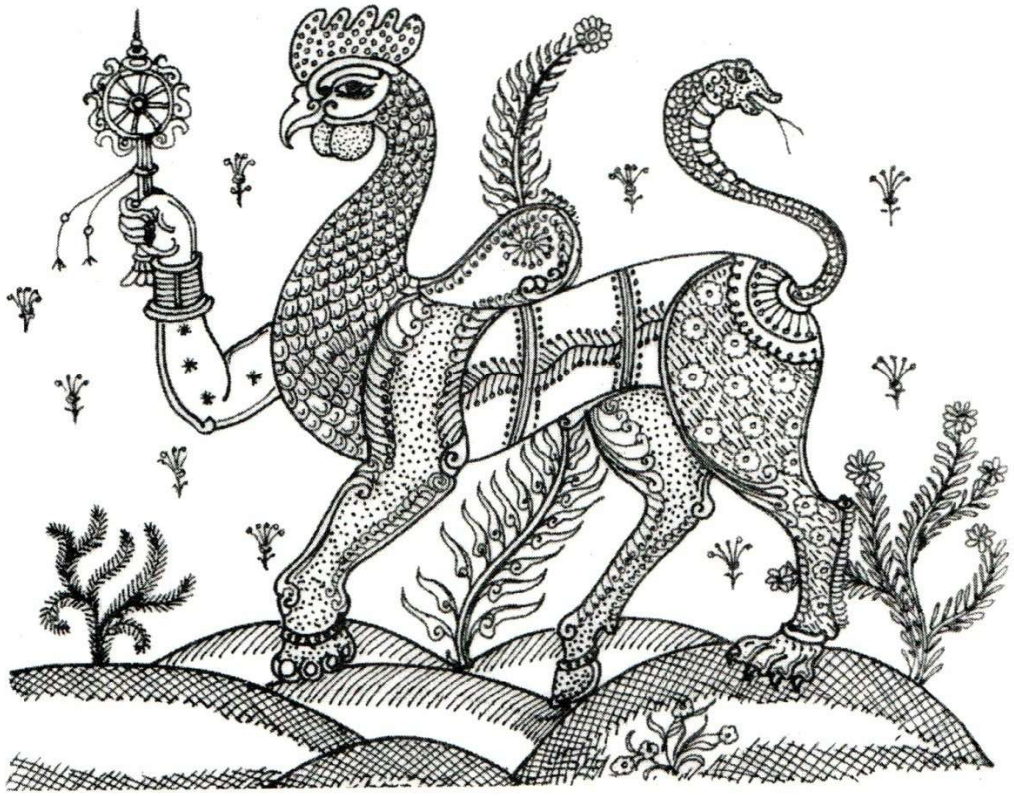
ISSN 0970-4450

PRANIKEE

Journal of Zoological Society of Orissa



Volume- XXX
2018

ISSN 0970-4450

PRANIKEE

Journal of Zoological Society of Orissa

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

Volume XXX, 2018

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

Zoological Society of Orissa has been attempting to provide effective communication among Zoologists through the publication of the Journal "PRANIKEE", which features the research work carried out by zoologists of the state and the country. The present edition of the journal (Volume XXX) contains five research papers. The first article describes Potential impact of climate change on the distribution of a frog species in Bhagirathi basin of Western Himalaya. Other research articles cover different aspects of zoological sciences including effects of indolylquinoline on functional fertility of male rats, cell proliferation during limb regeneration in tadpoles of Indian tree frog, applications of SDS-PAGE in differentiation of two earthworm genera, and feeding habits of the tadpoles of common Asian toad.

PK Mahapatra

PRANIKEE
Journal of Zoological Society of Orissa
Abbreviation: Pranikee
Volume 30, Year - 2018

Pranikee - Journal of Zoological Society of Orissa, ISSN 0970-4450
Volume XXX, 2018, pp 1-14

POTENTIAL IMPACT OF CLIMATE CHANGE ON THE DISTRIBUTION OF STOLICZKA'S TORRENT FROG, *NANORANA VICINA* IN BHAGIRATHI BASIN, WESTERN HIMALAYA.

Naitik Patel[1] and Abhijit Das[1*]

[1]Wildlife Institute of India, Chandrabani, Dehradun, Uttarakhand, India

*Corresponding author
Email: abhijit@wii.gov.in

ABSTRACT

We have used species distribution modeling to understand the potential impact of climate change on Stoliczka's Torrent Frog (*Nanorana vicina*). This frog is mid tohigh elevation dwelling species endemic to Western Himalaya. In this study, we have used bioclimatic variables combined with presence data of *N. vicina* to understand their current distribution and predict future distribution under the four pathways (RCP2.6, RCP4.5, RCP6 and RCP8.5) and two time periods (the 2050s and 2070s) for Bhagirathi River Basin. Suitable habitat of *N. vicina*en compasses 1006km^2 in the study area. Species distribution model suggest that global climate change will affect the suitable habitat area for *N. vicina* negatively in distant future (2070). It appears that suitable habitat of *N. vicina* will be reduced by 57% to 91% (492 km^2) in the Bhagirathi River Basin by 2070 under the global climate change, primarily due to the changes in temperature of the wettest quarter and precipitation. Future changes in temperature and precipitation may force *N. vicina* to move towards the higher elevation zone. Furthermore, suitable habitats need to be monitored for future monitoring.

Key Words: Amphibian, Species distribution modeling (SDM), Himalaya, Range shift

INTRODUCTION

Global amphibian decline is a prime concerns for the biodiversity crisis (Davidson et al., 2002).Amphibians are facing catastrophic population decline because of land use change (Hof et al., 2011), invasive species (Kats and Ferrer, 2003; Kenison et al., 2016), over exploitation (Stuart et al., 2004), infectious disease (Hof et al., 2011), and climate change (Pounds and Crump, 1994; Hof et al., 2011). Additionally, ubiquitous stream frogs likely to experience a greater peril owing to their specialized adaptation in mountain torrent streams (Yang, 1991). Such habitats are also more liable to get affected by climate change (Lui and Yang, 2000), and also by dam construction activities (Barinaga, 1990; Fei and Meng, 2005).

Stoliczka's Torrent Frog (*Nanorana vicina*) is endemic to Western Himalaya (Northern Pakistan, Kashmir, Himachal, Uttarakhand and Western Nepal) (Scheich and Kastle, 2002). This frog belongs to the family Dicroglossiineranoids. This group of frogs is thought to be evolved across Himalayan mountain chain (Bossuyt and Milinkovitch, 2001) which comprises three biodiversity hotspots (Myers, 2000).

N. vicina is a large growing species (>100g) adapted to a life in mountain streams. Females grow larger than males and are known to attain size up to 110mm. This stream breeding dicroglossidae frog is

uniquely adapted to fast flowing rocky mountain streams (Scheich and kastle, 2002). This species occurs in the elevation zone of 1500m to 3500m and likely to get affected by climate change (Patel and Das, 2015). High elevation amphibians are more vulnerable to climate change (Pounds et al., 1999). Some of the high elevation dwelling amphibian such as *Bufolatest* and *Scutiger occidentalis* has gone locally extinct from Ladakh, Western Himalaya (Vasudevan et al., 2002). Such study raises the concern for immediate monitoring and generating baseline information for high elevation amphibian species. *Namarari vicuna* has a high conservation value in Himalayan ecosystem (Rais et al., 2014). Although regarded as "Least Concerned", population of the species is assessed as "decreasing" fast in IUCN assessment (Ohler and Dutta, 2004). As many amhibian population declines have occurred in disturbed as well as in protected areas due to climate change and infectious. It is important to start identifying and mapping potential habitat for better management and conservation of the species (Guisan et al., 2013). We analyzed potential distribution and impact of climate change on *Nanorana vicina* by climate envelope modeling in Bhagirathi River Basin. Our study delineates the potential suitable habitat and climate change impact in study areaas first step towards its conservation measures.

METHODS

Study area

The study area lies in the catchment of Bhagirathi River in the Western Himalaya covering 7644 km^2, in the state of Uttarakhand, India (Fig. 1). The area is classified into different eco-climatic zones based on habitat types along an elevation gradient ranging from 500m-5000m (elevation 1500–2000 m). The forest type include tropical moist deciduous forest (below 1500 m), Himalayan sub-tropical forests Himalayan broadleaf forests (2000–2500 m), Himalayan conifer forest (2500–3000 m) and sub-alpine forest (3000–3600 m), moist alpine meadows (3600–5000 m) and alpine arid pastures or Desert Steppe (above 5000 m) (Rawat, 2017). Forest, agriculture and settlements are the major land use in this area (Nautiyal, 2010).

Species account

Nanorana vicina is a large sized frog(110mm) found in the Western Himalaya with an altitudinal range of 1500m to 3300m (Sathya kumar and Mathur, 2015) (Fig. 2). Characteristic features of the frog includes rounded finger and toe tips; prominent supra-tympanic fold; yellow to brown dark patches; on dorsal side of the body and small tubercles with spiny projections around vent (Scheich and Kastle, 2002).

Species occurrence data

We conducted nocturnal visual encounter survey (time constrained) at 91 plots along the elevation gradient from 500m to 4000m using Handheld GPS Garmin etrex 30 (Heyer and Heyer, 2014). We encountered *N. vicina* at 23 plots and for present data, we have used 16 locations after filtering clustered location (Fig. 1) (Norris, 2014).

Bioclimatic data

We used nineteen bioclimatic variables derived from www.worldclim.org (Hijmans et al., 2005). The baseline (current) climatic data used for running the models had a spatial resolution of 30 arc-seconds

(approximately 1 km) and was retrieved from the WorldClim for Bhagirathi River Basin. To determine the potential future distribution of the species under different climate scenarios, we used datasets of future climate using the RCPs (Representative Concentration Pathways) namely, RCP 2.6, RCP 4.5, RCP 6.0, and RCP 8.5 for both near future (2050s) and distant future (2070s) climate projections. Predictions from the Hadley Centre Global Environment Model version 2-Earth System (HadGEM2-ES) climate model was used for all four RCPs. HadGEM2-ES as an Earth system model, and it is preferred over other climate models since it simulates enhanced climate induced ecosystem and hydrological processes (Jones et al., 2011; Ahlstrom et al., 2012; Betts et al., 2015; Chakraborty et al., 2016).

Species Distribution modeling (SDM)

We used maximum entropy (MaxEnt 3.4.1) species distribution modeling (SDM; Phillips Steven and Dudík, 2008) to map the current and predicted future distribution of *Nanorana vicina* in the study area. MaxEntis a widely used tool for modeling species distributions using present data of species and various environmental parameters (Kramer Schadt et al., 2013). 13 presence records were used for training. 10000 points were used to determine the Maxent distribution (background points and presence points). MaxEnt uses Area under the receiver-operator curve (AUC) as a threshold, to predictive accuracy of the model (Merow et al., 2013). The generated output from MaxEnt estimates the habitat suitability (which is representative of a climatic niche) for species that generally varies from 0 (lowest) to 1 (highest) (Kumar et al., 2014). 10 percentile training presence Logistic threshold was used as threshold for suitable area (Norris, 2014). We calculated the area change to understand the range expansion or range contraction under different climate change scenarios (Duan, et al., 2016)

RESULTS

From 93 VES plots, a total of 23 plots were occupied by *N.vicina* within the elevation belt of 1500m to 3500m. The mean test data AUC > 0.95 for the Models (Table 2), was a good score for the model validation (Phillips et al., 2006). Distribution of *N.vicuna* in all scenarios is influenced by mean temperature of Wettest Quarter (BIO 8) variable with 41% contribution followed by Precipitation of Coldest Quarter (BIO 19) 36% , Annual Precipitation (BIO 12) 9% and Temperature Annual Range (BIO 7) 8%.

Future Potential distribution

Our study presents first current distribution and future climate prediction for *N.vicina* for Bhagirathi River Basin. In current scenario *N. vicina* occupy 13% areas (1006km^2) of the total area of Bhagirathi River Basin. The suitable area coverage for *N. vicina* in Bhagirathi basin shows decline for all future scenarios (Table. 3; Fig. 3). It appears to expand its range under near future 2050 climate change scenario with maximum increase 31% (1391km^2) in RCP 8.0 while three scenarios (4.5, 6.0, 8.0) of 2070 shows range contraction of *N.vicina* habitat in Bhagirathi basin. Area change will vary from -33.10 to +31.11% by the 2050 and from -91.58 to +11.13 by 2070.

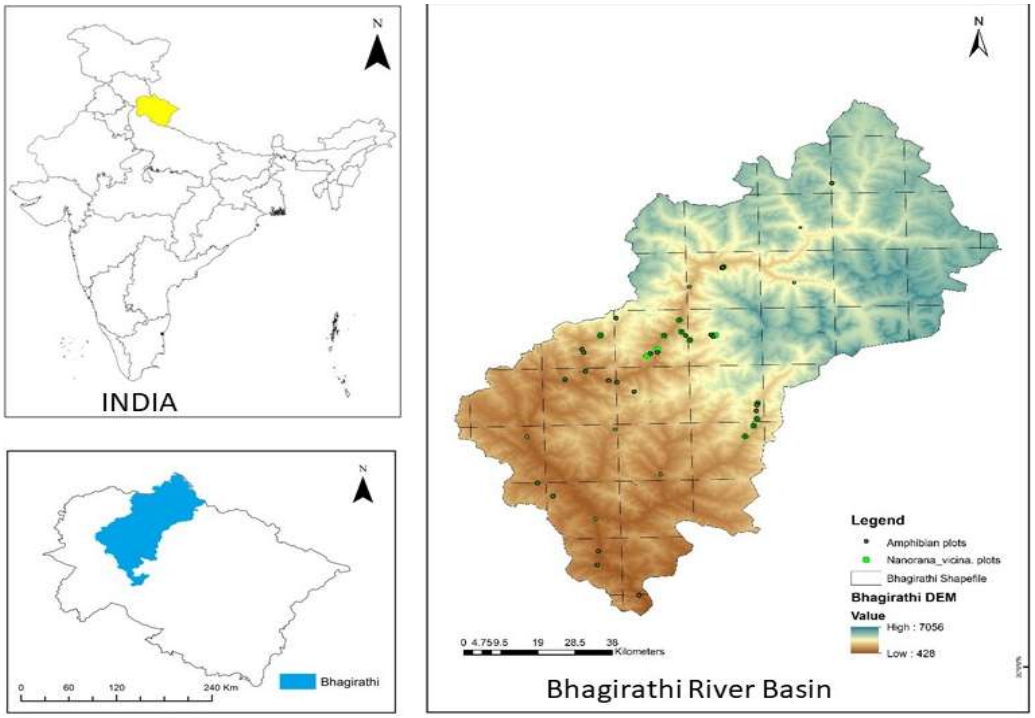


Fig. 1 Bhagirathi River Basin and presence location of *Nanorana vicina.*

Fig. 2: *Nanorana vicina* sitting on rock near torrent stream in Bhagirathi River Basin.

Table 1 Climatic variables used for modeling distribution.

Type	Variable Name	Code
Climatic	Annual Mean Temperature	BIO 1
Climatic	Mean Diurnal Range	BIO 2
Climatic	Isothermality (BIO 2/BIO 7) (*100)	BIO 3
Climatic	Temperature Seasonality	BIO 4
Climatic	Max Temperature of warmest Month	BIO 5
Climatic	Min Temperature of coldest Month	BIO 6
Climatic	Temperature Annual Range (BIO 5-BIO 6)	BIO 7
Climatic	Mean Temperature of Wettest Quarter	BIO 8
Climatic	Mean Temperature of Driest Quarter	BIO 9
Climatic	Mean Temperature of warmest Quarter	BIO 10
Climatic	Mean Temperature of Coldest Quarter	BIO 11
Climatic	Annual Precipitation	BIO 12
Climatic	Precipitation of Wettest Month	BIO 13
Climatic	Precipitation of Driest Month	BIO 14
Climatic	Precipitation seasonality	BIO 15
Climatic	Precipitation of Wettest Month	BIO 16
Climatic	Precipitation of Driest Quarter	BIO 17
Climatic	Precipitation of Warmest Quarter	BIO 18
Climatic	Precipitation of Coldest Quarter	BIO 19

Table 2 The percent contribution and permutation importance (in bold) of each climatic variable to the model building process and AUC value for each model.

Code	Current Scenario	RCP 2.6 2050	RCP 2.6 2070	RCP 4.5 2050	RCP 4.5 2070	RCP 6.0 2050	RCP 6.0 2070	RCP 8.0 2050	RCP 8.0 2070
BIO 1	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
BIO 2	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
	0.7	**0.7**	**0.7**	**0.7**	**0.7**	**0.7**	**0.7**	**0.7**	**0.7**
BIO 3	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
BIO 4	2.1	2.1	2.1	1.5	2.1	1.5	2.1	2.1	2.1
	0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
BIO 5	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
BIO 6	1.8	1.8	1.8	-	1.8	-	1.8	1.8	1.8
	0	**0**	**0**	-	**0**	**-**	**0**	**0**	**0**
BIO 7	8.3	8.3	8.3	7.8	8.3	7.8	8.3	8.3	8.3
	0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
BIO 8	41.4	41.4	41.4	44.3	41.4	44.3	41.4	41.4	41.4
	50.6	**50.6**	**50.6**	**46.2**	**50.6**	**46.2**	**50.6**	**50.6**	**50.6**
BIO 9	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
BIO 10	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-

BIO 11	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
BIO 12	8.9	8.9	8.9	8.6	8.9	8.6	8.9	8.9	8.9
	15.5	**15.5**	**15.5**	**16.9**	**15.5**	**16.9**	**15.5**	**15.5**	**15.5**
BIO 13	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
BIO 14	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
BIO 15	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
BIO 16	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
BIO 17	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
BIO 18	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
BIO 19	36.7	36.7	36.7	36.9	36.7	36.9	36.7	36.7	36.7
	33.2	**33.2**	**33.2**	**36.2**	**33.2**	**36.2**	**33.2**	**33.2**	**33.2**
AUC	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96

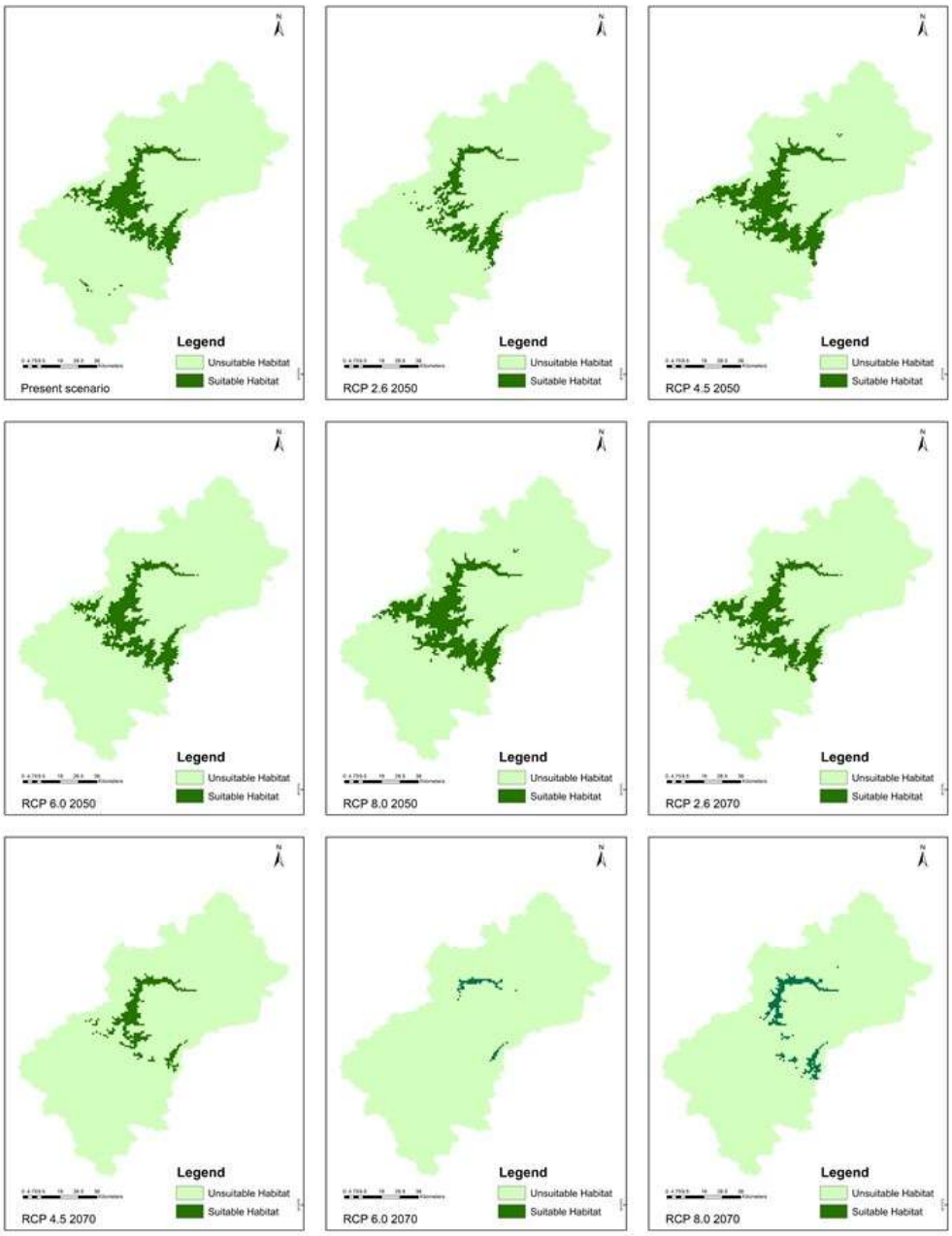


Fig. 3: Projection of suitable area for *Nanorana vicina* in Bhagirathi River Basin under current and future climate change scenarios.

Table 3 Suitable habitat for *N. vicina* under different Scenario

Scenario for predicting distribution of *N. vicina*	**Suitable Habitat in Bhagirathi River Basin**	**Area Change in future scenarios %**
Present Scenario	1006 km2	-
2.6 2050 CPIM5 HadGEM2-ES	673 km2	-33.1014
4.5 2050 CPIM5 HadGEM2-ES	1231 km2	22.36581
6.0 2050 CPIM5 HadGEM2-ES	983 km2	-2.28628
8.0 2050 CPIM5 HadGEM2-ES	1319 km2	31.11332
2.6 2070 CPIM5 HadGEM2-ES	1118 km2	11.1332
4.5 2070 CPIM5 HadGEM2-ES	431 km2	-57.1571
6.0 2070 CPIM5 HadGEM2-ES	81 km2	-91.9483
8.0 2070 CPIM5 HadGEM2-ES	316 km2	-68.5885

DISCUSSION

Impact of climate change has already been observed in Bhagirathi River Basin. There is an increase in temperature by 1.5° C in winters in this area since 1982 (Shrestha et al., 2012). Contraction of Gangotri glacier due to climate change is also reported in this region (Kulkarni and Karyakarte, 2014). However, there is no information available about impact of climate change on amphibians in this area. Furthermore, climate change-associated shifts in the distributions, body sizes and morphologies of amphibians in response to climate change have not been well studied (Yiming and Cohen, 2013).Therefore, here we provide the first insight into the climate change impact on *N. vicina* as a case study for amphibians in this region. Our results indicate that the suitable range of *N. vicina* would likely to shift to the higher elevation due to global climate change. There will be range expansion in near future scenarios 2050 (RCP

4.5, 6.0 and 8.0) as global climate change will provide advantage to amphibians with less intense winter (Araujo et al., 2006). However, distant future 2070 scenarios shows range contraction (-57% to -91%) in suitable habitat of *N.vicina* and range shift towards higher elevationas suggested by other studies (Mokhatla et al., 2015; Cunningham, 2016; Duan. Ren-Yan et al., 2016)

Although, modeling species distribution with respect to climate change is a complex process, bioclimatic envelope model can provide a useful first insight of such impacts (Pearson and Dawson, 2003). Most of the publications on climate change impact on species distribution reports the worst-case scenarios whereas impacts of such scenarios in reality are likely to be intermediate as multiple biotic and abiotic factors are governing species (Duan et al., 2016; Stegen et al., 2017). The movement of the species will also vary depending on their thermal tolerance limit and moisture requirement, which is a species specific parameter (Cunningham et al., 2016). Therefore, it is important to study the biology and life history traits of the species, that are likely to get affected by climate change such as change in body size (Sheridan et al., 2018), phenological modification (Gibbs and Breisch, 2001), early breeding (Beebee, 1995), expansion of fungal diseases (Rohr and Raffel, 2010) and in some cases extinction of the species (Pounds and Crump, 1994). As we have only used bioclimatic variables, our study provides the preliminary estimates of the climate change impact on *N. vicina* distribution.

It is difficult to address species distribution modeling of *N. vicina* at landscape level (Western Himalaya) as there are few published records available on amphibians of Western Himalaya (Patel and Das, 2016). Furthermore, there are taxonomic uncertainty about species identity from literature records about *N. vicina* (Rais et al., 2014). In many studies the taxonomic revisions have been questioned related to species identity and distribution records (Molur, 2008). Therefore, we have limited our study design to the Bhagirathi River Basin, as we were confident about species identity and its accurate geographic location. We need to increase our effort for generating baseline data for amphibian research in Western Himalaya to understand climate change impact on the distribution of *N. vicina*in that area. Presently, we have initiated with representation of potential distribution range and changes in the range with respect to global climate change. Present study is a starting point for the future research work on *N.vicina* and associated species. This information will be useful to discover new populations of *N.vicina*, and to identify survey sites for monitoring this species for more effective conservation.

ACKNOWLEDGMENTS

We acknowledge the financial assistance of the project from Department of Science and Technology, Govt. of India. We thank Uttarakhand forest department for proving study permission (UK/FD/702/5-6). We thank V.B. Mathur, G. S Rawat and S. Satyakumar of Wildlife Institute of India for facilitating research. We thank Bitupan, Preeti, Vishal and Pranoy for their support. We thank Jayant Borah, NeerajRawat, AnukulNath, Rupa Bhardwaj, Surya Sharma, Urjit Bhatt and Naresh Rawat for assisting in both on-field and off-field work.

REFERENCES

Ahlstrom A, Schurgers G, Arneth A, and Smith B (2012) Robustness and uncertainty in terrestrial ecosystem carbon response to CMIP5 climate change projections. *Environmental Research Letters*, 7(4):044008.

Araújo MB, Thuiller W and Pearson RG (2006) Climate warming and the decline of amphibians and reptiles in Europe. *Journal of biogeography*, 33(10):1712-1728.

Barinaga M (1990) Where have all the froggies gone? *Science*, 247(4946):1033-1035.

Beebee TJC (1995) Amphibian breeding and climate. *Nature* 374 (6519):219-220.

Betts RA, Golding N, Gonzalez P, Gornall J, Kahana R, Kay G and Wiltshire A (2015) Climate and land use change impacts on global terrestrial ecosystems and river flows in the HadGEM2-ES Earth system model using the representative concentration pathways. *Biogeosciences*, 12(5):1317-1338.

Bossuyt F and Milinkovitch MC (2001) Amphibians as indicators of early tertiary" out-of-India" dispersal of vertebrates. *Science*, 292(5514):93-95.

Chakraborty A, Joshi PK and Sachdeva K (2016) Predicting distribution of major forest tree species to potential impacts of climate change in the central Himalayan region. *Ecological Engineering*, 97:593-609.

Cunningham HR, Rissler LJ, Buckley LB and Urban MC (2016) Abiotic and biotic constraints across reptile and a00mphibian ranges. *Ecography*, 39(1):1-8.

Davidson C, Shaffer HB and Jennings MR (2002) Spatial tests of the pesticide drift, habitat destruction, UV-B, and climate-change hypotheses for California amphibian declines. *Conservation Biology*, 16(6):1588-1601.

Duan RY, Kong XQ, Huang MY, Varela S and Ji X (2016) The potential effects of climate change on amphibian distribution, range fragmentation and turnover in China. *PeerJ*, 4:e2185.

Fei L and Meng XL (2005) A Handbook of Recognizing Amphibians in China. Forestry Publishing House, Beijing, China.

Guisan A1, Tingley R, Baumgartner JB, Naujokaitis-Lewis I, Sutcliffe PR, Tulloch AI, Regan TJ, Brotons L, McDonald-Madden E, Mantyka-Pringle C, Martin TG, Rhodes JR, Maggini R, Setterfield SA, Elith J, Schwartz MW, Wintle BA, Broennimann O, Austin M, Ferrier S, Kearney MR, Possingham HP and Buckley YM (2013) Predicting species distributions for conservation decisions. *Ecology letters*, 16(12):1424-1435.

Gibbs JP and Breisch AR (2001) Climate warming and calling phenology of frogs near Ithaca, New York, 1900–1999. *Conservation Biology*, 15(4):1175-1178.

Heyer R, Donnelly MA, Foster M and Mcdiarmid R (Eds.) (2014) *Measuring and monitoring biological diversity: standard methods for amphibians*. Smithsonian Institution.

Hijmans RJ, Cameron SE, Parra JL, Jones PG and Jarvis A (2005) Very high resolution interpolated climate surfaces for global land areas. *International journal of climatology*, 25(15):1965-1978.

Hof C, Araújo MB, Jetz W and Rahbek C (2011) Additive threats from pathogens, climate and land-use change for global amphibian diversity. *Nature*, 480(7378):516-519.

Jones C and Coauthors D (2011) The HadGEM2-ES implementation of CMIP5 centennial simulations. *Geosci. Model Dev.*, 4:543–570, doi: 10.5194.

Kramer-Schadt S, Niedballa J, Pilgrim JD, Schröder B, Lindenborn J, Reinfelder V and Cheyne SM (2013) The importance of correcting for sampling bias in MaxEnt species distribution models. *Diversity and Distributions*, 19(11):1366-1379.

Kats LB and Ferrer RP (2003) Alien predators and amphibian declines: review of two decades of science and the transition to conservation. *Diversity and Distributions*, 9(2):99-110.

Kenison EK, Litt AR, Pilliod DS and McMahon TE (2016) Role of habitat complexity in predator–prey dynamics between an introduced fish and larval Long-toed Salamanders (*Ambystoma macrodactylum*). *Canadian Journal of Zoology*, 94(4):243-249.

Kulkarni AV and Karyakarte Y (2014) Observed changes in Himalayan glaciers. *Current Science*, 106(2):237-244.

Kumar S, Graham J, West AM and Evangelista PH (2014) Using district-level occurrences in MaxEnt for predicting the invasion potential of an exotic insect pest in India. *Computers and Electronics in Agriculture*, 103:55-62.

Liu W and Yang D (2000) A new species of Amolops (Anura: Ranidae) from Yunnan, China, with a discussion of karyological diversity in Amolops. *Herpetologica*, 56(2):231-238.

Merow C, Smith MJ and Silander Jr JA (2013) A practical guide to MaxEnt for modeling species distributions: what it does, and why inputs and settings matter. *Ecography*, 36(10):1058-1069.

Myers N, Mittermeier RA, Mittermeier CG, Da Fonseca GA and Kent J (2000) Biodiversity hotspots for conservation priorities. *Nature*, 403(6772):853-858.

Mokhatla MM, Rödder D and Measey GJ (2015) Assessing the effects of climate change on distributions of Cape Floristic Region amphibians. *South African Journal of Science*, 111(11-12):1-7.

Molur S (2008) South Asian amphibians: taxonomy, diversity and conservation status. *International Zoo Yearbook*, 42(1):143-157.

Nautiyal P (2010) Food chains of Ganga River ecosystems in the Himalayas. *Aquatic Ecosystem Health and Management*, 13(4):362-373.

Norris D (2014) Model thresholds are more important than presence location type: Understanding the distribution of lowland tapir (*Tapirus terrestris*) in a continuous Atlantic forest of southeast Brazil. *Tropical Conservation Science*, 7(3):529-547.

Ohler A and Dutta SK (2004) *Nanorana vicina*. The IUCN Red List of Threatened Species 2004: e.T58443A11781868. http://dx.doi.org/10.2305/IUCN.UK.2004.RLTS.T58443A11781868.en.

Pearson RG and Dawson TP (2003) Predicting the impacts of climate change on the distribution of species: are bioclimate envelope models useful? *Global ecology and biogeography*, 12(5):361-371.

Patel N and A Das (2016) Herpetofauna. (IN) Sathyakumar, S., (Ed.). Bibliography on the Fauna and Micro flora of the Indian Himalayan Region. ENVIS Bulletin: Wildlife and Protected Areas. Vol. 17. Wildlife Institute of India, Dehradun 248 002, India.128-157.

Phillips SJ, Anderson RP and Schapire RE (2006) Maximum entropy modeling of species geographic distributions. *Ecological modeling*, 190(3-4):231-259.

Phillips SJ and Dudík M (2008) Modeling of species distributions with Maxent: new extensions and a comprehensive evaluation. *Ecography*, 31(2):161-175.

Pounds JA and Crump ML (1994) Amphibian declines and climate disturbance: the case of the golden toad and the harlequin frog. *Conservation Biology*, 8(1):72-85.

Pounds JA, Fogden, MP and Campbell JH (1999) Biological response to climate change on a tropical mountain. *Nature*, 398(6728):611.

Rawat GS (2017) The Himalayan Vegetation along Horizontal and Vertical Gradients. In Prins, H.T. & T. Namgail (EDs). *Bird Migration across the Himalayas: Wetland Functioning amidst Mountains and Glaciers*, Cambridge Universiry Press, pp189-204.

Rais M, Abbassi S, Batool T, Jilani MJ, Assadi MA, Mubarak H and Baloch S (2014) A note on recapture of *Nanorana vicina* (Anura: Amphibia) from Murree, Pakistan. *Journal of Animal and Plant Sciences*, 24:455-458.

Rohr JR and Raffel TR (2010) Linking global climate and temperature variability to widespread amphibian declines putatively caused by disease. *Proceedings of the National Academy of Sciences*, 107(18):8269-8274.

Sathyakumar S and Mathur VB (Eds.) (2015) Wildlife watch- Series I.A user guide for monitoring wildlife species in the Indian Himalayan Region. Wildlife Institute of India, Dehradun. Pp 64.

Schleich HH and W Kästle (2002) Amphibians and reptiles of Nepal. Biology, Systematics, Field Guide. Koeltz Scientific Books. Koenigstein, Germany.

Sheridan JA, Caruso NM, Apodaca JJ and Rissler LJ (2018) Shifts in frog size and phenology: Testing predictions of climate change on a widespread anuran using data from prior to rapid climate warming. *Ecology and evolution*, 8(2):1316-1327.

Shrestha UB, Gautam S and Bawa KS (2012) Wide spread climate change in the Himalayas and associated changes in local ecosystems. *PLoS One*, 7(5):e36741.

Stegen G, Pasmans F, Schmidt BR, Rouffaer LO, Van Praet S, Schaub M and Haesebrouck F (2017) Drivers of salamander extirpation mediated by *Batrachochytrium salamandrivorans*. *Nature*, 544(7650):353-356.

Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues AS, Fischman DL and Waller RW (2004) Status and trends of amphibian declines and extinctions worldwide. *Science*, 306(5702):1783-1786.

Vasudevan KSP, Ganapathy C and Nadu T (2002) Amphibians in Ladakh: evidences and hypotheses on causal factors. Frog leg, *Newsletter of the Declining Amphibian Populations; Task Force-South Asia* No.10.

Yang DT (1991) Phylogenetic systematics of the Amolops group of ranid frogs of Southeast Asia and the Greater Sunda Islands. *Fieldiana:* Zoology, New Series, 63:1-42.

Yiming LI and Cohen JM (2013) Review and synthesis of the effects of climate change on amphibians. *Integrative Zoology*, 8(2):145-161.

Pranikee - Journal of Zoological Society of Orissa, ISSN 0970-4450
Volume XXX, 2018, pp 15-28

MODULATORY EFFECTS OF INDOLYL QUINOLINE ON FUNCTIONAL FERTILITY OF MALE RATS (*RATTUS RATTUS)*

SK Bhowal[1] and CR Sahu [2*]

[1] Post Graduate Department of Zoology, Maulana Azad College, Kolkata- 700013, India.
[2]Department of Zoology, University of Kalyani, WB – 741235, India.

*Corresponding Author
E mail: crsahu344@gmail.com

ABSTRACT

Functional fertility of indolyl quinoline 2-(2″-chloroacetamidobenzyl)-3-(3′-indolyl quinoline) was evaluated in male rats (*Rattus rattus*) treated by oral gavages for 12 weeks followed by mating exposures to proven proestrus females. Fertility rate, percentage of pregnancy and some biochemical parameters were assessed in the model. Further, male steroid hormone testosterone and serum gonadotropin (FSH and LH) levels were also evaluated to test the efficacy of the compound. Routine histological analysis of epididymis and testis were considered to examine any discrepancy arises out of the effect of the compound. Functional fertility in treated animals was decreased significantly as was evidenced by reduction in the fertility and pregnancy rates in the female rats which were mated with treated male rats. Level of testosterone hormone, protein, glycogen content as well as epididymal glycerophosphorylcholin (GPC) decreased whereas concentration of LH and FSH, alkaline phosphatase and cholesterol increased significantly. Histological examination revealed depletion in the number of different germinal cells, disruptions both in epididymal and testicular architecture as well as scanty spermatozoa. The results were discussed in the light of severity of the histological and biochemical responses made by the compound. The significant results revealed from the study indicate infertility activity of the said chemical agent on male rats.

Keywords: Male rat, indolyl quinoline, male reproductive rgans, infertility, gonadotropin, biochemical parameters, histological analysis, glycerophosphorylcholin.

Abbreviations: GnRH: Gonadotropic-Releasing Hormone; SRL: Sisco Research Laboratory; mL: milli litre; ANOVA: Analysis of Variance; NS: Not Significant; GPC: Glycerophosphorylcholine

INTRODUCTION

To fight the grave situation of population explosion in countries like India, effective family planning is need of the hour. A significant step towards this is the fertility management by means of contraception (Allag and Rangari, 2002). Though some extremely successful contraceptive modalities have been developed for females, yet no or very modest effective method of systemic contraception has been worked out for males till now. An array of synthetic chemicals and plant derived compounds in addition to the infertility inducing methods of hormonal, chemical and immunological approach have been explored for a long time (Gottwald et al., 2006, Kopf, 2008, Etribi et al., 1982, Vannucchi et al., 2015). Use of hormonal steroids or gonadotropic-releasing hormone (GnRh) agonists/ antagonists causing acute oligospermia or azoospermia is under investigation (Paulsen et al., 1994, Edwards et al., 2013). But these methods affect either secondary sex characters in male or are not cost effective. On the other hand a

number of nitrogen heterocyclic compounds derived from indole have been explored for their anti flagellated activities. Imidazole, carbazole, indazole, ornidazole are examples of such compounds. They also demonstrated anti proliferative as well as antispermatogenic activity or *vice versa* (Grima et al., 2001). The imidazole group of compounds which are clinically used in treatment against flagellated protozoa and anaerobic bacteria for genital tract infections of both men and women have also been experimentally demonstrated to possess potential antifertility activity in male rat (Lohiya et al., 2005, Gupta et al., 2011). Meanwhile, antispermatogenic and antisteriodogenic properties of some traditionally used contraceptive agents have also been reported (Corsi et al., 1976, Silvestrini et al., 1984). Other allied compounds have been reported as anti-spermatogenic (Dhanapal et al., 2012, Mishra and Singh, 2009, Singla et al., 2013). So exploring effective anti-spermatogenic agents that could rely on alternative methods to the hypothalamo-pituitary gonadal axis may appear to become effective in the development of male systemic contraceptive. The compound 2-(2″-chloroacetamidobenzyl) - 3-(3′-indolyl) quinoline or indolyl quinoline contains indole, and quinoline moieties that match with all of the mentioned compounds structurally and functionally (Cooper et al., 1997, Grima et al., 2001, Bone et al., 2000, Chakrabarty et al., 1999). The above information was the main thrust behind this effort of evaluating modulatory effects of the compound on the functional fertility in male rats.

MATERIALS AND METHODS

Chemicals:

Analytical grade chemicals and reagents were purchased from Merck India Ltd. Staining reagents were from SRL India Ltd., Mumbai, India. The tested chemical compound has been synthesised through the application of Friedel-Crafts reaction (Mahato et al., 1994). The purity of the compound was verified and the bioactivity was determined (Bhowal et al., 2008).

Animals:

All experiments on laboratory animals were performed as per the guidelines of the animal ethics committee of the University. Rats (*Rattus rattus*) from random bred colonies were housed on a fourteen hours light and ten hours dark cycle under standard husbandry conditions (temp. 22^0 C ± 2^0 C, relative humidity 55 % ± 5%) and were provided standard pellet food and sterile water *ad libitum.*

Design of experiment, mating and fertility tests

Eight different groups consisting of 5 animals in each group were allocated from a population of 80 male rats, where 50% of the total was considered as control without any treatment. The test material was orally gavaged to different groups of rats for 2, 4, 8 and 12 weeks at a dose of 250 mg / kg of body weight (5g/kg per day did not cause lethality, (unpublished observation). Mating schedules were designed to examine the infertility effect by the compound accurately as well as monitoring their health and weight throughout the experimental period. The males were kept in a cage with the cyclic females (at pro estrous stage) as 1:1 ratio to observe any copulation plug or the presence of spermatozoa in the vaginal smear on the next morning and in successful mating it was considered as Day -1 of pregnancy. Accordingly, the percentage of successful mating was considered with the number of sperm positive females per

number of exposed female. The so called successful mating females were separated on next morning while food and water was given *ad libitum.*

On day 10 of gestation, laparotomy of female rats was performed under ether anesthesia (through inhalation) and their ovaries were excised, uteri were exposed including counting of corpora lutea, implantation sites and number of normal live fetuses was done. Fertility rates were calculated by the percentage of implantation sites per number corpora lutea (representing the number of eggs ovulated) and pregnancy rate was considered to be the number of viable fetuses per female mated.

Histology of testis and epididymis

The tunica albugenia of one of the two testes was nicked at both the ends after immersing in Bouin's fixative for 12 hours and continued the fixation process for another 12 hours. Epididymides of five rats per group were fixed in Bouin's fixative. Tissues were dehydrated in ethanol following several changes in upgrades of alcohol, thereafter, embedded in paraffin for section cuttings (6-µm thick) and microscopic studies. The tissue slides were processed for staining through eosin-hematoxylin and finally photomicrographs were taken.

Estimation of Testosterone hormone

Testosterone was quantitated by Radio Immuno Assay (RIA) using the commercial Coat-A-Count total testosterone RIA kit. There was 3.3% cross-reactivity of the anti-sera with dihydrotestosterone. The sensitivity of the assay was found to be 4 ng/dl (0.14 nmol/L) and that of intra-assay coefficient of variation was 4.85%. The study samples were assayed in duplicate.

Estimation of FSH and LH

Concentrations of serum FSH and LH were estimated by RIA with the help of second antibody precipitation. Carrier-free I125 was used for iodination of hormone. Pure rat FSH (NIDDK-FSH-1-9) and LH (NIDDK-rLH-1-9) has been iodinated using chloramine- T (Greenwood et al., 1963). NIDDK-anti-rFSH-S-11 and anti-rLH-S-11 anti-sera were used at a final tube dilution of 1:125,000, 1:750,000 and 1:437,500 or greater, respectively. The sensitivities of the assays have been found to be 1 ng/ml for FSH and 0.75 ng/ml for LH. The intra-assay coefficients of variation for all assays were <10%. Hormone concentrations were expressed in terms of NIDDK reference preparations, RP-2 in the case of rFSH and RP-3 in the case of rLH.

Biochemical profiles

For estimation of protein, testicular tissue (100 mg/test) was homogenized in 2ml ice cold 0.02 mol/L Tris-HCl buffer (pH 7.8), centrifuged at 800×g for 10 min at 4°C. Protein concentration was estimated from the precipitate which was collected at 4°C from the supernatant with 2 ml of 10% (w/v) trichloro acetic acid. The precipitate was dried, dissolved in 1 N NaOH solution and was estimated spectrophotometrically at 660 nm (Lowry et al., 1951).

Homogenized testicular tissue in 2 mL of ice cold 0.02 mol/L Tris-HCl buffer (pH 9.0) was used for estimation of alkaline phosphatase concentration. The homogenate was centrifuged at 800×g for 10 min at 4°C. The supernatant was taken for estimation at 420 nm spectrophotometrically by paranitrophenol phosphate method (Chakraborty and Lala, 1998).

Cholesterol was extracted from testicular tissue (100 mg) homogenizing in 2 ml of glacial acetic acid; after centrifugation the supernatant fraction was taken for cholesterol estimation spectrophotometrically at 626 nm by the ferric chloride method (Chakraborty and Lala, 1998).

Glycogen was extracted from 100 mg of testicular tissue dissolved in 30% (w/v) potassium hydroxide solution in a boiling water bath. The solution was then cooled, glycogen precipitated with 2 volumes of ethanol, centrifuged, washed with ethanol and finally dried on a water bath. The precipitate was dissolved in 1 mL distilled water. The resultant solution was filtered through glass wool to remove extraneous polysaccharides. From this solution, glycogen was estimated spectrophotometrically at 490 nm using 80% (v/v) phenol and concentrated sulphuric acid (Chakraborty and Lala, 1998).

Glycerophosphorylcholine (GPC) was estimated from epididymis. One of the two epididymis was quickly placed in icecoldnormal saline 0.9% (w/v) and was used for extraction of GPC. Spermatozoa were taken out through puncture of the tubules with a needle and the epididymis was then chopped with a fine scissors and finally mixed in 2 ml normal saline, centrifuged at 550×g for 10 min at 4ºC. The supernatant was oxidized to formaldehyde by periodate. The formaldehyde was quantitatively converted to a purple colour compound by adding chromotropic acid and then measured spectrophotometrically at 580 nm (Chakraborty and Lala, 1998).

Statistical analysis

Results were expressed as mean ± standard deviation of mean of five repeated determination for 5 rats in each of the 4 groups of rats. The significance of difference in the mean data obtained were analysed and were compared using one-factor ANOVA. Values were considered significant when $P<0.05$ or less.

RESULTS

Effects on fertility status of the rats

The fertility status of the female rats mated with a normal male rat not underwent any treatment showed a normal implantation site (Fig.1 A). A significant reduction related to time of exposure in functional fertility as indicated by rate of pregnancy was noticed due to the introduction of the compound. The female rats mated with the compound treated male rats during treatment period showed a decreased numbers of implantation sites from 5.20 to 0.0 (Figs.1 B, C) whereas the females mated with control males showed average number of implantation site from 13.6 to 14.0 (Fig.1A). While the pregnancy rate for the control varied from 2.72 to 2.80 (Table 1) during the experiment period, the treated group of rats showed a value ranges between 1.04 and 0.0 in 12 weeks treatment period. Thus, pregnancy rate of treated female animals showed decreased value compared to control group animals (Figs. 4 A, B). However, there is a very little change in percentage of mating.

Effects on testicular histology

The section of the testis of control rats showed seminiferous tubules of variable size with spermatocytes in the lumen at different stages of maturation (Fig. 2 A). The cellular interstitial connective tissue was prominent. A few blood vessels were also visible in the interstitial region. In contrast to the control the section of the testis of compound treated male rats demonstrate a structural disorganization of the seminiferous tubules along with loss in number of differentiating germinal cells in 8 weeks of treatment (Fig. 2B). In addition to, hyperpycnotic nuclei in many cells and vacuolated cytoplasm were prominent features encountered in the treated section of the testis during 8 and 12 week. The tubules were found to be devoid of spermatozoa. However, no anomalies in the morphology of sperm were noticed.

Effects on epididymal histology

The epididymis in the section of the control groups revealed a lumen with normal cellular structure containing basal cells. The lumen also contains mature sperm cells (Fig. 3A). The treatment with the compound after 3 weeks revealed slightly smaller tubular diameters along with thicker tubular epithelium. Few spermatozoa were observed in treated animals compared to the controls. The degree of effects was very pronounced during 8 to 12 weeks (Fig. 3B).

Effects on hormonal profile

The effects of administration of the compound to male rats on the concentrations of reproductive hormone levels are shown in Fig 5. Serum FSH and LH increased significantly during the experimental period. On the other hand testosterone level declined significantly and the percentage of reduction is shown in Table 2 and Fig.5. Percentage of increase in FSH and LH concentration is also demonstrated in Table 2 and Fig. 5.

Effects on biochemical profile

The effects of treatment with the compound on the testicular and epididymal biochemistry of male rats are shown in Table 2. On treatment with the compound testicular cholesterol levels increased significantly (Fig. 7). There was decline in total protein concentration and increase in concentration of testicular alkaline phosphatase (Fig. 6). On the other hand reduction in testicular glycogen and epididymal GPC levels (Fig. 7) was observed. Percentages of change in other biochemical parameters of protein concentration and concentration of testicular alkaline phosphatase are given in Fig. 6 where as percentages of change in other biochemical parameters such as testicular cholesterol, glycogen and epididymal GPC concentration are given in Fig. 7.

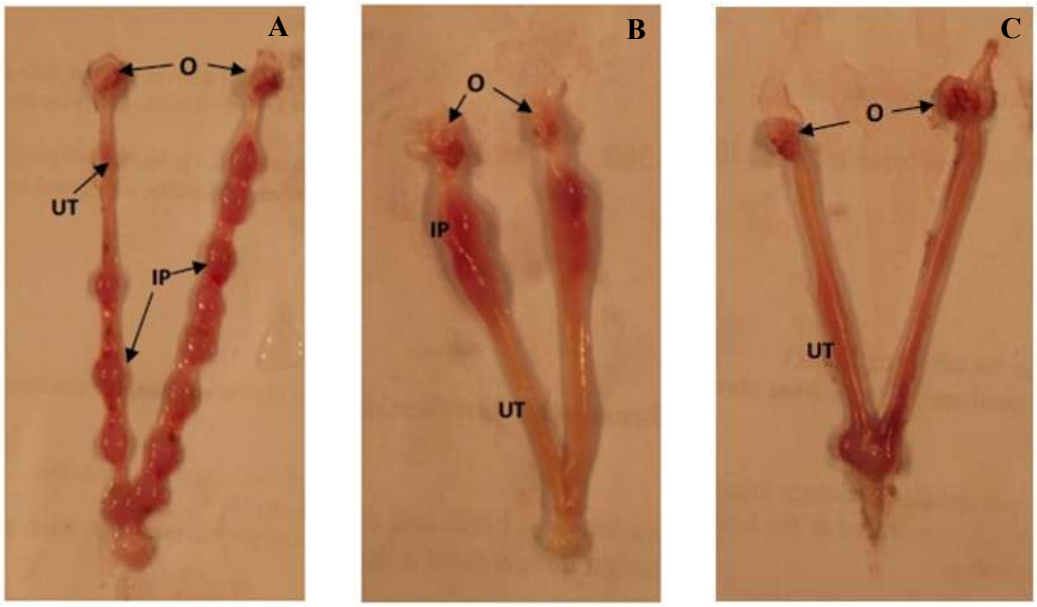


Fig. 1 (A) Normal implantation site in a normal female mated with normal male rat, (B) More reduction of implant site in a normal female mated with 4 weeks Indolyl quinoline treated male rat, (C) Few of implant sites in a normal female mated with 12 weeks indolyl Quinoline treated male rat. (IP: implantation site, O: ovary, UT: uterine tube)

Table 1 Percentage of pregnancy, number of implantation site, number of corpus luteum, fertility rate and pregnancy rate of female rats mated with male treated with indolyl quinoline at a dose of 250 mg / kg per day for 2, 4, 8 and 12 weeks.

Parameters	Experimental Groups	Treatment period			
		2 weeks	4 weeks	8 weeks	12 weeks
Percentage (%) of mating	Control	100	100	100	100
	Treated	100	75	75	75
No. of implantation sites (IP)	Control	13.6 ± 1.18	11.6 ±1.14	13.6 ±1.94	14.0 ± 1.58
	Treated	5.20 ±0.83	2.60 ±0.54	0.80 ±0.83	0.00±0.00
No. of corpus luteum (CL)	Control	13.9 ± 1.02	13.2 ± 0.90	14.5±1.23	15.3±1.24
	Treated	13.2 ± 1.03	12.2 ± 1.15	11.4±0.81	10.6±0.72*
Fertility rate	Control	97.84± 5.33	87.87± 6.17	93.79± 5.27	91.50± 8.02
	Treated	39.39± 2.01	21.31± 1.88	7.01± 0.24*	0
Pregnancy rate	Control	2.72± 0.16	2.64± 0.25	2.72± 0.19	2.80± 0.12
	Treated	1.04± 0.05*	0.65± 0.03	0.20± 0.01	0

Each value is expressed as the mean ± SD (n = 5 per group). Results were statistically analyzed with one-way ANOVA and compared with the control group. *P < 0.01 or 0.05; @ at P < 0.001, NS = Not significant.

Table 2 Hormonal and Biochemical profile expressed as percentage value of increase or decrease after treatment with indolyl quinoline for 2, 4, 8, 12 weeks.

Hormonal and Biochemical Parameters	Treatment period			
	2 weeks	4 weeks	8 weeks	12 weeks
Testicular testosterone	Percentage (%) of reduction control vs treated			
	41.27	43.06	46.51	43.57
Serum FSH	Percentage (%) of increase control vs treated			
	26.53	28.42	29.77	27.36
Serum LH	Percentage (%) of increase control vs treated			
	22.12	18.75	17.85	20.53
Testicular protein	Percentage (%) of reduction control vs treated			
	27.02	32.42	38.85	37.73
Alkaline phosphatase	Percentage (%) of increase control vs treated			
	67.10	58.82	57.50	55.73
Cholesterol	Percentage (%) of increase control vs treated			
	13.43	28.67	31.43	31.68
Glycogen	Percentage (%) of reduction control vs treated			
	48.42	43.13	43.21	38.36
Epididymal GPC	Percentage (%) of reduction control vs treated			
	52.94	34.90	33.38	33.33

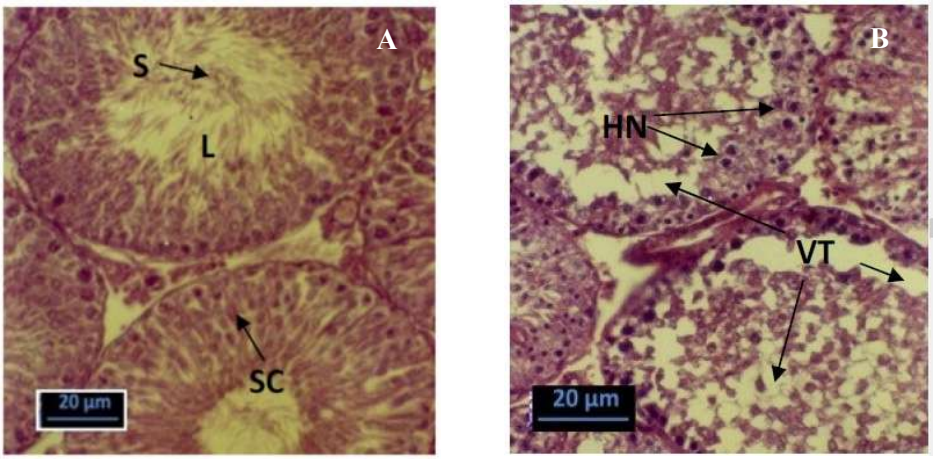


Fig. 2 (A) T. S. of the testis of normal rat showing normal architecture of the seminiferous tubules with differentiating spermatogenic cells. (B) T.S. of testis of 8 weeks indolyl quinoline treated male rat showing loss of differentiating germ cells. (HN: hyperpycnotic nuclei, L: lumen of seminiferous tubule, S: spermatozoa, SC: Sartoli cell, VT: vacuolated tubule)

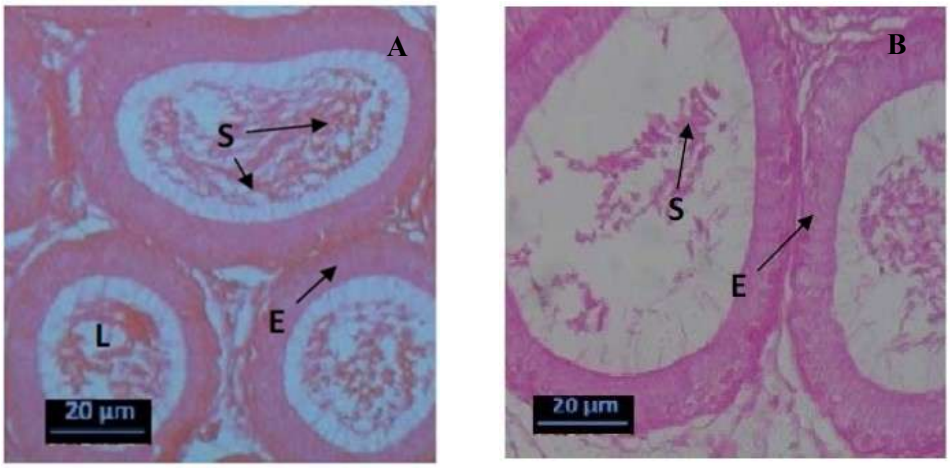


Fig. 3 (A) T.S. of epididymis of control rat showing normal architecture of tubules and spermatozoa. (B) T.S. of epididymis of 6 weeks indolyl quinoline treated male rat showing thicker epithelium. Note less number of spermatozoa. (E: epithelium, L: lumen of epididymis S: spermatozoa)

.

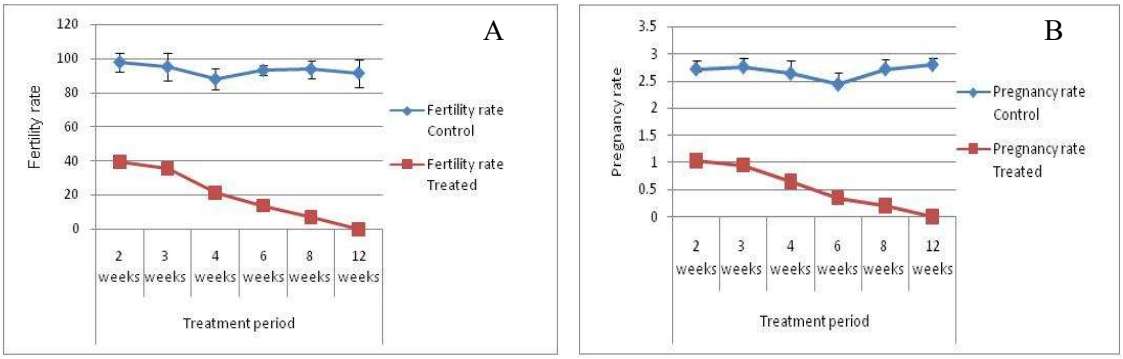


Fig 4 Fertility (A) and pregnancy rate (B) of control and treated animals administered with indolyl quinoline during the experiment period at a dose of 250 mg/kg in male rats.

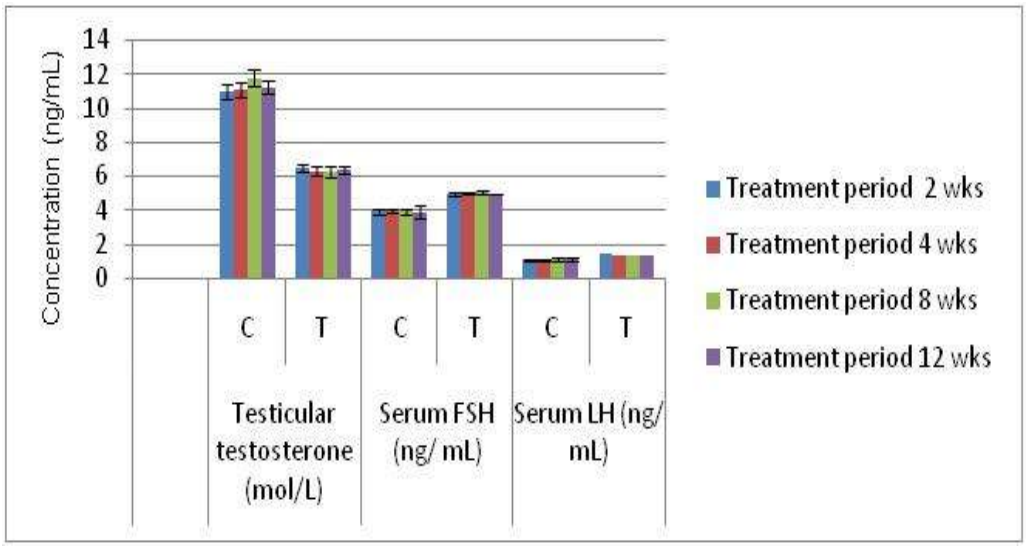


Fig 5 Testosterone, FSH and LH hormone profiles of control (C) and 2, 4, 8, 12 weeks indolyl quinolone treated (T) male rats.

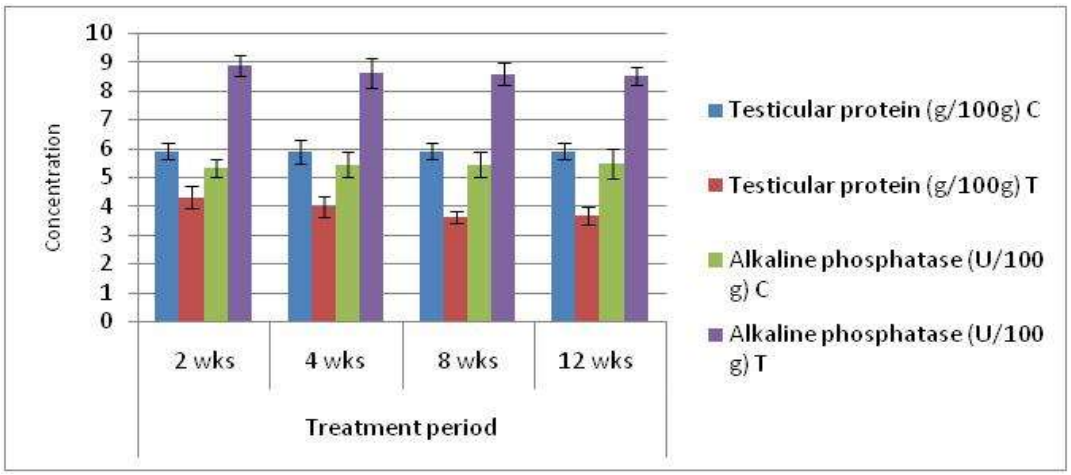


Fig 6 Biochemical profiles of protein and alkaline phosphatase that show % of reduction in treated (T) animals compared to control (C) after indolyl quinoline treatment for 2, 4, 8 and 12 weeks at a dose of 250 mg / kg in male rats.

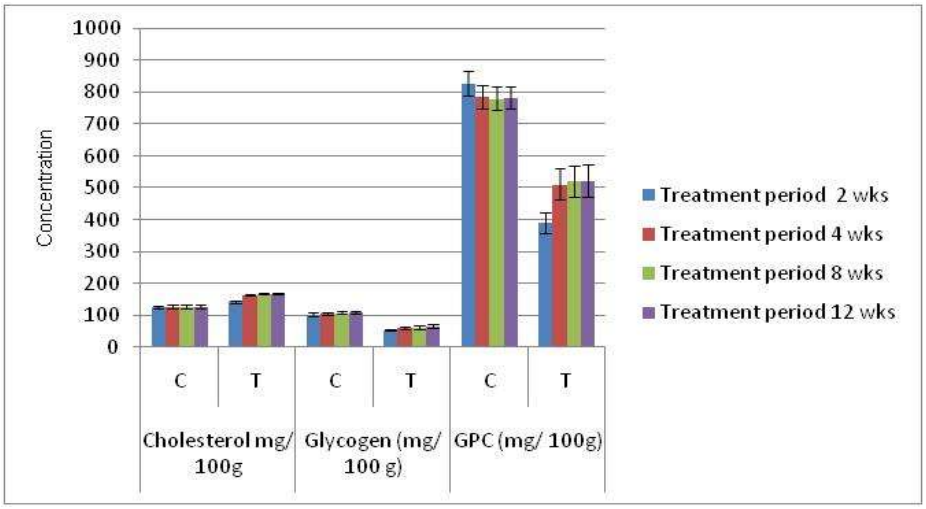


Fig 7 Biochemical profiles of cholesterol, glycogen and GPC that show % increase in treated (T) animals compared to control (C) after indolyl quinoline treatment for 2, 4, 8 and 12 weeks at a dose of 250 mg / kg in male rats.

DISCUSSION

Spermatogenesis is a process of both mitotic and meiotic divisions where spermatogonia divide to produce spermatocytes and subsequently haploid spermatids. Further, the spermatids undergo a notable change during their development through a process of spermiogenesis where the round shaped structure of the spermatids turned into elongating spermatids that transform into spermatozoa through certain steps. The whole process occurs into the seminiferous tubular lumen. The compound of interest studied here is capable of inducing harm to seminiferous epithelium of rat and thus changing the process of spermatogenesis along with other reproductive parts in question causing infertility.

Functional infertility was induced in male rats by the compound through exposed time period as substantiated by reduced fertility and the rate of pregnancy. To examine the compound to have an effect on which phase of spermatogenic process, epididymal maturation and or testicular events of spermatogenesis, a periodical mating schedule followed by sacrifice of the male rats has been planned. It is known that for maturation of spermatozoa the epididymis plays a very crucial role and a cascade of reactions take place in epididymal fluid during transportation of the sperm through the convoluted ductus system of the epididymis. The fertilizing ability of the sperm (Robaire and Viger, 1995, Dacheux et al., 2003, Gatti et al., 2004, Turner et al, 1995, Metayer et al., 2002, Blobel, 2000) is ensured by the factors derived from the epididymal fluid that rearrange and reorganize lipids and proteins in the spermatozoal membrane (Yanagimachi, 1994) Decline in GPC level, an indicator of epididymal function (Setchel et al., 1994), morphological changes in epididymis and sperm count indicate that epididymal dysfunction could be one of the causes of infertility induced by the compound during treatment period. Moreover, no distinct morphological change in the shape and structure of the epididymal sperms as seen under light

microscope indicates that the tested compound does not cause antifertility through morphological disruption of sperms.

Contrary to this, no significant change is observed in the mating rates between the control and treated rats, suggesting no harmful effects of the tested compound on the copulating potential of the rats. It has further been studied that the inhibition of spermatogenesis by the compound is attributed to the elevated levels of gonadotrophins. The events of spermatogenesis are mainly regulated by testosterone and the role of gonadotrophin also could not be ruled out (Clearmont and Hervay, 1965, Meitrich et al., 1994, McLachlan et al., 1994 a, b, O'Donnel et al., 1996, McLachlan et al., 1996).

The possible cause of induction of infertility by the tested compound might be a reduction in the level of testosterone, though the other possibilities may not be ruled out. The beginning and continuation of spermatogenesis in pre-pubertal and pubertal rats is LH and FSH dependant. Negative feedback regulation of FSH, LH and sex hormone to this process has also been studied extensively (Everett, 1994) and it has been shown that high level of FSH and LH are detrimental as sub- normal level (Kerr and Sharpe, 1989, Sharpe, 1994). Histological assessment in the present study is a useful parameter that offers a sensitive indicator of damage to the epididymis and the testis cells. The treatment made thereof through different weeks also provides information on the marked cells and degree of toxicity. FSH acts as a mitotic factor for Sertoli cells (Sharpe, 1994). Further, cooperation of testosterone and FSH brings about the binding of spermatids with the Sertoli cells. This is an indispensible step in the process of spermiogenesis (Muffy et al., 1993). Sertoli cells do not divide in adult under normal conditions. But it has also been shown that the terminally differentiated Sertoli cells can re-start mitotic cycle even after attaining maturity (Chaudhury et al., 2005). Thus it can be clearly stated that the compound imposed some change in the overall reproductive physiology that could lead to the inhibition of spermatogenesis. Therefore, the action of the test compound on the spermatozoa pursuing epididymal maturation suggests a condition of functional infertility during the treatment period.

ACKNOWLEDGEMENT

Our thanks goes to the Principal, Maulana Azad College, Kolkata-700013 for providing necessary infrastructural assistance; to UGC and DBT for providing financial assistance for the research work.

REFERENCES

Allag I and Rangari K (2002) Extragenomic action of steroids on spermatozoa Prospects for regulation of fertility. *Health Popul*, 25 (1):38-44.

Bhowal SK, Lala S, Hazra A, Paira P, Banerjee, Mondal NB and Chakraborty S (2008) Synthesis and assessment of fertility-regulating potential of 2-(2″-chloroacetamidobenzyl)-3-(3′-indolyl) quinoline in adult rats as a male contraceptive agent. *Contraception,* 77:214-222.

Blobel CP (2000). Functional processing of fertilin; evidence for a critical role proteolysis in sperm maturation and activation. *Rev Reprod*, 5:75-83.

Bone W, Jones NG, Kamp G, Yeung CH and Cooper TG (2000) Effect of ornidazole on fertility of male rats: inhibition of a glycolysis-related motility pattern and zona binding required for fertilization in vitro. *J Reprod Fertil*, 118:127-135.

Chakrabarty G, Basu A, Manna PP, Mahato SB, Mandal NB and Bandyopadhyay S (1999) Indolylquinoline derivatives are cytotoxic to *Leishmania donovani* promastigotes and amastigotes in vitro and are effective in treating murine visceral leishmaniasis. *J Antimicrob Chemother*, 43:359-366.

Chakraborty S and Lala S (1998) Assessment of the antifertility effect of phaseolinone, an anti-leishmanial agent, in male rats. *Contraception*, 58:183-191.

Chaudhury I, Sadler-Rggleman I, Ague JM and Skinner MK (2005) The helixloop-helix inhibitor of differentiation (ID) proteins induce post-mitotic terminally differentiated Sertoli cell to re-enter the cell cycle and Proliferate. *Biol Reprod*, 72:1205-1217.

Clearmont Y and Hervay SG (1965) Duration of the cycles of the epithelium of normal, hypophysectomized and hypophysectomized hormone treated albino rats. *Endocrinology*, 76:80-89.

Cooper TG, Yeung CH, Skupin R and Haufe G (1997) Antifertility potential of ornidazole analogues in rats. *J Androl*, 18:431-438.

Corsi G, Palazzo G, Germani C, Barcellona S and Silvestrini B (1976) 1-Halobenzyl-1H-indazole –3-carboxylic acids. A new class of antispermatogenic agents. *J Med Chem*, 19:778-783.

Dacheux JL, Gatti JL and Dacheux F (2003) Contribution of epididymal secretory proteins for spermatozoa maturation. *Microsc Res Tech*, 61:7-17.

Dhanapal R, Ratna JV, Saratchandran I and Gupta M (2012) Reversible antispermatogenic and antisteroidogenic activities of *Feronia limonia* fruit pulp in adult male rats. *Asian Pac J Trop Biomed*, 2(9): 684-690.

Edwards B, Smith A and Skinner DC (2013) Dose and durational effects of the gonadotropin-releasing hormone agonist, deslorelin: the malerat (*Rattus norvegicus*) as a model. *J. Zoo Wildl Med*, 44 (4 Suppl):S97-101.

Etribi A, Ibrahim A, Mahmoud K, El-Haggar S, Hamada T and El-Ahmadi I (1982) Antisperm antibodies and human infertility. *Fertil Steril*., 37(2):236-239.

Everett JW (1994) Pituitary and hypothalamus: perspective and overview. In: Knobil E, Neill JD, editors. The physiology of reproduction. New York: Raven Press; pp 1509-1526.

Gatti JL, Castella S, Dacheux F, Ecroyd H, Métayer S, Thimon V and Dacheux JL (2004) Post-testicular sperm environment and fertility, *Anim Reprod Sci*, 82-83:321-339.

Gottwald U, Davies B, Fritsch M and Habenicht U (2006) New approaches for male fertility control: HE6 as an example of a putative target. *Mol Cell Endocrinol*, 250(1-2): 49-57

Greenwood FC, Hunter WM and Glover JS (1963) The preparation of 131I labeled human growth hormone of high specific activity. *Biochemistry,* 89:114–123.

Grima J, Silvestrini B and Cheng Y (2001) Reversible inhibition of spermatogenesis in rats using a new male contraceptive, 1-(2,4′-dichlorobenzyl)- indazole-3-carbohydrazide, *Biol. Reprod*, 64:1500-1508.

Gupta RS, Saxena P, Gupta R and Kachhawa JB (2011) Evaluation of reversible contraceptive activities of *Cuminum cyminum* in male albino rats. *Contraception*, 84(1):98-107.

Kerr JB and Sharpe RM (1989) Focal disruption of spermatogenesis in testis of adult rats after a single administration of human chorionic gonadotrophin. *Cell Tissue Res*, 257:163-169.

Kopf G (2008) Approaches to the identification of new nonhormonal targets for male contraception. *Contraception*, (4):S18-22

Lohiya NK, Mishra PK, Pathak N, Manivannan B, Bhande SS, Panneerdoss S and Sriram S (2005) Efficacy trial on the purified compounds of the seeds of *Carica papaya* for male contraception in albino rat. *Reprod Toxicol*, 20(1):135-148.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem*, 193(1):265-275.

Mahato SB, Mandal NB, Chattopadhyay S, Nandi G, Luger P and Weber M (1994) Synthesis of indolylquinolines under Friedel–Crafts reaction conditions. *Tetrahedron*, 50: 10803-10812.

McLachlan RI, Wereford NG, deKrester M and Robertson DM (1994a) Testosterone effect on spermatogenesis in the gonadotropin-releasing hormone immunized rat. *Biol Reprod*, 50:271-280.

McLachlan RI, Wereford NG, Donnel L, de Krester M and Robertson D (1996) The endocrine regulation of spermatogenesis: independent roles for testosterone and FSH. *J Endocrinol*, 148:1-9.

McLachlan RI, Wereford NG, Tgouts C, de Krester M and Robertson DM (1994b) Effects of testosterone on spermatogenic cell populations in the adult rat. *Biol Reprod*, 51:945-955.

Meitrich ML, Wilson G, Ye WS, Kurdglu B, Parchuri N and Terry NHA (1994) Hormonal protection from procarbazine-induced testicular damage is selective for survival and recovery of stem spermatogonia. *Can Res*, 54:1027-1034.

Metayer S, Dacheux F, Dacheux JL and Gatti JL (2002) Comparison, characterization, and identification of protease and protease inhibitors in epididymal fluids of domestic animals. Matrix metalloproteinases are major fluid gelatinase. *Biol Repod*, 66:1219-1229.

Mishra RK and Singh SK (2009) Reversible antifertility effect of aqueous rhizome extract of *Curcuma longa* L. in male laboratory mice. *Contraception*, 79 (6):479-487.

Muffy KE, Nazian S and Cameron DF (1993) Junction-related Sertoli cell cytoskeleton in testosterone-treated hypophysectomized rats. *Biol Reprod*, 49:1122-1132.

O'Donnel L, McLachlan RJ, and Wereford NG (1996) Testerone withdrawal promotes stage specific detachment of round spermatid from the rat seminiferous epithelium. *Biol Repord*, 55: 895-901.

Paulsen C, Christensen R and Bagatell C (1994) Status of male contraception: hormonal approach. In: Corson SL, Derman RJ, Tyner LB, editors. Fertility control. 2nd ed. London: Golden Publishers; 281-292.

Robaire B and Viger RS (1995) Regulation of epididymal epithelial cell function. *Biol Reprod*, 52: 226-236.

Setchel BP, Maddocks S and Brooks DE (1994) Anatomy, vasculature, enervation and fluids of the male reproductive tract. In: Knobil E, Neill JD, editors. The physiology of reproduction. New York: Raven Press, pp 1063-1175.

Sharpe RM (1994) Regulation of spermatogenesis. In: Knobil E, Neill EK, editors. The Physiology of Reproduction. New York: Raven Press, pp 1363-1434.

Sharpe RM (1994) Toxicity of spermatogenesis and its detection. In: Kenneth SK, editor. Reproduction and development. New York: Marcel Dekker, Inc; pp 625-635.

Silvestrini B, Palazzo G and De Gregario M (1984) Lonidamine and related compounds. *Prog Med Chem,* 21:110-135

Singla N, Kaur G, Babbar BK and Sandhu BS (2013) Potential of triptolide in reproductive management of the house rat, *Rattus rattus*. *Integr Zool*, 8(3):260-276.

Turner TT, Miller DW and Avery EA (1995) Protein synthesis and secretion by the rat caput epididymis in vivo: influence of the luminal microenvironment. *Biol Reprod*, 52:1012-1019.

Vannucchi C, Angrimani D, Eyherabide A, Mazzei C, Lucio C and Maiorka P (2015) Effects of intratesticular administration of zinc gluconate and dimethyl sulfoxide on clinical, endocrinological, and reproductive parameters in dogs. *Theriogenology*, 84(7):1103-1110

Yanagimachi R (1994) Fertility of mammalian spermatozoa: its development and relativity. *Zygote*, 2:371–372.

Pranikee - Journal of Zoological Society of Orissa, ISSN 0970-4450
Volume XXX, 2018, pp 29-38

STUDY OF CELL PROLIFERATION DURING EARLY PHASE OF LIMB REGENERATION IN THE TADPOLES OF INDIAN TREE FROG

Neha Gupta and Pravati Kumari Mahapatra*

Cell and Developmental Biology Laboratory, Post Graduate Department of Zoology, Utkal University, Vani Vihar, Bhubaneswar, Odisha, 751004.

*Corresponding Author

Email: mpkzooluu@gmail.com

ABSTRACT

Animals such as mammals, birds, and lizards cannot restore lost limbs but instead merely undergo a process of wound healing. In contrast, urodele amphibians such as newts and salamanders can regenerate their amputated limbs, while anuran amphibians are intermediate between urodele amphibians and other vertebrates in terms of their regenerative capacity. Epimorphic regeneration in amphibian tadpoles occurs through the establishment of a balanced growth state in which a blastema gives rise to all the mesenchymal cells, whereas definite areas of the epidermis proliferate leading to its extension, thus, allowing the enlargement of the whole structure. This type of regeneration involves specific mechanisms that coordinate and regulate cell proliferation. We have investigated cell proliferation patterns during blastema formation in the regenerating hindlimbs of the tadpoles of Indian tree frog, *Polypedates maculatus*, based on immunofluorescence reactivity of blastemal cells towards two potent markers of cell proliferation, i.e., proliferating cell nuclear antigen (PCNA) and fibroblast growth factor 1 (FGF1).

INTRODUCTION

Epimorphic regeneration is the process by which complete regeneration of a complex structure such as an amphibian limb occurs through production of a proliferating blastema. Limb regeneration in amphibians takes place in two major phases namely, formation of a blastema that resembles the early limb bud, and growth and differentiation of the blastema into the missing limb parts. Formation of the limb regeneration blastema is a reverse developmental process that takes place by histolysis of the tissues, resulting in tissue disorganization and liberation of individual cells. Blastema cell proliferation depends on different factors, some of them are originating from the peripheral nerves while some from the epidermal cap (Boilly et al., 1990). But, the nature of factors those trigger blastema cell proliferation remain unknown. Several mitogens, assumed to be produced by nerves, such as substance P (Globus and Vethamany-Globus, 1985; Smith et al., 1995), transferrin (Mescher and Munaim, 1984) and fibroblast growth factor (FGF) (Gospodarowicz and Mescher, 1980; Jeong, 2008; Gilbert, 2010), were considered to be important during limb regeneration.

Proliferating cell nuclear antigen (PCNA) or DNA sliding clamp protein is also called as the “maestro of the replication fork” (Moldovan et al., 2007) because it coordinates the recruitment of many proteins to sites of DNA replication and regulates their activities. The PCNA plays a critical role in a wide range of nuclear processes including DNA replication, translesion DNA synthesis, base excision repair,

nucleotide excision repair, mismatch repair, recombination, chromatin assembly and remodeling, sister chromatid cohesion, and cell cycle control (Maga and Hubscher, 2003, Moldovan et al., 2007; Naryzhny, 2008; Tsurimoto, 1999; Zhuang and Ai, 2010).

The PCNA is considered to be a marker of cell proliferation. It is a subunit of the DNA-polymerase δ which is involved in DNA replication and DNA repair. Its rate of mRNA and protein synthesis is known to increase during the S-phase of the cell cycle (Bravo and Celis, 1980; Bravo and MacDonald-Bravo, 1985; Tan et al., 1986; Almandral et al., 1987). It was initially found to be a protein that was differentially expressed during the cell cycle (Bravo et al*.,* 1980*)* and has became a marker of cell proliferation (Van et al*.,* 1998).

Fibroblast growth factors (FGF) represent a large family of secreted molecules which function through the activation of specific tyrosine kinases receptors by activating different pathways including the Ras/MAP kinase and the phospholipase-C gamma pathways. During embryonic development, FGFs have diverse roles in regulating cell proliferation, patterning, morphogenesis, migration and differentiation. In the adult organism, they act as homeostatic factors and function in tissue repair and response to injury. Some FGFs when properly expressed are known for their contribution to the pathogenesis of cancer. A subset of the FGF family, expressed in adult tissue, is important for neuronal signal transduction in the central and peripheral nervous systems.

FGF-1 and FGF-2 stimulate the growth of many cell types and also have neurotrophic and angiogenic activities (Greenhalg et al., 1990) which make FGF a potent molecule for limb regeneration. It was first shown that FGF is able to stimulate cell proliferation of denervated blastemas both *in vivo* (Mescher et al., 1979; Chew and Cameron, 1983) as well as *in vitro* (Carlone et al., 1981; Merscher and Loh, 1981; Albert et al., 1987; Boilly and Albert, 1988). The presence of FGF-1 in blastemas was demonstrated using heparin sepharose chromatography and immunochemical methods in axolotl larva (Boilly et al., 1991). Expression of FGF receptors in limb blastemas of newts was also reported using *in situ* hybridization (Poulin et al., 1993). FGF-1 is also known as acidic FGF and is involved in regeneration processes (Hee-Jeong et al., 2008; Gilbert, 2010). As both PCNA and FGF are associated with cell division and differentiation, aim of the present study was to investigate proliferation of cells during limb regeneration in the tadpoles of the Indian tree frog *Polypedates maculatus*, a potent amphibian model from Indian context, based on immunohistochemical localization of these two proteins in the cells of regeneration blastema.

MATERIALS AND METHODS

Collection and rearing of tadpoles

Egg masses of the Indian tree frog, *Polypedates maculatus* were collected during the month of July-August from Utkal University campus, Bhubaneswar, Odisha, India (20º 16᾽ 35. 2᾽᾽ N 85º 50᾽ 35. 0᾽᾽ E) and reared in the laboratory following the standardized procedure (Mohanty-Hejmadi, 1977). Tadpoles following hatching were fed with boiled *Amaranthus* leaves and boiled yolk of egg *ad libitum*.

Amputation

The tadpoles of hindlimb developed stages (Gosner stage 35-37, Gosner, 1960) were anesthetized in 1/3000 MS 222 (Tricaine methane sulfonate) in conditioned tap water (tapwater aerated for72h). Hind limb amputation from mid of the shank region was made on a presterilized porcelain plate using a sharp blade. Following regeneration, the tadpoles were fixed in 4% buffered formalin at regular intervals for histological investigation.

Histology

Limb regenerates fixed in 4% buffered formalin were then washed in tap water for 12h and dehydrated with gradual series of increasing percentages of alcohol as per the standard procedure. Then, the tissues were embedded in wax and sections of 5-7µm thick were cut. The sections were placed onto gelatinized slides and stretched at 57ºC for 5 minutes. Then the slides were stained with the help of standard Mallory staining protocol.

Immunohistochemistry

Tissue sections were deparaffinised by dipping the slides in 2 changes of xylene and then transferred into series of changes in alcohol (100%, 95%, 70%) followed by water wash for rehydration. Antigen unmasking were performed by boiling the slides in glycine HCL buffer (P^H-3.5) for 15 minutes at temperature of 90-92˚. Then the slides were cooled by keeping them undisturbed at room temperature for 30 minutes and unspecific binding to tissues on the slide were prevented by treating them with blocking serum. The primary antibody (1˚Ab) (PCNA - Rabbit polyclonal IgG and FGF - Goat polyclonal IgG) was used at a dilution of 1:50 in NDS-PBS diluents. After application of 1˚Ab, the slides were kept in a humidified chamber at 4˚C for 24h. Then the 1˚Ab treated slides were washed in PBS buffer for 3 times (2 min each) on the next day before secondary antibody (2°Ab) incubation. 2˚Ab (PCNA - Donkey anti-rabbit IgG-R and FGF - Donkey anti-Goat IgG-FITC) was used at a dilution of 1:200 in NDS-PBS diluents. Then the treated slides were incubated for 30 minutes in dark. After incubation, they were washed in PBS buffer for 3 times (2 minutes each) and mounted by using one drop of temporary fluorescent mounting medium and a cover slip. The mounted slides were viewed under the fluorescent microscope (LEICA DM3000 LED) and suitable photographs were taken at 4X, 10X, 20X and 40X magnification.

RESULTS

HISTOLOGICAL ANALYSIS

Distinct limb regenerates was noted in 72h post amputation (Fig. 1A). Histological slides showed the covering of wound by epidermal cells (Fig. 1B). Below the epidermal layer a distinct basement layer was formed. The regeneration blastema was packed with loose mesenchymal cells. There was accumulation of cells beneath the apical ectodermal cap which was evident in 72 h (Fig. 1B) and 96 h (Fig. 2B) of limb regenerates. In 96h limb regenerates, the epidermal layer was thicker, mesenchymal cells were compact and basement membrane disappeared (Fig. 2B).

IMMUNOFLUROSENCE LOCALIZATION OF FGF1 AND PCNA

In 72h limb regenerates, a moderate level of FGF1 expression was visualised in the blastema cells underlying the wound epidermis (Fig. 1C). During 96h of post amputation, a very strong FGF-1 immuoreactivity was seen over the entire epidermis of the limb regenerate, especially in the epidermal cap of the blastema. In addition to the cells of epidermal cap, the blastema mesenchymal cells were also moderately labeled with FGF1 (Fig. 2C).

The expression of PCNA protein was very low in the limb regenerates of 72h post amputation (Fig. 1D). But, in 96h limb regenerate, a moderate level of expression of PCNA was distinctly seen in the blastemal cells. In contrast to the blastema cells, the apical ectodermal cap did not display a strong PCNA labelling (Fig. 2D).

Thus, the expression level of both PCNA and FGF-1 were low in 3day hind limb regenerate and was restricted to the lining of wound epidermis and few mesenchymal cells. However, a remarkable expression could be seen in 4 day amputated limb mainly in the epidermal cap (Figs. 3 and 4).

DISCUSSION

Histological preparations showed that during hind limb regeneration in amphibian tadpoles there was a continuous series of growth and proliferation of cells. This makes an amphibian limb to be considered as a research model which provides numerous insights into the principles and mechanisms of tissue and organ regeneration. Different stages of limb regeneration sharing mechanisms of growth control and patterning with limb development has been reported by Endo et al. (2004). We observed the expression level of FGF-1 to be very low in 3day hind limb regenerate and was restricted to few mesenchymal cells. However, a remarkable expression could be seen in 4day amputated limb mainly in the epidermal cap. In axolotls, expression of FGF1 has also been reported by Boilly (1989). In the newt, *Pleurodeles*, immunolocalization of FGF-1 concerns both the cytoplasm of epidermal cells as well as cell periphery during limb regeneration. The presence of intracellular FGF-1 filling the whole perinuclear space indicates that this factor is probably produced in these cells. The presence of FGF-1 around epidermal cell may be a storage form of this growth factor as described by Zenjari et al., (1996).

Similar to FGF1, the expression level of PCNA was low during 3day limb regenerates as compared to 4day limb regenerates. During forelimb regeneration of *Xenopus laevis* froglets, the level of expression of PCNA was low in the control limbs and the regenerates. The metabolism of PCNA protein has been reported to be most sensitive to neural influence in *Xenopus laevis* froglets (Lemaitre et al., 1992, Frontera et al., 2014).

CONCLUSION

Cell proliferation is an essential event occurring during hind limb regeneration in anuran tadpole. The immunofluorescence activity of FGF1 and PCNA serves as important methods to study proliferating cells during the process of regeneration. Based on the present findings, it can be concluded that both FGF1 and PCNA are expressed in the blastema cells, comparatively higher in 96 h limb regenerates indicating more mitotic activity as compared to 72h limb regenerates.

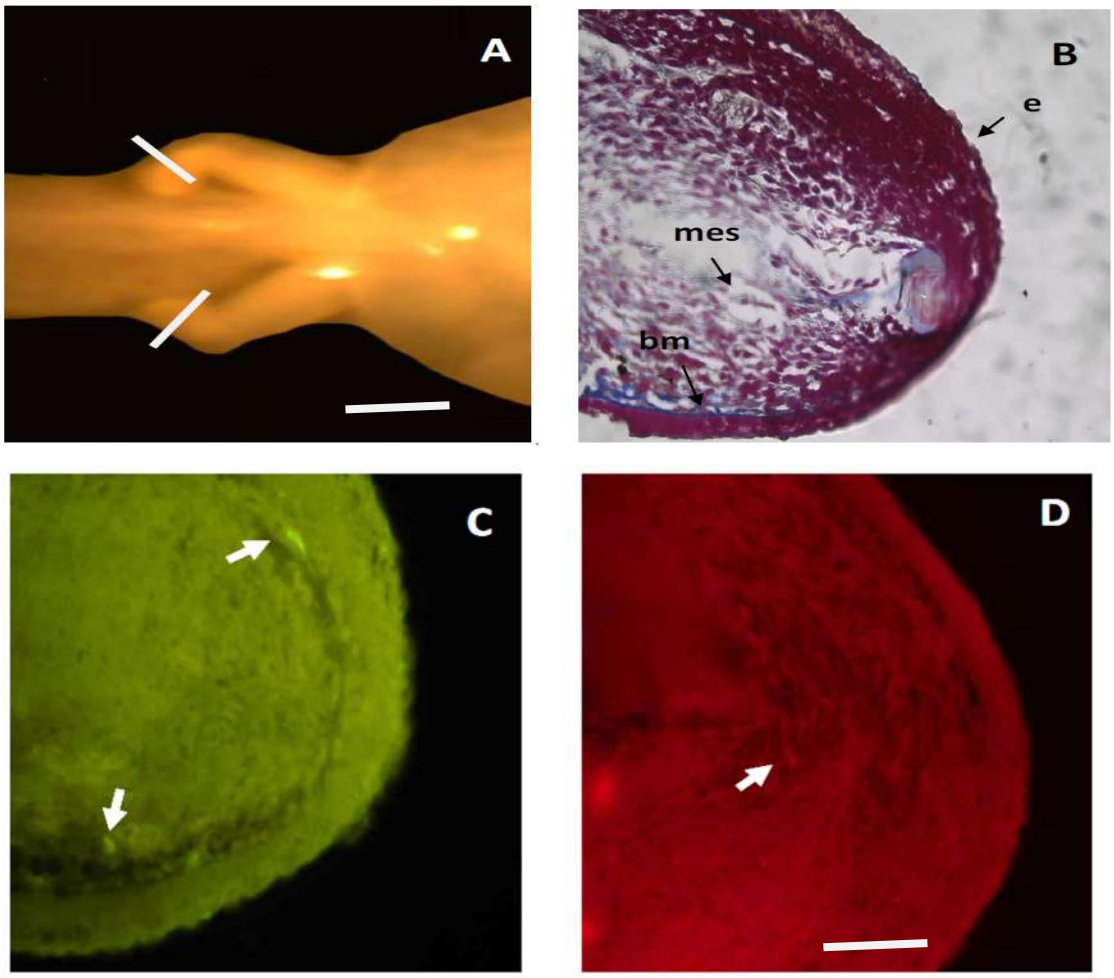


Fig. 1: Limb regenerates after 72h of amputation. (A) Limb regenerate in the form of a bud; (B) Mallory stained section; (C) Immunolocalization of FGF1; (D) Immunolocalization of PCNA, mes- Mesenchyme, e- Epidermis, bm- Basement membrane (Scale bar A=5mm, C-D= 50µm).

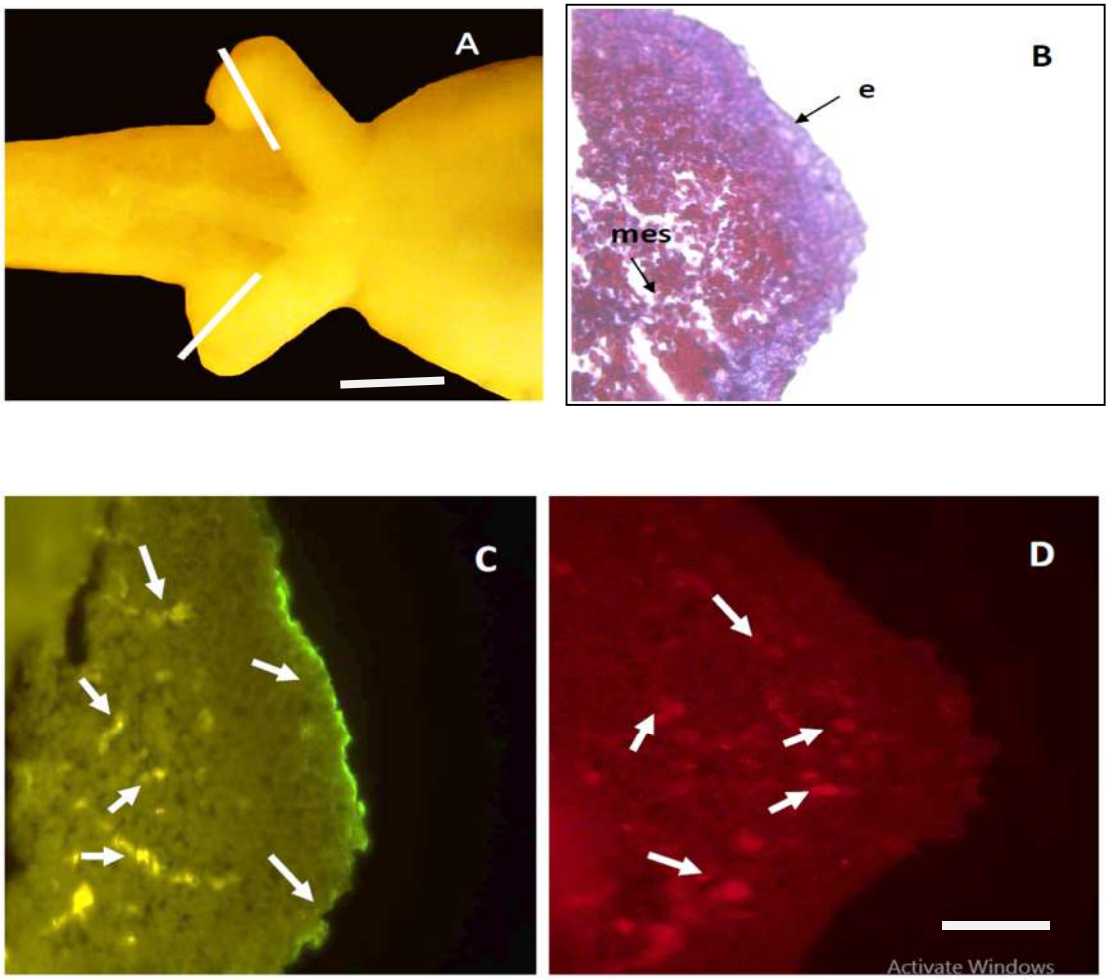


Fig. 2: Limb regenerates after 96h of amputation. (A) Limb regenerate in the form of a bud; (B) Mallory stained section; (C) Immunolocalization of FGF1; (D) Immunolocalization of PCNA, mes- Mesenchyme, e- Epidermis, bm- Basement membrane (Scale bar A=5mm, C-D = 50µm).

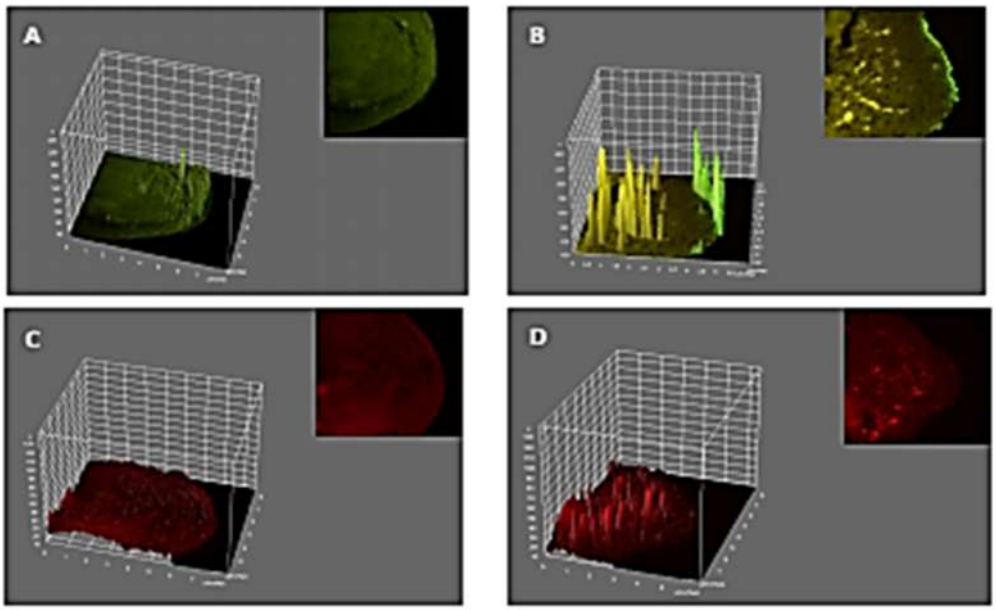


Fig. 3 Three dimensiional surface plot of FGF1 (A-B) and PCNA (C-D) immunodetection in hind limb regenerates of 72h and 96h respectively using Image J software.

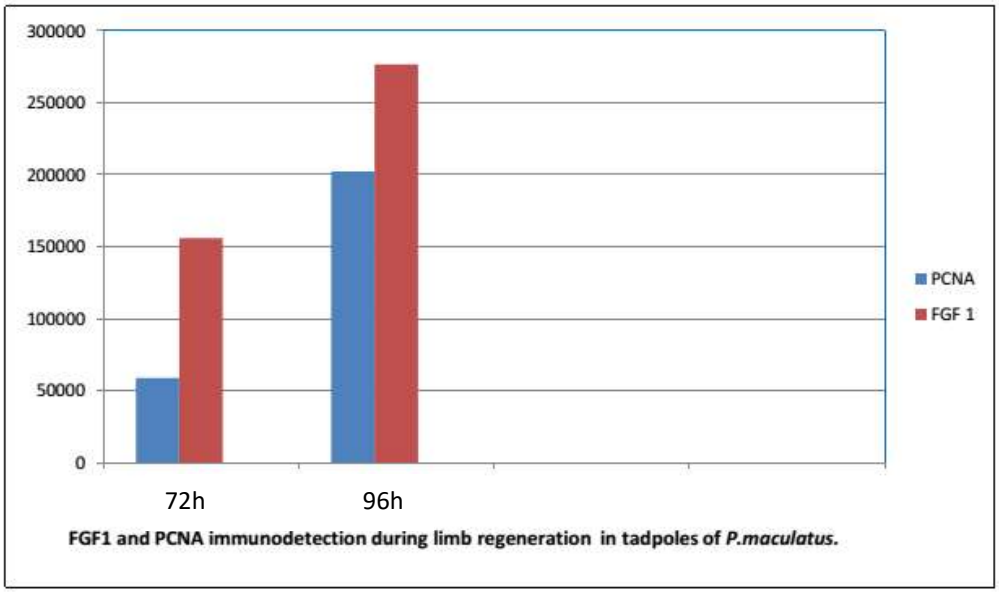


Fig. 4 Corrected Total Cell Fluorescence (CTCF) value of expression of PCNA and FGF1 in 72h and 96h hind limb regenerates using Image J software.

ACKNOWLEDGEMENTS

Authors would like to thank DST, Government of India for financial support under PURSE grant to Utkal University. Authors also thank CoE-IOCB of Utkal University.

REFERENCES

Albert P, Boilly B, Courty J and Barritault D (1987) Stimulation in cell culture of mesenchymal cells of newt limb blastema by EDGF I or II (basic or acidic FGF). *Cell Differentiation***,** 21:63-68.

Almandral JM, Huebsh D, Blundell PA, McDonald-Bravo H and Bravo R (1987) Cloning and sequence of the human nuclear protein cyclin. Homology with DNA binding proteins. *Proceedings of the National Academy of Sciences of the United States of America*, 84:1575-1579.

Boilly B (1989) Production of growth factors by the blastema during limb regeneration ofurodeles (Amphibia). In Recent Trends in Regeneration Research (Eds. V. Kior1sis, S.Koussoulakos and H. Wallace). Plenum Press, New York, pp.81-96.

Boilly B and Albert P (1988) Control of blastema cells proliferation during newt limb regeneration i*n vitro* cell culture study. *Monographs in Developmental Biology*, 21:1-8.

Boilly B, Cavanaugh KP, Hondermarck H, Bryant SV and Bradshaw AA (1991) Acidic fibroblast growth factor is present in regenerating limb blastemas of axolotl and binds specifically to blastema tissues. *Developmental Biology*, 145:302-310.

Boilly B, Lheureux E, Boilly-Marer Y, and Bart A (1990) Cell interactions and regeneration control.*The International Journal of Developmental Biology,* 34:219-231.

Bravo R and Celis JE (1980) A search for differential polypeptide synthesis throughout the cell cycle of HeLa cells. *Journal of Cell Biology*, 84:795-802.

Bravo R and MacDonald-Bravo H (1985) Changes in the nuclear distribution of cyclin (PCNA) but not its synthesis depend on DNA replication. *The EMBO Journal*, 4:655-661.

Carlone RL, Ganagarajah M and Rathbone MP (1981) Bovine pituitary Fibroblast Growth Factor has neurotrophic activity on newt limb regenerates and skeletal muscles in vitro. *Experimental Cell Research*, 132:15-21.

Chew KE and Cameroun JA (1983) Increase in mitotic activity of regenerating Axolotl limb by growth factor-impregnated implants. *Journal of Experimental Zoology*, 226:325-329.

Endo T, Bryant SV and Gardiner DM (2004) A stepwise model system for limb regeneration. *Developmental Biology*, 270:135-145.

Frontera L J, Cervino A S, Jungblut L D and Paz D A (2014) Brain-derived neurotrophic factor (BDNF) expression in normal and regenerating olfactory epithelium of Xenopus laevis. *Annals of Anatomy,* 198:41-48.

Gilbert SF (2010) Developmental Biology, 9th Ed, Sinauer Associates, Inc; Publishers. Sunderland, Massachusetts, USA, pp 1-740.

Globus M and Vethamany-Globus S (1985) In vitro studies of controlling factors in newt limb regeneration. In Regulation of Vertebrate Limb Regeneration (Ed. R.E. Sicard). Oxford University Press, New York, pp 106-127.

Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica.*16:183-190.

Gospodarowicz O and Mesher AL (1980) Fibroblast growth factor and the control of verebrate regeneration and repair. *The Annals of the New York Academy of Sciences*, 339:151-174.

Greenhalg DG, Spaugel KH, Murray MJ and Ross R (1990) PDGF and FGF stimulate wound healing in the genetically diabetic mouse. *The American Journal of Pathology,* 136:1235-1246.

Hee-Jeong IM, Xin Li, Prasuna Muddasani, Gun-Hee Kim, Francesca Davis, Jayanthi Rangan, Christopher B. Forsyth, Michael Ellman, and Eugene Jma Thonar (2008) Basic Fibroblast Growth Factor Accelerates Matrix Degradation Via a Neuro-Endocrine Pathway in Human Adult Articular Chondrocytes. *Journal of Cellular Physiology*, 215(2):452-463.

Lemaitre JM, Mechai M and Geraudie J (1992) Nerve-dependent expression of c-mycprotein during forelimb regeneration of *Xenopus laevis* froglets. *The International Journal of Developmental Biology*, 36:483-489.

Maga G and Hubscher U (2003) Proliferating cell nuclear antigen (PCNA): a dancer withmany partners. *Journal of Cell Science*, 116:3051-3060.

Mescher AL and Gospodarowicz D (1979) Mitogenic effect of a growth factor derived frommyelin on denervated regenerates of newt forelimbs. *Journal of Experimental Zoology,* 207:497-503.

Mescher AL and Loh JJ (1981) Newt forelimb regeneration blastemas in vitro: cellular response to explantation and effects of various growth-promoting substances. *Journal of Experimental Zoology*, 216:235-245.

Mescher AL and Munaim SI (1984) 'Trophic' effect of transferrin on amphibian limb regeneration blastemas. *Journal of Experimental Zoology*, 230:485-490.

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

Moldovan GL, Pfander B, and Jentsch S (2007) PCNA, the Maestro of the Replication Fork. *Cell,* 129(4):665-679.

Naryzhny SN (2008) Proliferating cell nuclear antigen: a proteomics view. *Cellular and Molecular Life Sciences,* 65:3789-3808.

Poulin ML, Patrie KM, Botelho MJ, Tassava RA and Chiu IM (1993) Heterogeneity in the expression of fibroblast growth factor receptors during limb regeneration in newts (*Notophthalmus viridescens*). *Development,* 119(2):353-361.

Smith MJ, Globus M, and Vethamany-Globus S (1995) Nerve extracts and substance P activate the phosphatidylinositol signalling pathway and mitogenesis in newt forelimb regenerates. *Developmental Biology,* 167:239-251.

Tan CK, Castillo C, SO, AG, and Downey KM (1986) An auxiliary protein for DNA polymerase δ from fetal calf thymus. *The Journal of Biological Chemistry*, 261:12310-12316.

Tsurimoto T (1999) PCNA binding proteins. *Frontiers in Bioscience*, 4:849-858.

Van DPJ, Brugal G and Baak JP (1998) Proliferation markers in tumours: interpretation and clinical value. *The Journal of Clinical Pathology*, 51:716-724.

Zenjari C, Boilly-Marer Y, Desbiens X, Oudghir M, Hondermarck H and Boilly B (1996) Experimental evidence for FGF-' control of blastema cell proliferation during limbregeneration of the Amphibian *Pleurodeles waltl, The International Journal of Developmental Biology*, 40: 965-971.

Zhuang ZH and Ai YX (2010) Processivity factor of DNA polymerase and its expanding role in normal and translesion DNA synthesis. *Biochimica et Biophysica Acta*, 1804:1081-1093.

Pranikee - Journal of Zoological Society of Orissa, ISSN 0970-4450
Volume XXX, 2018, pp 39-46

APPLICATIONS OF SDS-PAGE IN DIFFERENTIATION OF EARTHWORM GENERA

Harshita Swain, Ranjan Sahu and Sudhansu Sekhar Nishank

Post Graduate Department of Zoology, Utkal University, Bhubaneswar-751004

*Coresponding Author
Email: nishank25@gmail.com

ABSTRACT

Characterization of several invertebrate and vertebrate species have been done through SDS-PAGE and MALDI-TOF. However, use of SDS-PAGE for differentiating different genera, remains to be studied extensively. The objective of the present study was to use SDS-PAGE in differentiating different genera of earthworm, found in Odisha. Crude proteins were isolated from tissues of both intact and regenerated earthworms belonging to the genera, *Lampito mauritii* and *Drawida willsi*. The proteins were then resolved in 10% SDS-PAGE. From the protein profiles obtained, we found that a band of molecular weight around 61 kDa was specific for *Lampito mauritii.* Similarly, a protein band of molecular weight around 50 kDa was unique to the genus, *Drawida willsi.* Thus, these two bands, 61 kDa and 50 kDa can be used as markers to differentiate these two earthworm genera.

Keywords: SDS-PAGE, *Lampito mauritii, Drawida willsi*, differentiate, genera

INTRODUCTION

Earthworms (phylum Annelida) are widely distributed inhabiting both terrestrial and aquatic ecosystems. Till date, more than 6000 species are described worldwide out of which about 3000 to 3500 are valid. About 150 species are considered as peregrine on a global scale (Mubeen and Hatti, 2018; Edwards, 2004). They are considered to be important ecosystem engineers, comprising about 80% of the soil invertebrate biomass in most of the terrestrial ecosystems in the world (Lee, 1985).These invertebrates serve as excellent model organisms for studying the soil fauna, owing to their small size, easy handling and culture maintenance. Their role in indicating the soil quality has already been explored as indicators for the bioaccumulation of soil contaminants (Fründ et al*.,* 2011). However, the first step in any study is the identification of earthworm species. Currently, the key to differentiation of earthworms is based on their morphological and anatomical characters, which is considered as the gold standard method for identification, described by Julka and Senapati (1987). But many of these morphology based traits have been found to be overlapping between taxa, since these traits are not constant and create a varying degree of discrepancy (Pop et al*.,* 2003). Thus, variance can be seen in earthworm taxonomic systems among different researchers, as each considers different criteria for identification. In addition to the above taxonomic inconsistencies, this method of differentiation is not suitable when dealing with field collections, where several genera and species of earthworms are involved, as it is a time consuming, labour intensive and a difficult process, especially for non-specialists.

Since it is important for people working with earthworms as a test organisms, it is essential that a proper way of verifying as to which genus an earthworm belongs. There is an imperative need of fine tuning the classification method. Therefore, the objective of our study was to demonstrate the utility of sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE), the simplest proteomic approach, to distinguish different earthworm genera. In this study we have compared the tissue protein profiles of two earthworm genera, *Lampito mauritii* and *Drawida willsi*.

MATERIALS AND METHODS

Specimen collection

Specimens of *Drawida willsi* were obtained from different locations of Balangir district having coordinates 20°42'15.3"N 83°29'00.5"E, 20°42'31.0"N 83°28'58.0"E and 20°42'39.9"N 83°29'58.9"E (Fig. 1 A). Specimens of *Lampito mauriti* were collected from the campus of Utkal University, Vani Vihar, Bhubaneswar having coordinates 20°18'09.7"N 85°50'26.8"E, 20°18'13.3"N 85°50'32.9"E, 20°18'21.5"N 85°50'35.1"E and 20°18'24.9"N 85°50'16.0"E (Fig. 1 B). Both the species were collected by a random sampling method. All the earthworm samples were cultured in laboratory conditions in round plastic pots having 110 cm diameter and 200 cm height with adequate provision of food, moisture and temperature. A mixture of soil, cow dung, leaf litter and organic compost made up from kitchen wastes in a ratio of 50:30:10:10 was used in preparing the culture medium. In each pot 10 adult earthworms were kept and the pots were kept inside a dark room with good ventilation.

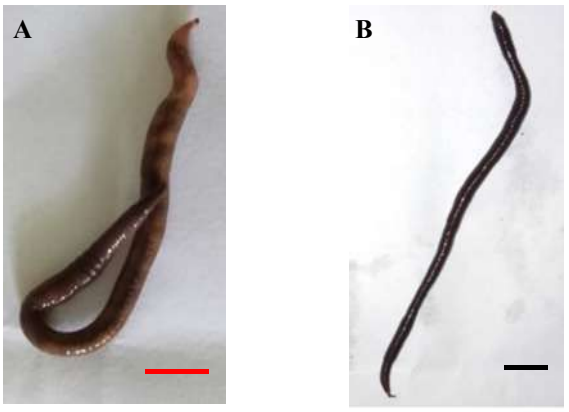


Fig. 1 (A) *Drawida wilsi* and (B) *Lampito mauritii* Scale bar = 1cm

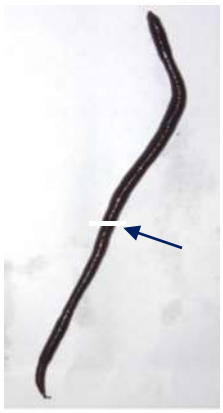


Fig. 2 Site of amputation (blue arrow)

Experimental design

For experiment two intact adult earthworms of the genus *Lampito mauritii* were cultured in pots in the first set. The second set consisted of two amputed earthworms of the genus *Lampito mauritii.* Similarly, the third and fourth culture pots consisted of two intact and two amputated earthworms of the genus *Drawida willsi,* respectively. The earthworms were amputated by cutting one-third portion of their length

posteriorly (Fig. 2). The earthworms of both the species were cultured and maintained in laboratory conditions. The amputated earthworms were allowed to regenerate and the regenerated tissue was taken for protein isolation.

Protein isolation

After around three weeks when blastema formation was seen in the amputated earthworms, the earthworms from all experimental setups were taken for protein isolation. The earthworms were first washed with tap water. The earthworms of both normal and regenerated forms were dissected, using sterilized instruments, to remove the gut. 100 mg of tissue was homogenised in 980 µl of GenePro™ Total Protein Isolation Buffer (Puregene Genetix) and 20 µl of 1X Protease Inhibitor Cocktail (Promega) by BI-50 tissue grinder (BR Biochem Life Sciences). The tissue extracts were incubated on ice for 5 minutes followed by centrifugation at 15,000 rpm for 10 minutes at 4℃. The supernatant containing the total protein fraction was then transferred to a fresh microcentifuge tube and stored at -20℃ until use.

Protein estimation

The concentration of protein in the supernatants was estimated using Lowry and Bradford method under the absorbance of 700 nm and 595 nm, respectively (Bradford, 1976; Lowry et al., 1951). A series concentration of bovine serum albumin was used to construct a standard curve.

Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed using 10% resolving gel, topped by 4% stacking polyacrylamide gel. The gels were prepared based on the method proposed by Laemmli (1970). Both gels contained 10% SDS. The gel slab contained 10 wells. Except for the first well and the last well, each well was loaded with the tissue supernatant with sample volume ranging from 6.7 to 11.4 µl. The volume of the supernatant was then adjusted to 15 µl with double distilled water. The final volume of each slot was finally adjusted to 30 µl by adding 15 µl of loading dye (supernatant: loading dye = 1:1). PageRuler™Plus Prestained Protein Ladder, 10-250 kDa (Thermo Fisher Scientific), was used as protein marker and loaded in the first well. The electrophoresis was carried out at 40V for 4 hours in a cold room. The gel was then stained with Coomassie Brilliant Blue R-250 and destained with methanol/glacial acetic acid/distilled water (1:1:8, w/w/w) to detect the protein bands.

RESULTS AND DISCUSSION

In *L. mauritii* protein concentration remained either same in the intact and regenerated individual (LM 1 and LMA1. In the second case the level declined in the regenerated in *L. mauritii* than the intact one or declined in the regenerated individual (LM2 and LMA2). However, in *D wilsi* concentration of protein remain high in the intact individual as compared to the regenerated individuals. A cumulative account of protein concentration of tissue supernatants is presented in table 1.

Table 1 The concentration of protein in different earthworm tissue supernatants

SAMPLE	PROTEIN CONCENTRATION (in mg/ml)
LM1	4.3
LM2	3.7
LMA1	4.3
LMA2	3.5
DW1	5.8
DW2	5.9
DWA1	3.6
DWA2	3.6

Note: LM1 = First intact individual of *L. mauritii,* LM2 = Second intact individual of *L. mauritii,* LMA1 = First regenerated individual of *L. mauritii,* LMA2 = Second regenerated individual of *L. mauritii,* DW1 = First intact individual of *D. willsi,* DW2 = Second intact individual of *D. willsi,* DWA1 = First regenerated individual of *D. willsi,* DWA2 = Second regenerated individual of *D. willsi.*

SDS-PAGE analysis

SDS-PAGE analysis revealed that the tissue extracts of earthworms of both *L. mauritii* and *D. willsi* contained several proteins. Visual observation of protein profiles of intact and regenerated earthworms of *Lampito mauritii* revealed constant 14 band patterns, including 5 intense bands, with an apparent molecular weight ranging from 100 kDa to < 10 kDa (as seen in lanes 2, 3, 4 and 5 in Fig 4).

Protein profiles of intact and amputated *D. willsi* were found to be similar revealing 7-9 band patterns, including 6 intense bands, and having molecular weights ranging from 100 kDa to < 10 kDa (as seen in lanes 7, 8 and 9 in Fig 4). However, in lane 6 only five bands were seen.

Bands of molecular weight 61 kDa and 50 kDa can be used for differentiation of the two genera.

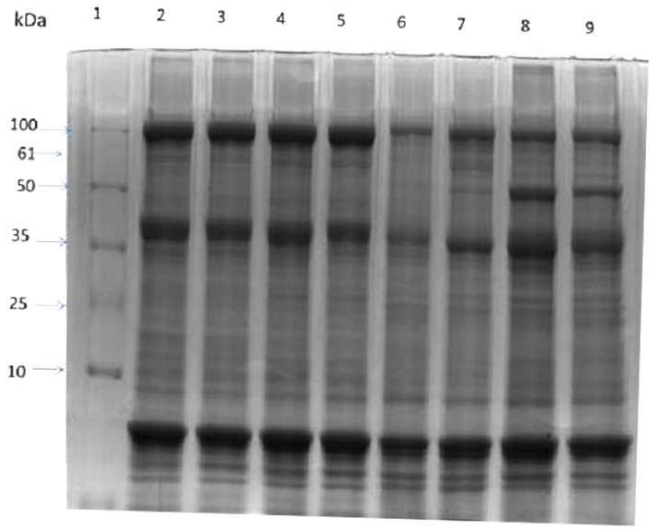


Fig.4 SDS PAGE profile of earthworms *L. mauritii* and *D. willsi*.

Lane 1: Molecular marker

lane 2: LM1 (First intact individual of *L. mauritii*)**, Lane 3: LM2 (**Second intact individual of *L. mauritii*)**, Lane 4: LMA1 (**First regenerated individual of *L. mauritii*)**, Lane 5: LMA2 (**Second regenerated individual of *L. mauritii*)**, Lane 6: DW1 (**First intact individual of *D. willsi*)**, Lane 7: DW2 (**Second intact individual of *D. willsi*)**, Lane 8: DWA1 (**First regenerated individual of *D. willsi*) and Lane 9: DWA2 (**Second regenerated individual of *D. willsi*).**

SDS-PAGE analysis of protein profiles clearly showed that all bands below 50 kDa are common in both the earthworm genera. In lanes 2, 3, 4 and 5 a protein band of around 61 kDa, just below the 100 kDa intense band, can be seen in Fig. 4. However, this band was absent in lanes 6, 7, 8 and 9. Thus, this band of around 61 kDa is a characteristic of *L. mauritii*. In lanes 8 and 9, an intense band of around 50 kDa was seen. In lane 7, also the band of approximately 50 kDa was present. However, it was not as intense as that visualised in lanes 8 and 9. This protein band of molecular weight 50 kDa is therefore, specific for *D. willsi*.

Thus, protein bands of molecular weights of approximately 61 kDa and 50 kDa in *L. mauritii* and *D. willsi*, respectively, allowed the discrimination between the protein profiles of the two genera. These two bands are peculiar in the protein profiles and can serve as the markers for differentiating between the earthworm genera, *L. mauritii* and *D. willsi*.

In our study, we have shown the applicability of SDS-PAGE to differentiate earthworm genera. However, certain studies in different parts of the world have also demonstrated the utility of this technique in distinguishing species which is comparable to the results we have obtained. Oien and Stenersen (1984) have distinguished two morphologically indistinct species, *Eisenia fetida* and *Eisenia unicolor* from each other by the following esterase electromorph characteristics obtained by PAGE. They found that proteins

having a molecular weight of 56,000 and 52,000 were seen in *Eisenia fetida* only and not in *Eisenia unicolor.* Also Engelstad and Stenersen (1991) studied SDS-PAGE analysis of acetylesterases patterns allowed the differentiation among *Eisenia fetida*, *Eisenia andrei* and *Eisenia veneta.* Five bands were found in *Eisenia fetida*, of which four bands were also found in *Eisenia Andrei* while in *Eisenia veneta* only three bands were found which was unique to this species. The reliability of the esterase patterns obtained suggested the use of electrophoretic method as a practical approach to establish pure cultures or to verify which individual belongs to which genus or species. Bøgh (1992) have also reported that gel electrophoresis of three to four different enzymes to be a suitable technique for routine taxonomic classification of earthworms. Reports on the analysis of SDS-PAGE has been proved to be useful in differentiation of other genera such as chromonids larvae of dipterans (chironomids from a group, *Thienemannimyia* and four genera *Chironomus, Cricotopus, Dicrotendipes* and *Glyptotendipes*), shrimps (rock shrimp, pink shrimp and white shrimp), fishes (cod, haddock, whiting, pollack, Alaska pollack, saithe, hake, plaice, lemon sole, dab, halibut, witch, brown trout, rainbow trout, sea trout, salmon, pink salmon, chum salmon, sockeye salmon, chinook salmon, coho salmon, scampi, monkfish, herring and mackerel; red snapper and 11 other fish species; Atlantic salmon, sea trout, rainbow trout, turbot, Alaska pollack, pollack, pink salmon, Arctic char, chum salmon, and New Zealand hake; hoki, cod, red fish, Alaska pollack, Atlantic salmon and saithe; *Channa gachua,C. striatus*, *C. marulius* and *C. punctatus*) and snakes (*Micrurus, Bothrops, Bothriopsis, Crotalus* and *Lachesis*) (An et al., 1988; Etienne et al., 2000; Etienne et al., 2001; Hasnain et al., 2005; Huang et al., 1995; Oh et al., 2014; Scobbie and Mackie, 1988; Soares et al., 1998). Piñeiro *et al.* (1999) have also studied SDS-PAGE protein profiles of 25 commercially valuable fish genera which showed discriminating specific protein profiles for each genus. Etienne et al. (2000), Etienne et al. (2001) and Rehbein et al. (1999) have identified different fish genera by studying specific sarcoplasmic proteins.

Our present study is limited by the fact that only two genera have been used for differentiation by SDS-PAGE and we have used 10% separating gel. These limitations can be resolved by inclusion of more number of genera of earthworms and conducting MALDI-TOF (Matrix Assisted Laser Desorption Ionization - Time of Flight) of the proteins isolated from SDS-PAGE for further confirmation of variation among different genera of earthworms.

CONCLUSION

SDS-PAGE is a useful tool to obtain a discrete method of differentiating different genera and species of earthworms. It is a relatively simple method which gives useful results. The preliminary approach of this technique involves detecting the unique bands of the whole protein-genera by visual analysis.

ACKNOWLEDGEMENTS

Authors would like to thank DST-SERB, Government of India for financial support.

REFERENCES

An H, Marshall MR, Otwell WS and Wei CI (1988) Electrophoretic identification of raw and cooked shrimp using various protein extraction systems. *Journal of Food Science*, 53 (2):313-318.

Bøgh PS (1992) Identification of earthworms (Lumbricidae): choice of method and distinction criteria. *Megadrilogica*, 4:163-174.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72 (1-2):248-254.

Edwards CA (2004) Earthworm ecology, 2nd edition. CRC Press LLC, New York, pp 3-11.

Engelstad F and Stenersen J (1991) Acetylesterase pattern in the earthworm genus *Eisenia* (Oligochaeta, Lumbricidae): implications for laboratory use and taxonomic status. *Soil Biology and Biochemistry*, 23 (3):243-247.

Etienne M, Jérôme M, Fleurence J, Rehbein H, Kündiger R, Mendes R, Costa H, Perez-Martin R, Piñeiro-González C, Craig A, Mackie I, Yman IM, Ferm M, Martinez I, Jessen F, Smelt A and Luten J (2000) Identification of Fish Species after Cooking by SDS−PAGE and Urea IEF: A Collaborative Study. *Journal of Agricultural and Food Chemistry*, 48 (7):2653-2658.

Etienne M, Jérôme M, Fleurence J, Rehbein H, Kündiger R, Mendes R, Costa H and Martínez I (2001) Species identification of formed fishery products and high pressure-treated fish by electrophoresis: a collaborative study.*Food Chemistry*, 72 (1):105-112.

Fründ HC, Graefe U and Tischer S (2011) Earthworms as Bioindicators of Soil Quality. In: Karaca A (ed) Biology of Earthworms. Soil Biology. Vol 24. Springer, Berlin, Heidelberg, pp 261-278.

Hasnain A, Arif SH, Ahmad R and Pandey RB (2005) Identification of species-marker bands in native and SDS-PAGE patterns of soluble muscle proteins of four species of genus *Channa* (Channidae: Channiformes) with evidence of some intraspecies heterogeneity. *Asian Fisheries Science*, 18 (1/2):49-58.

Huang TS, Marshall MR and Wei CI (1995) Identification of red snapper (*Lutjanus campechanus*) using electrophoretic techniques. *Journal of Food Science*, 60 (2):279-283.

Julka J M and Senapati B K (1987) Earthworms (Oligochaeta: Annelida) of Orissa, India. *Records of the Zoological Survey of India.* Occasional Paper No. 92, pp 1-49.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head ofbacteriophage T4. *Nature*, 227 (5259):680-685.

Lee KE (1985) Earthworms, their Ecology and Relationships with Soil and Land Use. Academic Press, Sydney.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the phenol folin reagent. *Journal of Biological Chemistry*, 193 (1):265-275.

Mubeen H and Hatti SS (2018) Earthworms diversity of Koppal district with the updated information ongenus Thatonia of Hyderabad-Karnataka region, Karnataka, India.*Journal of Asia-Pacific Biodiversity*, 11:482-493.

Oh JT, Epler JH and Bentivegna CS (2014) A rapid method of species identification of wild chironomids (Diptera: Chironomidae) via electrophoresis of hemoglobin proteins in sodium dodecyl sulfate polyacrylamide gel (SDS–PAGE). *Bulletin of Entomological Research*, 104 (5):639-651.

Oien N and Stenersen J (1984) Esterases of earthworms--III. Electrophoresis reveals that *Eisenia fetida* (Savigny) is two species. *Comparative Biochemistry and Physiology. C, Comparative Pharmacology and Toxicology,* 78 (2):277-282.

Piñeiro C, Barros-Velázquez J, Pérez-Martín RI, Martínez I, Jacobsen T, Rehbein H, Kündiger R, Mendes R, Etienne M, Jerome M, Craig A, Macki IM and Jessen F (1999) Development of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis reference method for the analysis and identification of fish species in raw and heat-processed samples: A collaborative study. *Electrophoresis*, 20 (7):1425-1432.

Pop AA, Wink M and Pop VV (2003) Use of 18S, 16S rDNA and cytochrome c oxidase sequences in earthworm taxonomy (Oligochaeta, Lumbricidae). *Pedobiologia,* 47 (5-6):428-433.

Rehbein H, Kundiger R, Yman IM, Ferm M, Etienne M, Jerome M , Craig A, Mackie I, Jessen F, Martinez I , Mendes R , Smelt A, Luten J, Pineiro C, Perez-Martin R (1999) Species identification of cooked fish by urea isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis: a collaborative study. *Food Chemistry*, 67 (4):333-339.

Scobbie AL and Mackie IM (1988) The use of sodium dodecyl sulphate polyacrylamide gel electrophoresis in fish species identification – a procedure suitable for cooked and raw fish. *Journal of the Science of Food and Agriculture*, 44:343-351.

Soares AM, Anzaloni Pedrosa LH, Fontes MR, Da Silva RJ and Giglio JR (1998) Polyacrylamide gel electrophoresis as a tool for the taxonomic identification of snakes from the Elapidae and Viperidae families. *Journal of Venomous Animals and Toxins*, 4 (2):137-142.

Pranikee - Journal of Zoological Society of Orissa, ISSN 0970-4450
Volume XXX, 2018, pp 47-57

ASSESSING FEEDING HABITS OF TADPOLES OF COMMON ASIAN TOAD *DUTTAPHRYNUS MELANOSTICTUS*

Jasmin Rout, Subhasish Prusty and Gunanidhi Sahoo*
Post Graduate Department of Zoology, Utkal University, Bhubaneswar - 751 004, India
*Corresponding Author
E-mail: gunanidhi.nou@gmail.com

ABSTRACT

The study of the diet of tadpoles can provide important information about the maintenance and dynamics of populations and communities of tropical amphibians. We characterized the diet of 175 tadpoles of *Duttaphrynus melanosticus*, collected from 35 sites of Jagatsinghpur district during rainy seasons (July to October) of 2017 and 2018. After morphometric measurements (total length and gut length), the complete intestine of each tadpole was analyzed for food items. The food spectrum of tadpoles included mostly detritus followed by phytoplanktons (represented by five classes and 38 genera). The dominant diatoms in this region are high in protein because they host nitrogen fixing cyanobacterial endosymbionts. The present study provides evidence for the voracious feeding behaviour of *D. melanostictus*.

Keywords: Anuran, diet, *Duttaphrynus melanostictus*, tadpoles, phytoplankton.

INTRODUCTION

Anuran tadpoles are gregarious predators capable of differentiating food items among diverse types of prey through varied feeding mechanisms and specific oral structures. Generally they are the key consumers and play an important role in the food chain of aquatic ecosystems. Diet is especially important in tadpoles because many species develop in short-lived aquatic environments (ephemeral ponds, temporary ponds). Such places add constraint on these tadpoles to consume foods which will ensure that they reach metamorphosis prior to the drying of the pond. Some tadpoles even rely on carnivory of other tadpole species to reach their metamorphic state (Heyer et al., 1975), whereas some species feed on conspecific eggs or tadpoles (Hero and Galatti, 1990; Mahapatra et al., 2017). The amount of food a tadpole consumes directly affects its growth (Kiffney and Richardson, 2001) and the quality of food consumed affects the rate of growth (Kupferberg et al., 1994). Hence, tadpoles of different species that live together are subjected to both intra- and inter-specific competition for food, space and to predation pressure.

The dietary habits of tadpoles have been studied by Wassersug et al. (1981). Lajmanovich (1997) showed that tadpoles feed on what is available in their habitat. Heyer (1976) and Lajmanovich (2000) concluded that food plays no important role in habitat partitioning among species, whereas Inger (1986) observed that partition food was intertwined with distribution in space. Differences in the internal oral characteristics may be related to preferences to different sizes of food particles in the microhabitats of tadpoles (Wassersug, 1980).There is a dearth of information on the diet of tadpoles especially from eastern part of India. Most of the studies on amphibians have been concentrated in the Western Ghats (biodiversity hotspot); on the west coast of India, and other areas remain understudied (Aravind and

Gururaja, 2011). Information on food provisions and feeding behaviour of the tadpoles is very essential for their successful breeding and continued existence. *Duttaphrynus melanostictus* (Schneider, 1799) is a widespread species that occurs in many south Asian countries such as India, Pakistan, Nepal, Bhutan and Myanmar (Dutta et al., 2009). It is a prolific breeder that utilizes different habitats ranging from lentic (e.g., ephemeral pools, temporary ponds, permanent ponds, gutters, puddles and cement cisternsin parks) to lotic systems (e.g., rivers, creeks and streams) for breeding (Saidapur and Girish, 2001; Rout et al., 2019).The objective of the present study was to verify the feeding spectrum of tadpoles of *Duttaphrynus melanostictus* collected from Jagatsinghpur district of Odisha, India.

MATERIALS AND METHODS

The tadpoles were collected from 35 different sites of Jagatsinghpur district (20.266°N 86.166°E), Odisha, India in order to investigate the food and feeding habits. The sampling was carried out during the rainy seasons (July to October) of 2017 and 2018 using dip net (mesh size 1mm). Soon after collection, the specimens (5 from each sampling point) were euthanized in MS-222 and preserved in 4% formaldehyde in the field itself in order to prevent complete digestion of the ingested food materials. After fixation, the tadpoles were brought to the laboratory and were sorted stage-wise according to Gosner (1960). A total of 175 tadpoles were used for diet analyses.

The gut of each tadpole was removed carefully; gut length was recorded with the help of a digital vernier caliper (Mitutoyo™) to the nearest 0.1mm. The first four centimeter of the gut was used for diet analyses. The gut contents were flushed with distilled water, taken on a Sedgewick rafter chamber and analyzed under a compound microscope (Laboscope, CMS-2). Photographs of the gut contents were taken with the help of a Sony cyber shot camera (5.1 megapixels, DCSW5) attached to the microscope. The food items were identified up to the genus level and quantified following standard procedures (Smith, 1994).

RESULTS

Intestine of tadpoles of *D. melanostictus*

The intestine of tadpoles gets drastically remodelled during metamorphosis. The intestinal epithelium is a complex structure that provides an enormous luminal surface area for efficient food processing and absorption, the primary function of the organ. The tadpole intestine has a long and simple structure. The morphology of the intestine of the *D. melanostictus* tadpoles was as under:

Stage 25: The length of the intestines ranged from 38.36 mm to 40.12 mm (39.20 ± 0.60 mm) as compared to the total length of the tadpoles (8.9 - 10.17 mm, 9.36 ± 0.52 mm). There was significant positive correlation between the lengths of tadpole and the gut ($r = 0.89$; $p<0.05$, Table 1, Fig. 1).The tadpole starts feeding at this stage.
Stage 32: The length of the intestine ranged from 48 to 89.2 mm (73.48 ± 14.06 mm) as compared to the total lengths of the tadpole (9.85 to 12.87 mm, 12.07 ± 1.48 mm). Both correlated positively ($r = 0.92$; $p<0.03$, Table 1, Fig. 1). The tadpole intestine was longer as compared to that of stage 25.

Stage 38: The length of the intestines increased considerably (r = 0.96; p<0.009, Table 1, Fig. 1) by this stage (67-123 mm, 97.6 ± 19.72 mm). The intestine continued to grow with the growth in size of the tadpole.

Stage 41: The length of the intestine at this stage ranged from 90 to 144 mm (112.8 ± 17.90 mm) as compared to the total length of the tadpoles which ranged from 17.02 to 18.32 mm (17.73 ± 0.47 mm). There was significant positive correlation between the tadpoles length and the gut lengths (r = 0.90; p<0.04, Table 1, Fig. 1).

Stage 46: This was the last stage (Gosner, 1960) in the larval development of *D. melanostictus.* The length of the intestine (32.06-52.3 mm; 42.05 ± 8.84 mm, Table 1, Fig. 1) was greatly reduced by this stage and resembled that of the adults.

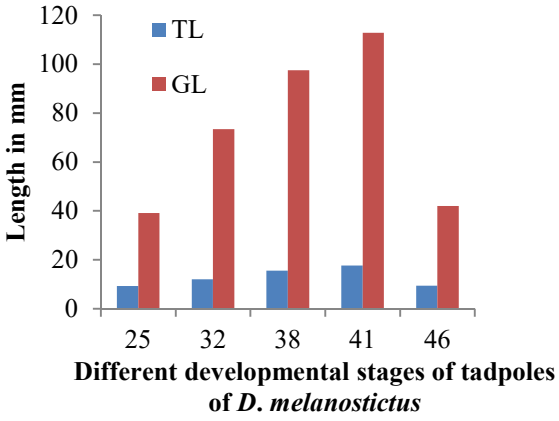


Fig. 1 Comparison of total length (TL) and gut length (GL) of tadpoles of *D. melanostictus* at different stages of development.

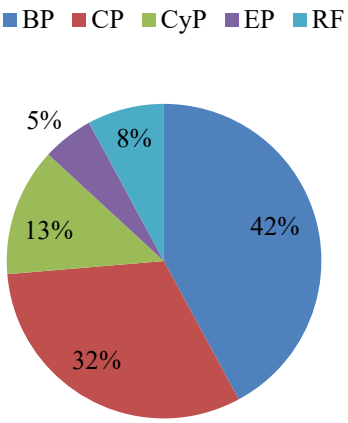


Fig.2 Percentage of food items (BP: Bacillariophyceae, CP: Chlorophyceae, CyP: Cyanophyceae, EP: Euglenophyceae, RF: Rotifera) in the diet of tadpoles

Food items of tadpoles of *Duttaphrynus melanostictus*

Qualitative analysis of gut contents of the tadpoles of *D. melanostictus* revealed that tadpoles started feeding from stage 25 onwards. The gut contents of the tadpoles mainly composed phytoplanktons like Bacillariophyceae, Cyanophyceae, Euglenophyceae, Chlorophyceae and zooplankton like Rotifera (Table 2).

Table 1 Diet of tadpoles of *Duttaphrynus melanostictus* in different developmental stages.

Food items		**Stage 25**	**Stage 32**	**Stage 38**	**Stage 41**
Phytoplankton					
Bacillariophyceae	*Amphora*	+	+	+	+
	Actinella	+	+	+	+
	Cymbella	+	+	+	+
	Cymatopleura	+	+	+	+
	Diatoma	+	+	+	+
	Fragillaria	+	+	-	-
	Frustulia	+	-	-	-
	Gomphonema	+	+	+	+
	Naviculla	+	+	+	+
	Nitzschia	+	+	+	+
	Penium	+	+	+	+
	Pinnularia	+	+	+	+
	Placoneis	+	+	+	+
	Sellaphora	+	+	+	+
	Synedra	+	+	-	-
	Tabellaria	+	+	+	+
Chlorophyceae	*Ankistrodesmus*	+	+	-	-
	Closterium	+	+	+	+
	Cosmarium	+	+	+	-
	Oedogonium	+	+	+	+
	Oocystis	+	+	+	+
	Pediastrum	+	+	+	+

	Scenedesmus	+	+	+	-
	Spirogyra	+	-	-	-
	Staurastrum	+	+	+	+
	Ulothrix	+	+	-	-
	Volvox	+	+	+	+
	Zygnema	+	+	+	-
Cyanophyceae	*Choococcus*	+	+	-	-
	Gloecapsa	+	+	+	-
	Merismopedia	+	+	+	-
	Nostoc	-	+	+	+
	Oscillatoria	+	+	-	-
Euglenophyceae	*Phacus*	+	+	-	-
	Trachelomonas	-	+	+	+
Zooplankton					
Rotifera	*Lecane*	+	+	+	-
	Lepadella	-	+	+	+
	Monostyla	-	+	-	-

+= Occurrence, - = Non occurrence

Stage 25: The phytoplankton's in the diet included Bacillariophyceae like *Amphora, Actinella, Cymbella, Cymatopleura, Diatoma, Fragillaria, Frustulia, Gomphonema, Navicula, Nitzschia, Penium, Pinnularia, Placoneis, Sellaphora, Synedra*and *Tabellaria*; Chlorophyceae like *Ankistrodesmus, Closterium, Cosmarium, Oedogonium, Oocystis, Pediastrum, Scenedesmus, Spirogya, Staurastrum, Ulothrix, Volvox* and *Zygnema*; Cyanophyceae like *Choococcus, Gleocapsa, Merismopedia*, and *Osillatoria*. Euglenophyceae like *Phacus*. (Table 2). Zooplankton includes Rotifera like *Lecane* (Table 2).

Stages 32: The Bacillariophyceae were represented by 15 genera (*Amphora, Actinella, Cymbella, Cymatopleura, Diatoma, Fragillaria, Gomphonema, Navicula, Nitzschia, Penium, Pinnularia, Placoneis, Sellaphora, Synedra*and *Tabellaria*); Chlorophyceaeby 11 genera (*Ankistrodesmus, Closterium, Cosmarium, Oedogonium, Oocystis, Pediastrum, Scenedesmus, Staurastrum, Ulothrix, Volvox* and *Zygnema*); Cyanophyceae by five genera (*Choococcus, Gleocapsa, Merismopedia, Nostoc* and *Osillatoria)* and Euglenophyceae by two genera (*Phacus* and *Trachelomonas)* (Table 2). Zooplanktons were represented by rotifers like *Lecane, Lepadella* and *Monostyla*. (Table 2).

Stages 38: *Amphora, Actinella, Cymbella, Cymatopleura, Diatoma, Gomphonema, Navicula, Nitzschia, Penium, Pinnularia, Placoneis, Sellaphora,* and *Tabellaria* (all Bacillariophyceae); *Closterium, Cosmarium, Oedogonium, Oocystis, Pediastrum, Scenedesmus, Staurastrum, Volvox and Zygnema* (Chlorophyceae); *Gleocapsa, Merismopedia and Nostoc* (Cyanophyceae); and *Trachelomonas* (Euglenophyceae) were identified from the larval gut (Table 2). Zooplankton includes Rotifers included *Lecane* and *Lepadella* (Table 2). Cannibalism was observed in the tadpole of *D. melanostictus* during the time of food shortage in the laboratory condition.

Stages 41: The tadpoles cease to feed from stage 42 onwards which coincide with the emergence of the forelimbs. From stage 46 onwards, the froglet start to feed on small invertebrates. The gut contents at this stage consists of phytoplanktons of class Bacillariophyceae (*Amphora, Actinella, Cymbella, Cymatopleura, Diatoma, Gomphonema, Navicula, Nitzschia, Penium, Pinnularia, Placoneis, Sellaphora,* and *Tabellaria*); Chlorophyceae (*Closterium, Oedogonium, Oocystis, Pediastrum, Staurastrum, Volvox*); Cyanophyceae (*Nostoc*);andEuglenophyceae (*Trachelomonas*)(Table 1). Zooplankton includes Rotifera like *Lecane* and *Lepadella* (Table 1).

Stage 46: This was the last stage (Gosner, 1960) in the larval development of *D. melanostictus.* The length of the intestine (32.06-52.3 mm; 42.05 ± 8.84 mm, Table 1, Fig. 1) was greatly reduced by this stage and resembled that of the adults.

Table 2 Species diversity in the food spectrum of tadpoles of *Duttaphrynus melanostictus* at four developmental stages (Gosner, 1960).

Food items	Developmental stages			
	25	32	38	41
	Number of species			
Baccilariophyceae	16	15	13	13
Chlorophceae	12	11	9	6
Cyanophyceae	4	5	3	1
Euglenophceae	1	2	1	1
Zooplankton	1	2	2	1

Diversity of diet in different plankton classes of *D. melanostictus* tadpoles

Among phytoplanktons, members of class Bacillariophyceae constituted the principal food items (42%) of tadpoles followed by Chlorophyceae (32%), Cyanophyceae (13%), and Euglenophyceae (5%). Zooplanktons (8%) were represented by rotifers only (Fig. 2).

DISCUSSION

Anuran larvae are some of the least understood in terms of their trophic relations (Altig et al., 2007). They act as a link between aquatic and terrestrial ecosystems since the material consumed by the larvae in the aquatic habitat will be carried to the terrestrial environment by the adults (Wassersug, 1972). The tadpoles need to be metamorphosed in a short period utilizing the available sources of nutrients and escaping from desiccation. Diet has a crucial role in the natural history of an animal, because not only does it reveal the source of the animal's energy for growth, maintenance, and/or reproduction (Dunham et al., 1989; Zug et al., 2001), but also it indicates part of the ecological roles of the animal. Tadpoles of various species are often morphologically different and feed on different food items to reduce competition in single water bodies (Harrison, 1987). They feed at many sites throughout the water column (benthic, mid water, surface) and have characteristic morphologies and behaviour (McDiarmid and Altig, 1999). Dietary descriptions of a species from different localities are essential as it records temporal and spatial variations in the diet of a species (Mahan and Johnson, 2007; Araujo, et al., 2009; Quiroga et al., 2011).

The selection of microhabitat is an important aspect of *D. melanostictus* as it plays a key role in the survival and growth (Rout et al., 2019). With a less developed tail and weak tail musculature, they are

not good swimmers and thus adopted to survive in shallow waters**.** The result of the gut content analyses revealed that apart from a large amount of detritus, the tadpole diet was largely based on microalgae which was also corroborated by several other workers (Lajmanovich, 2000; Rossa-Feres et al., 2004). Detritus, packed along the length of larval intestine, is mostly composed of degraded plant materials, which often bears little resemblance to the original plant tissue in terms of its structure and nutritional content. Much of the nutritional value of detritus may come from associated microbes than its particles *per se* (Cummins and Klug, 1979). We identified prey items mainly from class Bacillariophyceae**,** Chlorophyceae, Euglenophyceae and Cyanophycae. Diet composition of tadpoles revealed members of class Bacillariophyceae to be the most important prey category, an observation similar to Sinha et al., 2001 and Lalremsanga et al., 2013. The importance of Bacillariophyceae as a food source has also been reported for other anuran genera such as *Lithobates, Dendrosophus, Eupemphix* and *Scinax* (Rossa-Feres et al., 2004). Bacillariophyceae can be richer in calories, mainly as a form of lipids and they are more easily accessible for consumption than filamentous algae (Kupferberg et al., 1994). Being a source of carbohydrates, chlorophytic algae also form another important food source (Bold and Wynne, 1985). The zooplanktons as seen from tadpole diet were represented by class Rotifera, an observation similar to Narzary and Bordoloi, 2012. The diet preference and choice of algae as food indicates that the conservation of habitat in terms of algal diversity is essential for the survival and successful completion of life cycle of amphibian tadpoles.

The development of tadpole is dependent on a number of variables such as predation, competition, food availability etc. The amount of food consumed by a tadpole directly affects its growth (Kiffney and Richhandson, 2001). Sahu and Khare (1983) described that tadpoles, in the early part of life history, are herbivorous which changes to carnivory in the post metamorphosis stages. Studies of gut contents by Sinha et al. (2001) revealed that tadpoles are both phyto- and zoo-plankton feeder. Hota and Dash (1981) experimentally found that in *Bufo stomaticus,* a mixed diet of animal and plant products promoted the best larval growth but there was a decline in growth rate and a delay in metamorphosis of tadpoles grown only on animal products. The present study established that the tadpoles of *D. melanostitus* use both phytoplankton and zooplanktons. The wide spectrum of food choices indicated that they are highly adapted to various habitats: stream, ponds, lake, and lotic connected shallow standing pools.

Altig and Kelly (1974) measured gut lengths and examined gut organic contents of thirteen species of tadpoles. They suggested that species feeding primarily on animal material should have relatively shorter guts while those feeding on plant materials and detritus should have relatively longer guts. Tadpoles of *D. melanostictus* have short gut length (40-43mm). In the present study only diversity of food items present in the gut were studied. The study suggested only a qualitative analysis of food ingested in the habitat under the prevailing ecological condition.

In tropical aquatic ecosystems, the study of the natural diet of resident species is an important tool in understanding the biotic and abiotic interrelationships. Diet analysis of larvae provides valuable information on foraging pattern, nutritional requirements and trophic interaction in aquatic food webs which is critical for successful conservation and management. Habitat degradation and scarcity of food may result in poor survival which, in turn, will affect the population of anurans in nature. Globally, the amphibians are more threatened as compared to other groups of animals with approximately 48% of the more than 8,000 amphibian species on the planet are threatened with extinction. It is therefore essential

to conserve the aquatic habitats which form the most important component for reproduction and successful completion of lifecycle of anurans. Awareness among the people needs to be generated regarding the significance of anurans in ecosystem so that conservation strategies can be formulated and executed with the help of the local people. Further, such knowledge also indicates the susceptibility of the species in light of the current environmental alterations. Understanding the natural diet of tadpoles can help in developing management strategies for them.

REFERENCES

Altig R and Kelly JP (1974) Indices of feeding in anuran tadpoles as indicated by gut characteristics. *Herpetologica*, 31:67-69.

AltigR, Whiles MR and Taylor CL (2007) What do tadpoles really eat? Assessing the trophic status of an understudied and imperilled group of consumers in freshwater habitats. *Freshwater Biology*, 52:386-395.

Araújo MS, Bolnick DI, Martinelli LA, Giaretta AA and Dos Reis SF (2009) Individual-level diet variation in four species of Brazilian frogs. *Journal of Animal Ecology*, 78(4):848-856.

Aravind NA and Gururaja KV (2011) Theme paper on the amphibians of the Western Ghats. Report Submitted to Western Ghats Ecology Expert Panel. Ministry of Environment and Forests (MoEF), Govt. of India.

Bold HC and Wynne MJ (1985) Introduction to the Algae. Structure and Reproduction. Englewood Cliffs. New Jersey, Prentice-Hall.

Cummins KW and Klug MJ (1979) Feeding ecology of stream invertebrates. *Annual Review of Ecology and Systematics*, 10:147-172.

Dunham AE, Grant BW and Overall KL (1989) Interfaces between biophysical and physiological ecology and population ecology of terrestrial vertebrate ectotherms, *Physiological Zoology*, 62(2):335-355.

Dutta SK, Nair MV, Mohapatra PP and Mahapatra, AK (2009) Amphibians and reptilesof Similipal Biosphere reserve. Regional Plant resource Centre, Nayapalli, Bhubaneswar.

Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*, 16:183-190.

Harrison JD (1987) Food and feeding relations of common frogs and common toad tadpoles (*Rana temporaria* and *Bufobufo*) at a pond in mid-Wales. *Journal of Herpetology*, 1:141-143.

Hero JM, Galatti U (1990) Characteristics distinguishing Leptodactylus pentadactylus and Leptodactylus knudseni in the Central Amazonian Rainforest. J Herpetol 24:227-228.

Heyer WR, McDiarmid RW, Weigmann DL (1975) Tadpoles, predation, and pond habitats in the tropics. Biotropica 7:100-111.

Heyer WR (1976) Studies in larval amphibian habitat partitioning. *Smithsonian Contributions to Zoology*, 242:1-27.

Hota AK and Dash MC (1981) Growth and metamorphosis of *Rana tigrina* larvae: Effects of food level and larval density. *Oikos*, 37:349-352.

Inger RF (1986) Diets of tadpoles living in Bomean rain forest. *Alytes*, 5:153-164.

Kiffney PM and Richardson JS (2001) Interactions among nutrients, periphyton, and invertebrate and vertebrate (*Ascaphustruel*) grazers in experimental channels. *Copeia*, 2:422-429.

Kupferberg SJ, Marks JC and Power ME (1994) Effects of variation in natural algal and detrital diets on larval anuran (*Hylaregilla*) life-history traits. *Copeia*, 1994:446-457.

Lajmanovich RC (1997) Alimentación de larvas de anurosenambientestemporalesdelsistema del río Paraná, Argentina. *DoñanaActa Vertebrata*, 24:191-202.

Lajmanovich RC (2000) Interpretaciónecológica de unacomunidadlarvaria de anfibiosanuros. *Interciencia*, 25:71-79.

Lalremsanga HT, Hooroo RNK andLalronung S (2013) Oral morphology of the tadpoles of *Euphlyctiscyanophlyctis* (Schneider, 1799) with notes on feeding behaviour. *Science and Technology Journal*, 1(2):58-69.

Mahan RD and Johnson JR (2007) Diet of the gray tree frog *(Hyla Versicolor)* in relation to foraging site location. *Journal of Herpetology*, 41(1):16-23.

Mahapatra, S, Dutta SK and Sahoo G (2017) Opportunistic predatory behaviour in *Duttaphrynus melanostictus* (Schneider, 1799) tadpoles. *Current Science,* 112(8):1755–1759

McDiarmid RW and Altig R (1999) Tadpoles: The Biology of anuran larvae. Chicago, London, University Chicago Press.

Narzary J and Bordoloi S (2012) A study on certain common pond breeding anurans and their tadpoles in a pond of western Assam, India. *International Journal of Advanced Biological Research,* 2(2):342-348.

Quiroga MF, Bonansea MI, and Vaira M (2011) Population diet variation and individual specialization in the poison toad, *Melanophryni scusrubriventris* (Vellard, 1947). *Amphibia-Reptilia*, 32(2):261-265.

Rossa-Feres DC, Jim J and Fonseca MG (2004) Diets of tadpoles from a temporary pond in south-eastern Brazil (Amphibia, Anura). *Rrevista Brasileira de Zoologia* 21(4):745-754.

Rout J, Mahapatra S, Asrafuzzaman S, Dutta SK and Sahoo G (2019) Oviposition site selection by the Asian Common Toad *Duttaphrynus melanostictus* (Schneider, 1799), in an Indian biosphere reserve. *Herpetozoa* 31:157-171.

Sahu AK and Khare MK (1983) Field key of *Rana alticola* Annalldale (Anura: Ranidae) tadpoles. Journal of Bombay natural History Society 80(1):144-148.

Saidapur SK and Girish S (2001) Growth and metamorphosis of *Bufo melanostictus* tadpoles: Effects of kinship and density. *Journal of Herpetology*, 35:249-254.

Sinha BP, Chakravarty P, Borah MM and Bordoloi S (2001) Qualitative analysis of food spectrum of five species of anuran tadpoles from Arunachal Pradesh, India. *Zoos' print Journal*, 16:514-515.

Smith JL (1994) Arthritis and food borne bacteria. *Journal of Food Protection,* 57:935-941.

Wassersug R (1972) The mechanism of ultraplanktonic entrapment in anuran larvae. *Journal of Morphology*, 137:279-288.

Wassersug RJ (1980) Internal oral features of tadpoles from eight anuran families: Functional, systematic, evolutionary and ecological considerations. *Miscellaneous publication, Museum of Natural History*, 68:1-146.

Wassersug RJ, Frogner KJ and Inger RF (1981) Adaptations for life in tree holes by rhacophorid tadpoles from Thailand. Journal of Herpetology 15:41-52.

Zug GR, Vitt LJ and Caldwell JP (2001) Herpetology. An Introductory Biology of Amphibians and Reptiles, San Diego, Academic Press.

INSTRUCTIONS TO AUTHORS

Scope of the Journal

Pranikee is published by the Zoological Society of Orissa, Post Graduate Dept. of Zoology, Utkal University, Bhubaneswar-751 004, India and accepts English-language manuscripts concerning all kinds of biological processes including biochemistry and biosynthesis, physiology and metabolism, molecular genetics, molecular biology, genomics, proteomics, molecular farming, biotechnology/genetic transformation, nanobiotechnology, bioinformatics and systems biology, cell and developmental biology, stem cell biology, and reproductive biology.

Submission Process

All manuscripts must be submitted electronically to the editor of the journal. Papers are accepted for publication on the understanding that they have not been published and are not going to be considered for publication elsewhere. Authors should certify that the manuscript has already been published or submitted for publication in another journal. Authors are required to fill the Author's Agreement form and the form must accompany all papers submitted. Manuscripts may be rejected if they are beyond the scope of the journal.

Preparation of Manuscript

Style and format:

Manuscripts should be double-spaced with 3-cm margins on all sides of the page, in Times New Roman font. Every page of the manuscript, including the title page, references, tables, etc., should be numbered. All text, tables and figures should be sent in MS word document. After the acceptance of the manuscript, authors will be contacted to provide the jpeg format of the figures.

Symbols, units, and abbreviations

The journal follows the general conventions of Scientific Style and Format. If symbols such as χ, μ, η or ν are used, they should be added using the Symbols menu of Word. Degree symbols (°) must be used from the Symbol menu, not superscripted letter o or number 0. Multiplication symbols must be used (×), not the letter x. Spaces must be inserted between numbers and units (e.g., 3 kg) and between numbers and mathematical symbols (+, –, ×, =, <, >), but not between numbers and percent symbols (e.g., 45%). Please use SI units. All abbreviations and acronyms should be defined at first mention. Latin terms such as et al., in vitro, or in situ should not be italicized.

Manuscript content

Research articles should be divided into the following sections - Introduction, Materials and Methods, Results, Discussion, and References. Acknowledgements, if given, may be added after Discussion.

Title and contact information

The first page should contain the full title in sentence case, the full name and affiliations of all authors, and the contact e-mail address for the clearly identified corresponding author.

Abstract
The abstract should provide clear information about the research and the results obtained, and should not exceed 200 words. The abstract should not contain citations.

Key words
Please provide no more than 5 key words.

Introduction
This should argue the case for your study, outlining only essential background, and should not include the findings or the conclusions. It should not be a review of the subject area, but should finish with a clear statement of the question being addressed.

Materials and methods
Please provide concise but complete information about the materials and the analytical and statistical procedures used. This part should be as clear as possible to enable other scientists to repeat the research presented.

Results
The same data or information given in a Table must not be repeated in a Figure and vice versa. It is not acceptable to repeat extensively the numbers from Tables in the text or to give lengthy explanations of Tables or Figures.

Discussion
Statements from the Introduction and Results sections should not be repeated here. The final paragraph should highlight the main conclusions of the study; do not include "Conclusions" as a separate heading.

Acknowledgements and/or disclaimers, if any
Names of funding organisations should be written in full.

References
References should be cited in the text by the last name(s) of the author(s) and year of publication with a comma between them: for example, (Das, 1987) or (Maden and Maden 2012). If the citation is the subject of the sentence, only the date should be given in parentheses: "According to Gardiner (1987)…" For citation of references with 3 or more authors, only the first author's name followed by et al. (not italicised) should be used: (Pattnaik et al., 2002). If there is more than one reference in the same year for the same author, please add the letters a, b, etc. to the year: (Mishra et al., 2004a, 2004b). References should be listed in the text chronologically, separated by semicolons: (Dutta, 1987; Pattnaik et al., 2002; Mishra et al., 2004a, 2004b). References should be listed alphabetically at the end of the text without numbering. Journal titles should be abbreviated according to ISI Web of Science abbreviations.

References should be formatted as follows

Journal articles

Janda JM and Abbott SL (2010) The genus Aeromonas: taxonomy, pathogenicity, and infection. *Clin Microbiol Rev.*, 23:35–73.

Books

Novitski E (1977) Human Genetics. 3rd edition. New York, USA, Macmillan Publishing Co. Inc., pp 6-7.

Chapter in book

Lafont F, Ikonen E and Simons K (2010) Permeabilized epithelial cells to study exocytic membrane transport. In: Kreitzer G, Celis JE, editors. Cell Biology Assays: Essential Methods. 1st ed. London, UK, Academic Press, pp 3–12.

Conference proceedings

Zahar M, Benkerroum N, Guerouali A, Baou SA and Alahiane L (2002) Biological ensiling of sardine wastes in sugarcane molasses for their valorization in animal feeding: Microbiological study. In: *Proceedings of International Symposium on Environmental Pollution Control and Waste Management 7-10 January 2002, Tunis, Tunisia,* pp 304-311.

Thesis

Das M (2013) A Comparative study on biochemical and morphological aspects of blood in Rhacophorid anurans of Orissa. Ph. D. Thesis, Utkal University, Odisha, India.

Tables and Figures

All illustrations (photographs, drawings, graphs, etc.), not including tables, must be labelled "Figure." Figures must be submitted in the manuscript. All tables and figures must be numbered consecutively as they are referred to in the text. The tables and figures themselves should be given at the end of the text only, after the references, not in the running text. For all tables, please use Word's "Create Table" feature. Figures must be submitted in jpeg format, if manuscript is accepted.

Correspondence Address

Only electronic copy of the manuscripts should be submitted to editorpranikee@gmail.com

for correspondence

Secretary
Zoological Society of Orissa,
P. G. Department of Zoology,
Utkal University, Vani Vihar,
Bhubaneswar,
Odisha, India.
PIN 751 004

Zoological Society of Orissa

Brief History

Pranikee, the annual journal of Zoological Society of Orissa, publishes original research articles on Zoology.

The Society was founded in 1958 in order to promote effective communication between Zoologists through its publications, seminars and annual meetings.