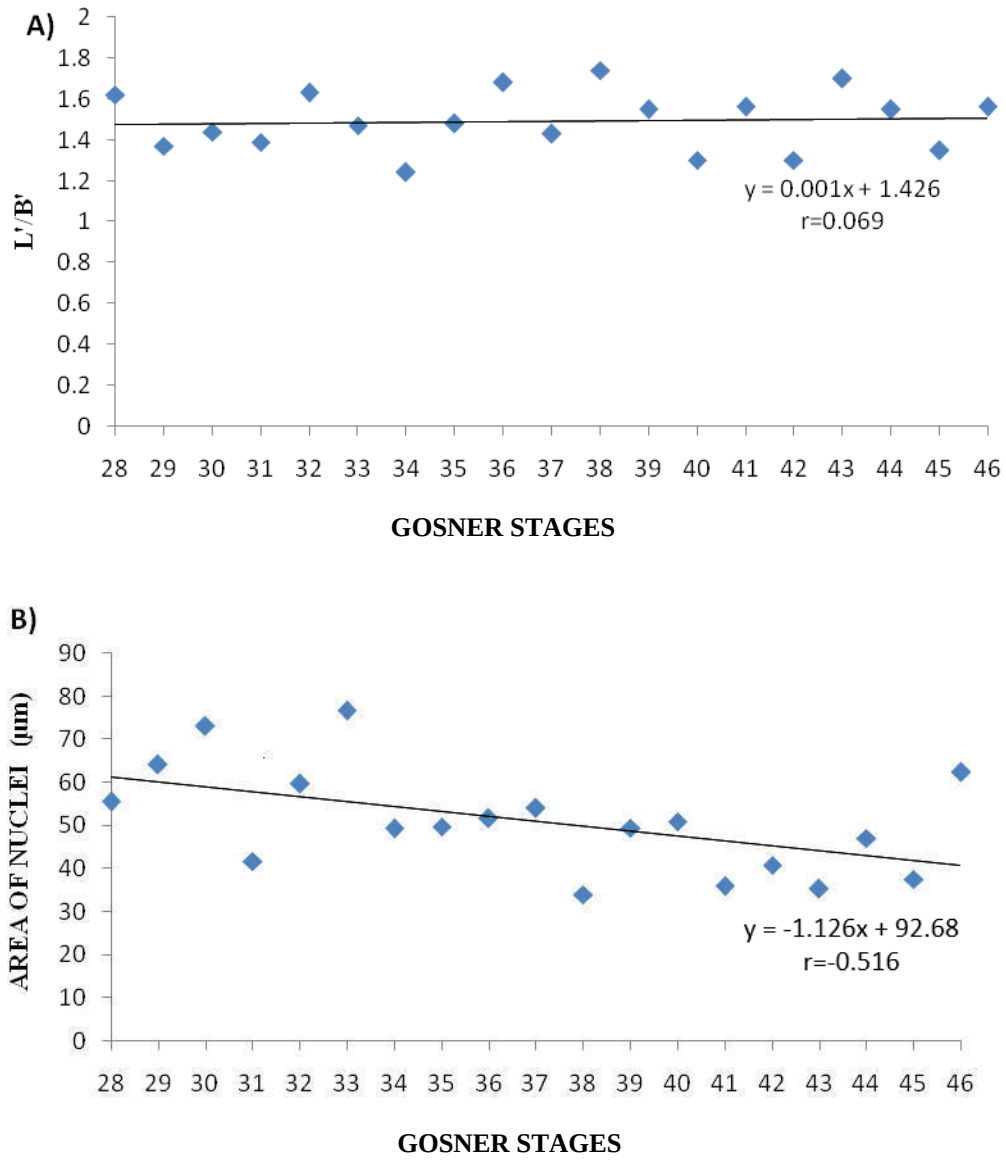

Fig. 9 (A) Correlation between different developmental stages and length: breadth of nuclei of RBCs; (B) Correlation between different developmental stages and area of nuclei of RBCs in the tadpoles of *D. melanostictus*.

Table 2: Leukocyte profile of different developmental stages of tadpoles of *Duttaphrynus melanostictus*

GOSNER STAGE	LYMPHOCYTE (%)	MONOCYTE (%)	EOSINOPHIL (%)	NEUTROPHIL (%)	BASOPHIL (%)
28	84	8	0	0	4
29	95	0	5	0	0
30	80	16	4	0	0
31	80	10	0	5	5
32	92	4	4	0	0
33	88	4	4	4	0
34	75	10	5	10	0
35	75	10	0	10	5
36	85	5	5	5	0
37	76	8	4	8	4
38	72	12	0	16	0
39	65	20	5	10	0
40	68	8	8	8	8
41	60	10	10	15	5
42	65	5	10	20	0
43	65	15	5	15	0
44	72	4	8	12	4
45	60	24	4	8	4
46	64	20	4	8	4
Mean (M)	74.78	10.36	4.47	7.68	1.84
Standard deviation (SD)	±10.61	±6.22	±3.06	±6.17	±2.24
Correlation coefficient (r)	-0.843	+0.445	+0.480	+0.852	+0.237

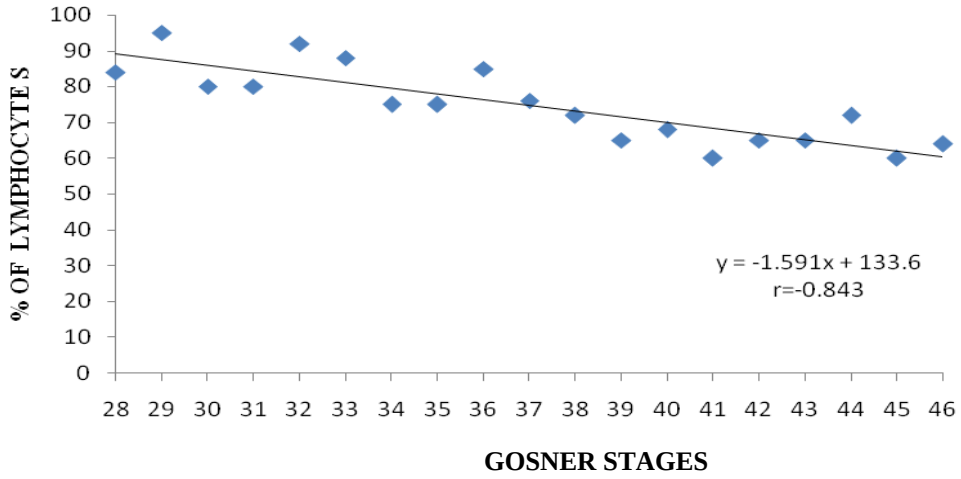

Fig. 10 Correlation between different developmental stages and percentage of lymphocytes in the tadpoles of *D. melanostictus.*

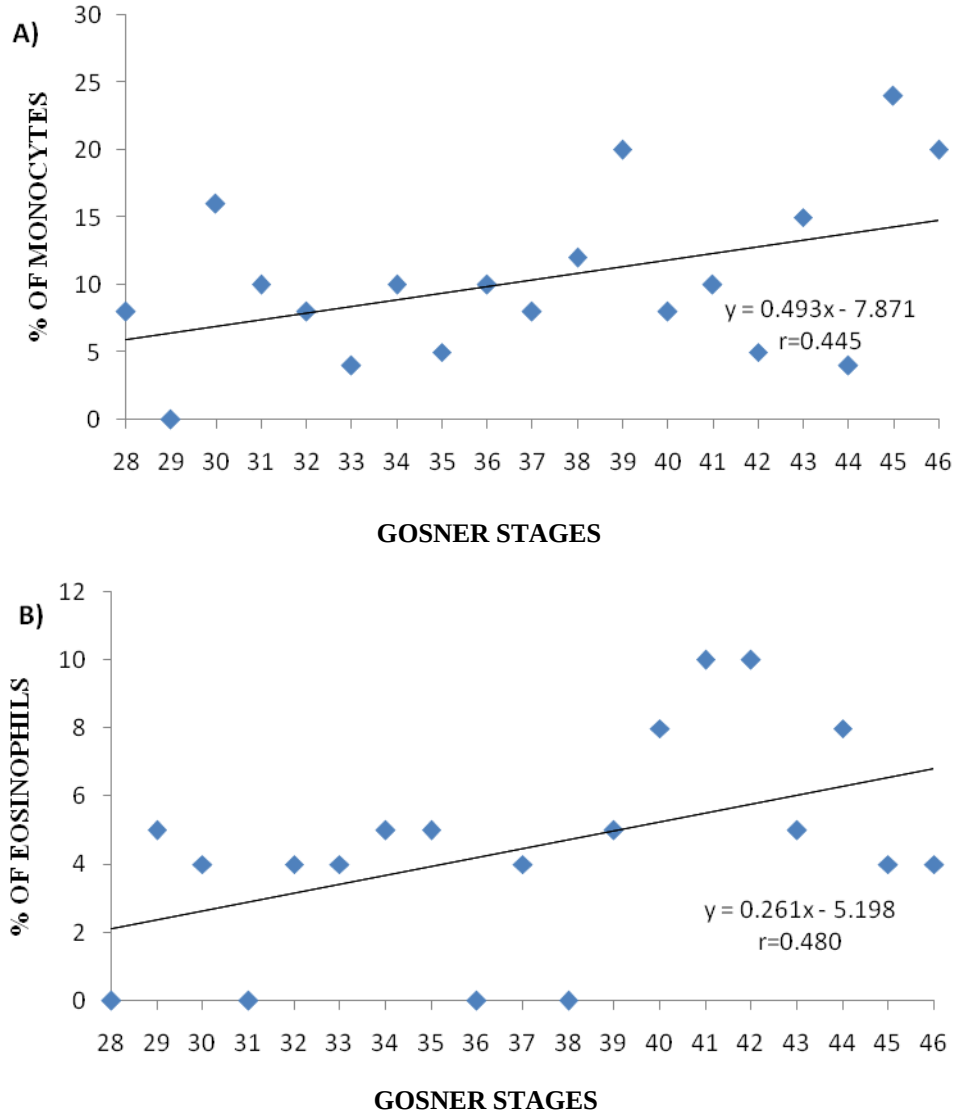

Fig. 11 (A) Correlation between different developmental stages and percentage of monocytes; (B) Correlation between different developmental stages and percentage of eosinophils in the tadpoles of *D. melanostictus*.

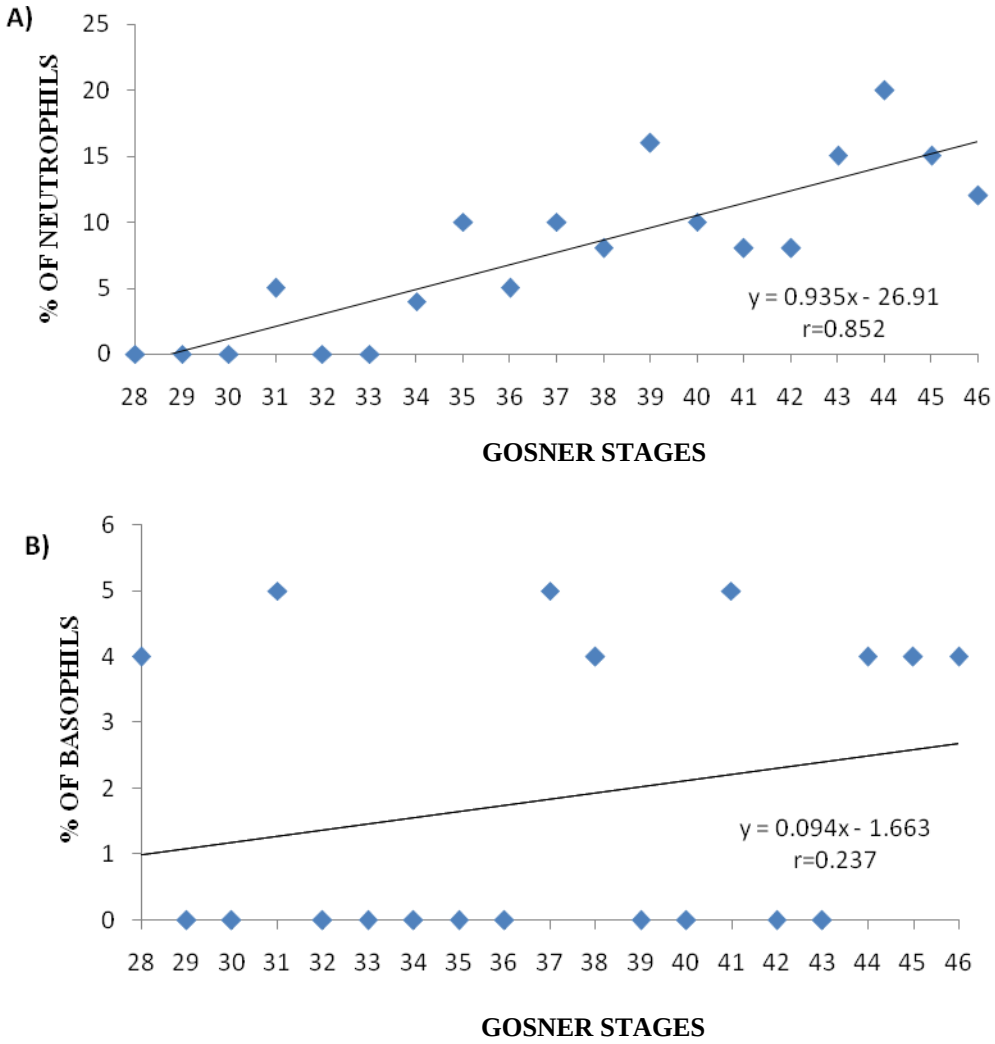

Fig. 12 (A) Correlation between different developmental stages and percentage of neutrophils; (B) Correlation between different developmental stages and percentage of basophils in the tadpoles of *D. melanostictus*.

DISCUSSION

Based on the present study it is evident that not only shape and size of RBCs but also numbers of WBCs change during development in tadpoles of *Duttaphrynus melanostictus*. The erythrocytes observed in the present study were mostly oval while round cells were found towards the later stages (Figs. 4A and B). The measurement of erythrocytes (L, B and L/B) supports the morphological observation (Figs. 6A, B and 7A). The L and L/B decreased with development with a negative correlation coefficient. Breadth of RBCs had a positive correlation with progress in development. This observation supports the previous findings, i.e. two general forms of erythrocytes (larval and adult forms) present in larval amphibians (Hollyfield, 1966; Benbassat, 1970; Broyles et al., 1981). The larval form is large and elongated while the adult form is smaller and rounder. The area of RBCs was negatively correlated (r= -0.244) with advancement of development. The maximum area of the erythrocyte in tadpoles of *Polypedates teraiensis* was observed to be 403.90±43.16µm (Gosner stage 27) (Das and Mahapatra, 2012). But, in the present study the maximum area of the erythrocyte was observed to be 226.81±48.7µm in stage 29 tadpoles (Table 1). Present study also showed a decrease in area towards the metamorphic climax with a negative correlation coefficint value, 'r'= -0.471. The nuclei of erythrocytes observed in this study were oval, centrally placed and their size decreased towards the metamorphic stages with a less change in their oval shape. The L' and B' were negatively correlated, but the L'/B' was positively correlated (Table 1). Variation in nuclei position i.e, from central to an eccentric position has reported in the RBCs of tadpoles of *Polypedates teraiensis* (Das and Mahapatra, 2012) and *Microhyla ornata* (Hota et al., 2013). But, such changes were not observed in general excepting few RBCs. Pokilocytosis was observed in stages 28-39 (Fig. 4F) and their disappearance during later stages was also a normal phenomenon. This was also observed in other anuran tadpoles (Firkin et al., 1989), which supports its universality. The abnormal shaped erythrocytes found were comma (Fig. 4C, stages 37-41) and spindle shaped (Fig. 4D stages 34-42). Other abnormal shaped erythrocytes i.e., several forms of tear drop, pencil shaped also documented in the tadpoles of *Polypedates teraiensis* (Das and Mahapatra, 2012) and *Microhyla ornata* (Hota et al., 2013), which were not found in this species. Rouleaux formation was found in the stages 39-44 (Fig. 4H). Dividing cells were found in all the stages investigated, indicating active role of these cells during the development. Erythrocytes undergoing degeneration and apoptosis were also observed (Fig. 4G).

Five types of leukocytes were observed in the present study as described earlier in other anuran species (Firkin et al., 1989). Lymphocytes were most abundant during the growth phase of larval development and appeared to have little association with metamorphic climax (Davis, 2009; Jordan and Speidel, 1922). In this study a similar observation was also found, that is, lymphocytes were found in large number during the growth phase and their percentage decreased towards end of metamorphosis. This observation is consistent with the data found in tadpoles of *P. teraiensis* (Das and Mahapatra, 2012) and *M. ornata* (Hota et al., 2013). Percentage of neutrophils remained positively correlated with respect to development. Hota et al. (2013) also founded similar observation in the tadpoles of *M. ornata*. Higher percentage of neutrophils in this species may be due to physiological condition as explained by Hota et al. (2013). Jordan and Speidel (1922,1924) believed that eosinophil produce substances that aided in the breakdown of tissues, thereby acting as a precursor to phagocytosis by monocytes. A positive correlation of percentage of monocyes was observed with progress in development. Elevation

in the percentage of monocytes is suggested to be due to increase in cellular debris during remodeling of larval structure. The percentage of eosinophils remained positively correlated with the progress in development. It is described that a number of chemicals are produced by eosinophil and these are known to play the function of initiation and modulation of the immune and inflammation process (Adamko et al., 2005; Rothenberg and Hogan, 2006). Thus, the rise in eosinophil number during metamorphosis is to modulate the lysis of tissue, which behaves in a way similar to the inflammation process. Eventhough there was fluctuation in the percentage of basophils, a positive correlation was evident between these cells and developmental stages. Davis (2009) suggested that increase in basophil number in *Rana catesbeiana* is due to their formation and entrance in circulation rather than a direct association with metamorphosis. Similar reason for increase in basophil number is suggested during development of this species.

CONCLUSION

The RBCs of earlier stages (28-42) tadpoles of *D. melanostictus* were oval while both oval and rounded RBCs were found during later stages (42-46). Different developmental stages showed a less number of varied form of erythrocyte i.e., comma shaped (37-41) and spindle shaped (34-42). Dividing cells and degenerating cells were found throughout development. The statistical study of different parameters of erythrocyte i.e., L, L/B, A, L' B' and A' showed a positive correlation coefficient with respect to different developmental stages. For B and L'/B' of RBC, the correlation coefficient was negative. The lymphocytes were abundant in early stages and their number decreases towards the climax stages with a correlation coefficient value 'r'= -0.843. Percentage of monocytes increased towards the metamorphic climax with the highest (24) at stage 45. Monocytes, eosinophils and neutrophils showed a positive correlation with respect to different developmental stages. Eventhough percentage of basophils fluctuated throughout development, a positive correlation was found between these cells and progress in development.

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ISSN 0970-4450