Evaluation of peptide-based approach for estimation of NSE and S-100$\beta\beta$ towards the development of a cost-effective test for prognosis of AIS patients

Amit R. Nayak[1], Neha H. Lande[1], Anuja P. Kawle[1], Dinesh P. Kabra[1], Nitin H. Chandak[1], Hemant J. Purohit[2], Girdhar M. Taori[1], Hatim F. Daginawala[1] and Rajpal S. Kashyap[1,*]

[1]Biochemistry Research Centre, Central India Institute of Medical Sciences, Nagpur 440 020, India
[2]Environmental Genomics Unit, National Environmental Engineering Research Institute, Nehru Marg, Nagpur 440 020, India

Here we compare the in-house ELISA technique for estimation of neuron-specific enolase (NSE) and S-100$\beta\beta$ (glial-specific protein) peptides with a commercial kit in serum samples of acute ischaemic stroke (AIS) patients. Nineteen improved and four expired AIS patients were included for the study. NSE concentrations were significantly higher ($P < 0.05$) in expired as compared with improved AIS patients by both in-house ELISA and commercial kit method. Estimation of S-100$\beta\beta$ by only in-house ELISA showed significantly high ($P < 0.05$) levels in expired AIS patients. Peptide-based estimation of NSE and S-100$\beta\beta$ may be used for prognosis of AIS patients.

Keywords: Acute ischaemic stroke, glial-specific protein, neurological disease, neuron-specific enolase, prognosis.

EARLY diagnosis and prognosis of stroke is of utmost importance for clinicians in planning appropriate management strategies for acute ischaemic stroke (AIS) patients. In the current scenario, diagnosis and prognosis of AIS patients basically rely upon computed tomography (CT) scanning/magnetic resonance imaging (MRI), which is somewhat unsatisfactory. CT scan has limited sensitivity in detecting minor changes in the brain. MRI is known to be superior to CT scan, and overcomes all the limitations associated with CT[1]. However, MRI is a costly and cannot be performed repeatedly, and is unavailable in most hospital settings. There is a need for alternative methods which can be used for early diagnosis and prognosis of AIS patients.

During the last two decades, numerous biomarkers of the central nervous system (CNS), as well as non-CNS origins, have been evaluated in the body fluids of AIS patients for predicting any adverse neurological prognosis in them[2]. Some of these are neuron-specific enolase (NSE), S-100$\beta\beta$ (glial-specific protein), myelin basic protein, creatine kinase isoenzyme BB, tau protein, polyamines and matrix metalloproteinase-9 (MMP-9) which are found to be promising markers in predicting the prognosis of AIS patients[3,4]. Out of these, NSE and S-100$\beta\beta$ are the most widely studied biomarkers for evaluating the prognosis of AIS patients[5]. Although NSE and S-100$\beta\beta$ estimation is economical compared to CT and MRI, its use is limited in the developing countries because of unavailability and high import cost. Therefore, development of indigenous and more cost-effective experimental protocols is required for the estimation of NSE and S-100$\beta\beta$ on a routine basis for prognosis of AIS patients.

In the present study, we compare our in-house cost-effective enzyme-linked immunosorbent assay (ELISA) developed using anti-peptide antibodies produced against selected peptides of NSE (i.e. NSE peptides 1 and 2) and S-100$\beta\beta$ (i.e. S-100$\beta\beta$ peptides 1 and 2) with a commercially available ELISA kit for the evaluation of predictive values of the test in serum samples of improved and expired AIS patients.

Forty AIS patients who were admitted to the Central India Institute of Medical Sciences (CIIMS), Nagpur, from December 2010 to May 2011 were included in the study. Diagnosis of AIS patients was based on the WHO definition of stroke, i.e. 'rapidly developing signs of focal (or global) disturbance of cerebral function lasting >24 h (unless interrupted by surgery or death), with no apparent non-vascular cause or history, neurological examination and CT'. All patients were admitted to the intensive care unit (ICU), where the ambient temperature was maintained between 20°C and 25°C. The protocol for this study was reviewed and approved by the Institutional Ethics Committee of CIIMS.

Patients with haemorrhagic stroke, transient ischaemic attack, brain malignancies, and those who had undergone brain surgery, severe systemic disease, dementia, psychiatric disease and active infection were excluded from the study. Patients who refused to participate and who took discharge against medical advice were also excluded from the study.

Detailed history was taken and CT scan was performed within 12 h of admission to exclude patients with stroke mimic, and severity of stroke was evaluated using National Institute of Health Stroke Scale (NIHSS). Based on the NIHSS score AIS patients were classified into four groups, i.e. score of 0 = no stroke, 1–6 = minor stroke, 7–18 = moderate stroke, 19–42 = severe stroke. The modified Rankin scale (mRS) was used for evaluation of prognosis of AIS patients by the clinician at the time of discharge.

Blood samples were taken from AIS patients at the time of admission. Similarly, blood samples were also taken from a healthy individual as control sample ($n = 10$). Blood was allowed to clot and after centrifugation (100 *g*

*For correspondence. (e-mail: raj_ciims@rediffmail.com)

Table 1. List of anti-neuron-specific enolase (NSE) peptide and anti-S-100$\beta\beta$ (glial-specific protein) peptide antibodies produced against the selected antigenic sequence of NSE and S-100$\beta\beta$

Antibody produced	Start position	Selected antigenic sequence of NSE and S-100$\beta\beta$	End position
Anti-NSE peptide-1	18	PTVEVDLYTA-C	27
Anti-NSE peptide-2	58	LGKGVLKAVDH-C	68
Anti-S-100$\beta\beta$ peptide-1	5	EKAMVALIDVFHQ-C	17
Anti-S-100$\beta\beta$ peptide-2	49	C-KEQEVVDKVM	58

Table 2. Sample dilution and antipeptide antibody dilution used for the estimation of NSE and S-100$\beta\beta$ in the serum sample of acute ischaemic stroke (AIS) patient

Type of marker	Anti-NSE peptide-1	Anti-NSE peptide-2	Anti-S-100$\beta\beta$ peptide-1	Anti-S-100$\beta\beta$ peptide-2
Sample dilution	1 : 200	1 : 200	1 : 500	1 : 200
Primary Ab dilution	1 : 5000	1 : 1000	1 : 10000	1 : 5000
Secondary Ab dilution	1 : 10000	1 : 10000	1 : 10000	1 : 10000

for 10 min) the serum was separated and stored at –20°C until further use.

Serum NSE levels were estimated using Can Ag NSE EIA kit (Sweden) according to instructions of the manufacturer. This test is based on a solid-phase, non-competitive immunoassay using two monoclonal antibodies (derived from mice) directed against two separate antigenic determinants of NSE molecule. The monoclonal antibodies (MAbs) used bind to the γ-subunit of the enzyme and thereby detect both $\gamma\gamma$ and $\alpha\gamma$ which are iso-enzymes of NSE. All the analyses were carried out in triplicate.

Quantitative determination of S100$\beta\beta$ in human serum was also performed Can Ag S100$\beta\beta$ EIA (Sweden). This is a two-step enzyme immunometric assay (EIA) based on two monoclonal antibodies derived from mouse, specific for two different epitopes of S100$\beta\beta$. All the analyses were carried out in triplicate.

The antigenic peptides of NSE and S100$\beta\beta$ were determined based on the method of Kolaskar and Tongaonkar (1990) using on-line software (Molecular Immunology Foundation (MIF)-Bioinformatics software) and reference sequences of NSE (sequence ID: NP_001966.1) and S100$\beta\beta$ (sequence ID:NP_006263.1) available in the National Centre for Biotechnology Information (NCBI) reference sequence database are mentioned as above. These antigenic peptide sequences were then subjected BLAST analysis of NCBI, to obtain the sequence similarities with other non-redundant protein database sequences. Based on the results of the BLAST analysis, four antigenic sequences (two of NSE and two of S-100) were finally selected, namely NSE peptide 1 (PTVEVDLYTA-C), NSE peptide 2 (LGKGVLKAVDH-C), S-100$\beta\beta$ peptide 1 (EKAMVALIDVFHQ-C) and S-100$\beta\beta$ peptide 2 (C-KEQEVVDKVM). These peptide sequences were sent for synthesis followed by antibody production in GenicBio Limited, Shanghai, China. The purity of the synthesized peptides was checked using HPLC and their quantification was done using mass spectrometry. All the designed peptides were synthesized with purity >90%. Specificity of the produced antibodies was checked by evaluating them in comparison with the pre-immune serum collected before immunizing the rabbits with the peptides. The produced antibodies were not cross-reacting with any other serum protein (data not given). The anti-peptides produced were named based on their peptide name (Table 1).

For the estimation of NSE and S-100$\beta\beta$ using anti-peptide antibody, a microtiter ELISA well plate was coated with 100 µl serum samples and incubated for 45 min at 37°C. The plate was then washed once with wash buffer, i.e. 0.5% Tween-20 in phosphate buffered saline (PBST). Microtitre wells were then blocked by adding 200 µl of blocking buffer (0.5% BSA in PBST) and incubated at room temperature for 90 min. The wells were washed again three times with PBST. Then 100 µl (concentration of the antibody stock is 1 mg/ml, which is then further diluted to working concentration as mentioned in Table 2) of primary antibodies (anti-peptide antibody raised in rabbit) was added and incubated for 45 min. The wells were again washed three times with PBST. Then 100 µl anti-rabbit HRP conjugated secondary antibody (dilution 1 : 10,000) was added and incubated at 37°C for 45 min. After incubation, the microtitre wells were washed four times with PBST and antibody reactivity was detected by adding 100 µl tetramethylbenzidine hydrogen peroxide (TMB/H_2O_2) substrate. The reaction was then stopped 5 min after adding 100 µl of 2.5 N sulphuric acid (H_2SO_4) and intensity of the developed colour was measured at 450 nm in an ELISA reader.

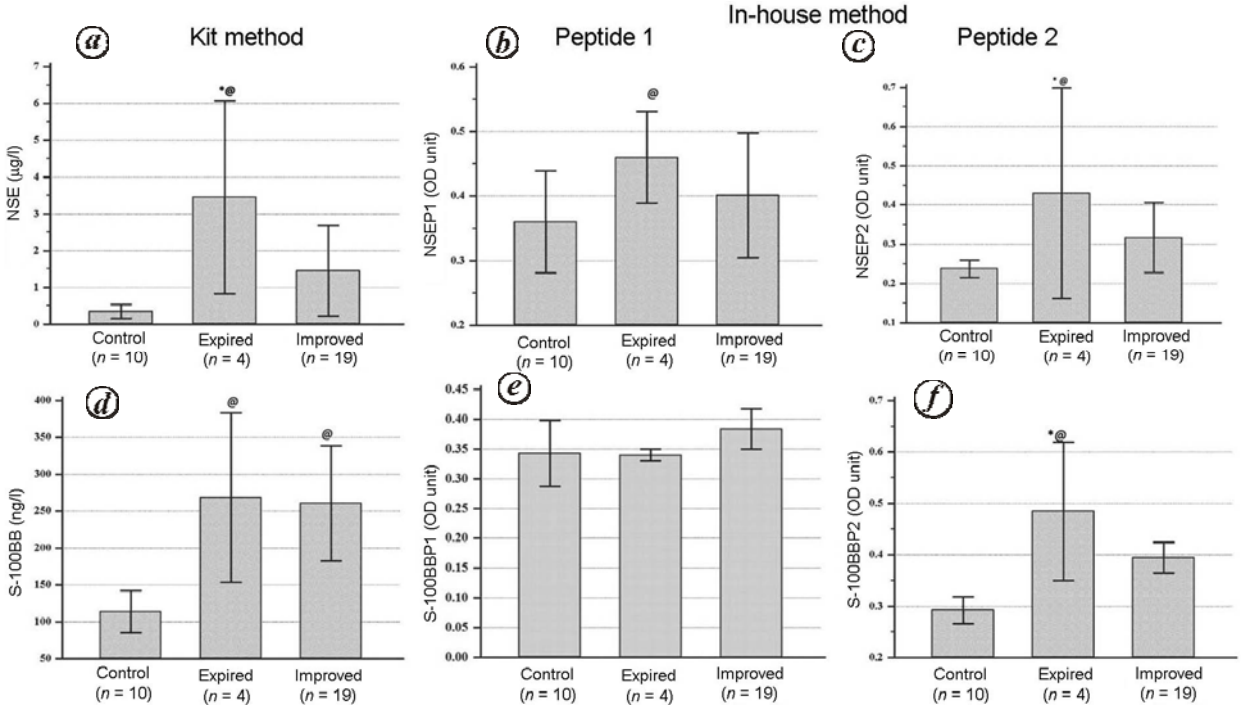

Figure 1. Estimation of serum level of neuron-specific enolase (NSE) and glial-specific protein (S-100$\beta\beta$) in control individuals and acute ischaemic stroke (AIS) patient. *$P < 0.05$ versus improved AIS patients; $^{@}P < 0.05$ versus control.

Table 3. Clinical characteristics of the studied AIS patients ($n = 23$)

Characteristics	Improved	Expired	P value
n	19	4	
Age (years)	51 ± 17	53 ± 13	0.8
Sex (M/F)	13 (68)/6 (32)	3 (75)/1 (25)	0.7
Admission within 24 h	14 (74)	3 (75)	0.5
Duration in the hospital (days)	13 ± 15	4 ± 3	0.2
Associated risk factors			
HTN	8 (42)	2 (50)	0.8
DM	8 (42)	1 (25)	0.9
CVE	10 (53)	2 (50)	0.7
IHD	2 (11)	0	0.8
Past history of stroke	2 (11)	0	0.8
Smoking habit	2 (11)	1 (25)	0.9
Drinking habit	3 (16)	2 (50)	0.4
Other disorders	9 (47)	2 (50)	0.6
Thrombolysis	3 (16)	3 (75)	0.07
Decompression surgery	2 (11)	0	0.8
NIHSS score at admission	12 ± 5	19 ± 10	0.04

Informed consents were taken from all enrolled participants and their kin for the study. As mentioned earlier, the study was approved by the Institutional Ethical Committee of CIIMS.

All the statistical analysis was performed using MedCalc (version 13.1). Test for proportion was used to compare baseline characteristics associated with the improved and expired AIS patients. Similarly, a t-test was used to compare the mean values of NSE and S100$\beta\beta$ between expired and improved AIS patients. The statistical level of significance was set at $P < 0.05$.

Out of 40 patients, 23 were finally included in the study which comprised of 19 improved and four expired AIS patients. The baseline and clinical characteristics of improved and expired AIS patients showed that there was no significant difference between them except for the severity score (NIHSS). Expired AIS patients showed significantly higher ($P < 0.05$) NIHSS score (18 ± 3) compared to improved AIS patients (12 ± 5) (Table 3).

Figure 1 shows the mean levels of NSE protein estimated by the kit method and peptide-based approach in serum samples of improved and expired AIS patients and control individuals. The mean levels of NSE concentration (kit method) were significantly higher in expired AIS patients compared to improved AIS patients and control samples (Figure 1 *a*). Similar results were observed when NSE was estimated by in-house ELISA using anti-NSE anti-peptides 1 and 2 (Figure 1 *b* and *c*).

Figure 1 *d–f* shows the mean levels of S-100$\beta\beta$ protein estimated by the kit method and peptide-based approach. The result shows that although the level of S-100$\beta\beta$ increases ($P < 0.05$) in the serum samples of AIS patients, there is no significant difference in the mean S-100$\beta\beta$ concentration (kit method) in expired and improved AIS patients (Figure 1 *d*). On the contrary S-100$\beta\beta$ estimation using in-house ELISA with S-100$\beta\beta$ anti-peptide shows significantly high ($P < 0.05$) S-100$\beta\beta$ levels in expired compared to improved AIS patients when tested with S-100$\beta\beta$ peptide 2 (Figure 1 *f*).

During the past few years, use of anti-peptide antibodies has been widely reported in the diagnosis of various diseases. Pattnaik *et al.*[6] have shown that anti-peptide antibodies produced against peptides of conserved regions of flaviviruses selectively recognized flaviviruses. Dambinova *et al.*[7] evaluated the anti-*N*-methyl-D-aspartate receptor subunit NR2 (anti-NR2) peptide antibody for the detection of NR2 protein levels for diagnosis of AIS patients. Santana *et al.*[8] reported monospecific antibody generated against synthetic peptides of interleukin-8 (IL-8) as a versatile tool for detecting IL-8 by different immune techniques such as ELISA, dot blot, Western blotting and immunocytofluorescence. Although peptide designing and antibody production is a costly affair, if the peptide and anti-peptide are prepared in bulk, it will reduce the cost of synthesis. Similarly, the amount of antibody produced will be sufficient to perform the test on a large number of samples without the problem of batch-to-batch variation. Further, the development of in-house protocol using peptide and anti-peptide further reduces the cost of the test, since there is no involvement of dealers, distributors or manufactures. Thus, adapting the peptide-based approach is more cost-effective than the market kit. Apart from this, use of synthetic peptides and anti-peptide antibodies instead of whole antigen avoids the possibilities of non-specific cross-reactivity and increases the possibility of specific reactivity. Therefore, the development of antipeptide antibody-based diagnostic or prognostic test could be a cost-effective approach.

In the present study, we compared the levels of NSE and S-100$\beta\beta$ in serum samples of AIS patients using anti-peptide antibodies and a commercial kit for the development of cost-effective prognostic test. We found that the NSE concentration using the kit method is significantly higher in expired patients compared with improved AIS patients at the time of admission. Similarly, anti-NSE-peptides 1 and 2 antibodies are able to differentiate between expired and improved AIS patients. There is no difference in S-100$\beta\beta$ concentration between improved and expired AIS patients when estimated by the kit method. However, estimation of S-100$\beta\beta$ concentration using anti-S-100$\beta\beta$ peptides shows significantly higher S-100$\beta\beta$ levels in expired AIS patients when tested with anti-S-100$\beta\beta$ peptide 2 antibodies.

Normally NSE is present only in negligible amounts in the peripheral blood. Elevated NSE levels were reported within 4 h after the onset of stroke, where it has a half-life of around 48 h (ref. 9). It has been reported to be an indicator of infarction volume and worse neurological outcome after ischaemic stroke[10]. Tiainen *et al.*[11] have reported that a decrease in serum NSE levels after therapeutic hypothermia attenuates neuronal death in cardiac arrest patients. We observed higher NSE concentration among expired AIS patients. This confirms the current findings which suggest that serum NSE levels could be an indicator of severity and a bad prognosis. Estimation of NSE using anti-peptides has also shown similar results.

Similar to NSE, serum S100$\beta\beta$ protein levels were known to increase 8 h after the onset of stroke[12,13], which then reached peak values at 24 h (ref. 14). Blood S-100$\beta\beta$ levels were also reported to correlate with severity and functional outcome in stroke patients[15]. However, the difference is best described in the blood samples collected between 48 and 72 h of a stroke[15,16]. In our earlier study, we also reported higher levels of S-100$\beta\beta$ in an expired patient at 72 h (ref. 17). We did not observe any difference in S-100$\beta\beta$ levels between improved and expired AIS patients using the kit method, while the estimation of S-100$\beta\beta$ by anti-S-100$\beta\beta$ peptide-2 antibody showed a higher concentration in expired AIS patients even at the time of admission. S-100$\beta\beta$ is an acidic calcium-binding protein found in glial and Schwann cells[18]. It has also been reported in other cell types[19]. Expression of NSE is reported only in neurons and cells of neuroendocrine origin[20]. The higher pool of S-100$\beta\beta$ proteins from all cell types could be possibly masking the alteration in S-100$\beta\beta$ concentrations specifically occurring due to neuronal injury. The antibody produced against the specifically designed peptides and anti-peptides of S-100$\beta\beta$ showed higher specificity compared to the kit method for estimation of S-100$\beta\beta$ at the time of admission.

The results of the present study suggest that in-house ELISA developed using the selected peptides and anti-peptides of NSE and S-100$\beta\beta$ can be used in a cost-effective manner for prediction of severity and mortality in AIS patients. The study also opens an avenue for the development of less expensive diagnostic protocols.

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ACKNOWLEDGEMENT. This work was supported by the Department of Biotechnology, Government of India grant under the project no. BT/PR14368/MED/30/525/2010.

Received 21 April 2015; revised accepted 5 July 2015

doi: 10.18520/v109/i8/1466-1470

Molecular analysis of genetic stability in *in vitro* regenerated plants of broccoli (*Brassica oleracea* L. var. *italica*)

Pankaj Kumar, Geetika Gambhir, Ayesh Gaur and D. K. Srivastava*

Department of Biotechnology, Dr Y. S. Parmar University of Horticulture and Forestry, Solan 173 230, India

Genetic integrity of tissue culture-raised plantlets of broccoli cv. Solan green head from India was assessed using RAPD. First, highly efficient, reliable and high-frequency shoot regeneration was achieved in leaf (62.96%) and petiole (91.11%) explants on MS medium containing 4.5 mg/l BAP + 0.019 mg/l NAA, and 4.0 mg/l BAP and 0.5 mg/l NAA respectively. Maximum rooting ability (93.99%) with healthy and vigorous roots was observed on MS medium containing 0.20 mg/l NAA. The regenerated plantlets with well-developed shoot and root system were acclimatized successfully. For genetic stability studies, a total of 66 amplicons were amplified using 15 informative primers with a high degree of monomorphism (88.45%) across the mother plant and 20 randomly selected *in vitro* regenerated plantlets.

Keywords: Broccoli, genetic fidelity, leaf and petiole explants, plant regeneration, RAPD–PCR.

BROCCOLI is an important vegetable crop of the cabbage family Brassicaceae (formerly Cruciferae) with chromosome number $2n = 18$. It is nutritionally rich with medicinal property and classified as the *italica* cultivar group of the species *Brassica oleracea.* It is high in vitamins C and A, soluble fibre and contains the medicinally important anticancerous compound sulphoraphane with potential application in the pharmaceutical industry[1,2]. Plant tissue culture is an important aspect of plant biotechnology because genetic manipulation is now necessary to harness its potential to overcome crop yield losses due to biotic and abiotic stresses. So establishment of a highly efficient, reliable and stable plant regeneration system without the risk of genetic instability is a major step in genetic improvement. Scaling-up of any micropropagation protocol is severely hindered due to incidence of somaclonal variations[3]. The occurrence of somaclonal variations is a potential drawback when the propagation of elite plant is intended[4], where assessment of the tissue culture-raised variations using clonal fidelity is required to maintain the advantages of the desired elite genotypes such as superior growth, resistance to abiotic and biotic stresses and other horticultural and agronomically important

*For correspondence. (e-mail: dksuhf89@gmail.com)

traits. Several techniques have been developed to assess the genetic purity of the tissue culture-raised plants such as morphological, physiological and cytological studies, isozymes[5], field assessment and molecular studies[6]. Several DNA markers have been successfully employed to assess the genomic stability in regenerated plants, including those with no obvious phenotypic alterations[7].

In this communication, we report the development of a reproducible high-frequency plant regeneration protocol of broccoli (*Brassica oleracea* L. var. *italica* cv. Solan green head) using leaf and petiole explants. Broccoli cv. 'Solan green head' was developed at the Department of Vegetable Science, Dr Y. S. Parmar University of Horticulture and Forestry, Solan, Himachal Pradesh, India and is used in our breeding programme because of its good yield potential and early maturity. The ability of this regeneration protocol to deliver genetically uniform and stable regenerants was evaluated by RAPD markers. Presently, this regeneration protocol is being used in our laboratory for the development of transgenic broccoli (*Brassica oleracea* L. var. *italica* cv. Solan green head) with insect resistance gene (*cryIAa*) through *Agrobacterium*-mediated gene transfer technique.

Certified, uniform and healthy seeds of broccoli were germinated in pots in the glasshouse in our Department. *In vivo*-grown seedlings (18–20-days-old) were used as a source of explants. Young tender leaves and petiole explants were excised and surface-sterilized using 0.1% $HgCl_2$ for 1 min and 30 sec and washed thrice with distilled water. After treatment with $HgCl_2$, the explants were treated with 0.2% bavistin for 2 min and 30 sec and again washed thrice with sterilized distilled water in order to remove traces of sterilants. The explants were cut into pieces of 0.5–1.0 cm size and cultured on Murashige and Skoog (MS) basal medium[8] supplemented with various concentrations and combinations of benzyl amino purine (BAP) and napthalene acetic acid (NAA) for multiple-shoot induction. The pH of the medium was adjusted to 5.8 before adding agar agar to it. The medium was poured into culture vessels and sterilized at 1.08 kg/cm^2 for 15 min in an autoclave. All the aseptic manipulations were carried out in a laminar airflow chamber. After inoculation, all the cultures were kept in our culture room at 26 ± 2°C and 70–80% humidity under 16 h photoperiod with a light intensity of 40 m mol/m^2/s provided by cool white fluorescent lamps.

Leaf explants were cut and their surface was gently tapped with scalpel blade to cause injury and the petiole explants were cut into small pieces. The explants were then cultured on MS basal medium supplemented with different combinations and concentrations of BAP and NAA (mg/l). Five flasks with five explants each were inoculated for every combination and kept in the culture room. Each experiment was repeated thrice and observations were recorded at weekly intervals. Explants were evaluated for percentage shoot regeneration and average number of shoots per explant. Shoot proliferation and elongation were obtained on the same best shoot regeneration medium. After shoot multiplication, the *in vitro* developed shoots were transferred to root regeneration medium containing different concentrations of NAA. After the regeneration of roots, the percentage of shoots forming roots was calculated.

The regenerated plantlets with well-developed shoot and root system were carefully taken out and washed gently under running tap water to remove traces of medium from the roots. The *in vitro*-regenerated plantlets were transferred to earthen pots containing a mixture of sand, soil, and farmyard manure (1 : 1 : 1 ratio). During initial days of acclimatization, high humidity (70–80%) was maintained by covering the pots with polythene bags. The percentage survival of the hardened plants was recorded after 4–5 weeks of transfer to the pots.

For RAPD analysis, 20 hardened plantlets were randomly selected and evaluated for genetic similarity with each other and with the mother plant. The modified CTAB (cetyl trimethyl ammonium bromide) method with some modifications[9] was used to extract genomic DNA from leaves of the mother plant and *in vitro*-regenerated clones. PCR amplification of genomic DNA of the mother plant and *in vitro*-regenerated plantlets of broccoli was carried out using 21 random decamer oligonucleotide primers (Sigma Alderich, USA) in BIORAD MJ Mini DNA amplification system. The PCR reaction mixture of 25 µl contained 14.20 µl autoclaved double-distilled water, 2.50 µl *Taq* DNA polymerase buffer, 2 µl random primer, 2 µl dNTPs mixture, 0.3 µl *Taq* DNA polymerase and 4 µl genomic DNA. PCR was carried out in a thermocycler with a total of 40 cycles. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 2 min. All the PCR samples were given 5 min pre-PCR amplification at 94°C and 10 min post-amplification at 72°C. The amplification products were resolved by electrophoresis on a 1.2% agarose gel, stained with ethidium bromide, photographed in Alpha-imager gel documentation system and the images were saved for analysis. The size of the amplicon was determined from a 100–10,000 bp DNA ladder. Random primers, each consisting of 10 nucleotides, were obtained and used for RAPD–PCR studies. Out of 21 primers, only 15 gave amplification and clearly identifiable amplicon. These were used for genetic fidelity studies.

Data were analysed for different parameters of plant regeneration and genetic fidelity studies. Plant regeneration studies based on mean values per treatment were made using analysis of variance for completely randomize design[10]. Data analysis of genetic fidelity studies was carried out only for those primers which gave scorable patterns for the clones under study. After amplification, total scorable bands were calculated. The number of monomorphic and polymorphic bands, percentage of monomorphism and size range of amplicon in base pairs

Table 1. Effect of various concentrations and combinations of BAP and NAA (in MS basal medium) on shoot regeneration from leaf explants of broccoli (*Brassica oleracea* L. var. *italica*)

Medium composition	Average number of shoots regenerated per explant	Per cent shoot regeneration
MS medium + 3.0 mg/l BAP + 0.5 mg/l NAA	1.063	44.44 (41.80)
MS medium + 3.5 mg/l BAP + 0.5 mg/l NAA	0.930	37.22 (37.58)
MS medium + 4.0 mg/l BAP + 0.5 mg/l NAA	1.490	62.96 (52.52)*
MS medium + 4.5 mg/l BAP + 0.5 mg/l NAA	0.997	57.78 (49.48)
MS medium + 5.0 mg/l BAP + 0.5 mg/l NAA	1.353	53.33 (46.92)
MS medium + 5.5 mg/l BAP + 0.5 mg/l NAA	2.483*	62.22 (52.13)
$CD_{0.05}$	0.317	9.060 (5.287)
SE ±	0.145	4.158 (2.426)

Values in parenthesis are arc sine transformed. *Denotes highly significant values.
BAP, Benzyl amino purine; NAA, Napthalene acetic acid; MS, Murashige and Skoog.

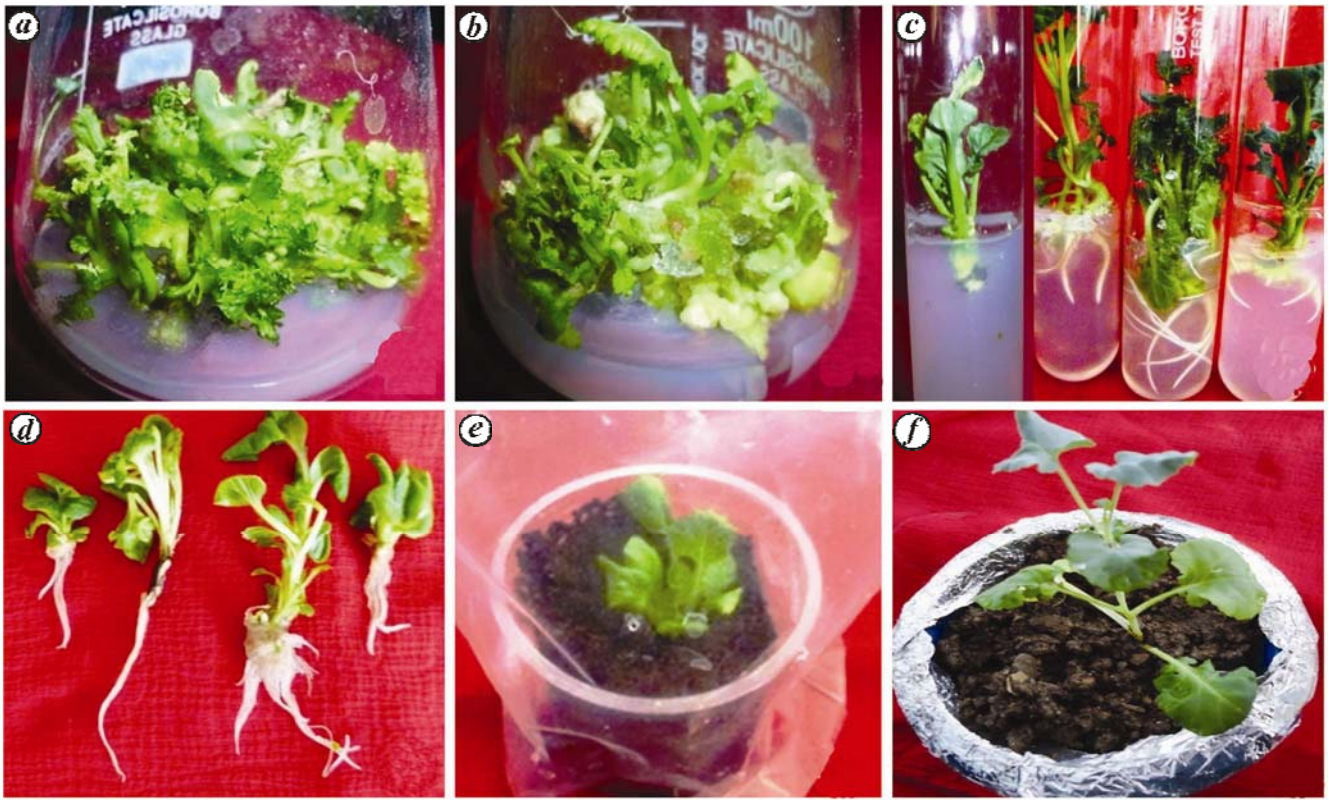

Figure 1. *In vitro* plant regeneration studies in broccoli (*Brassica oleracea* L. var. *italica* cv. Solan green head). ***a***, Shoot regeneration from leaf explants after 32 days in culture on MS medium supplemented with 4.0 mg/l BAP and 0.5 mg/l NAA. ***b***, Shoot regeneration from petiole explants after 28 days in culture on MS medium supplemented with 4.5 mg/l BAP and 0.019 mg/l NAA. ***c***, Root regeneration in *in vitro* regenerated shoots after 18 days in culture on MS medium supplemented with 0.20 mg/l NAA. ***d***, Development of complete plantlet. ***e***, Hardening of *in vitro* regenerated plantlet. ***f***, Successful acclimatization of plant in the pot.

were also calculated separately to assess the genetic fidelity of tissue culture-raised plants.

The requirement of various plant growth regulators for inducing shoot-bud differentiation varies from organ to organ and from plant to plant. It depends on the intrinsic status of these growth-promoting substances within the plant species. In our experiments, we used young, tender, completely green leaf and petiole explants from *in vivo*-grown seedlings for efficient shoot regeneration. The explants began to expand after one week of culturing and callus proliferation occurred after two weeks of culturing for both explant types. After 6–7 weeks, significant differences were observed among the treatments for percentage of explants forming shoots. Multiple shoot induction was achieved using different concentrations and combinations of BAP and NAA for both explant types. In the case of leaf explants, maximum shoot regeneration frequency, i.e. 62.96% and 62.22% was obtained on MS basal medium supplemented with 4.0 mg/l BAP and 0.5 mg/l NAA, and 5.5 mg/l BAP and 0.5 mg/l NAA

Table 2. Effect of various concentrations and combinations of BAP and NAA (in MS basal medium) on shoot regeneration from petiole explants of broccoli (*B. oleracea* L. var. *italica*)

Medium composition	Average number of shoots regenerated per explant	Per cent shoot regeneration
MS medium + 2.0 mg/l BAP + 0.019 mg/l NAA	1.013	51.11 (45.64)
MS medium + 2.5 mg/l BAP + 0.019 mg/l NAA	1.177	46.66 (43.09)
MS medium + 3.0 mg/l BAP + 0.019 mg/l NAA	1.620	60.37 (51.02)
MS medium + 3.5 mg/l BAP + 0.019 mg/l NAA	2.053	69.63 (56.61)
MS medium + 4.0 mg/l BAP + 0.019 mg/l NAA	2.573	83.33 (65.97)
MS medium + 4.5 mg/l BAP + 0.019 mg/l NAA	3.617*	91.11 (72.88)*
$CD_{0.05}$	0.356	7.809 (5.248)
SE±	0.163	3.584 (2.408)

Values in parenthesis are arc sine transformed. *Denotes highly significant values.

Table 3. Effect of various concentrations of NAA (in MS half strength basal medium) on per cent root regeneration from *in vitro* developed shoots of broccoli (*B. oleracea* L. var. *italica*)

Medium composition	Per cent root regeneration
MS (half strength) medium + 0.05 mg/l NAA	78.15(8.837)
MS (half strength) medium + 0.10 mg/l NAA	81.11(9.006)
MS (half strength) medium + 0.20 mg/l NAA	93.99(9.693)*
$CD_{0.05}$	8.429
SE ±	3.444

Values in parenthesis are arc sine transformed.
*Denotes highly significant values.

respectively (Table 1 and Figure 1 *a*). For the petiole explants, the highest shoot regeneration (91.11%), with highest mean number of shoots (3.61) per explant was recorded on MS medium supplemented with 4.5 mg/l BAP and 0.019 mg/l NAA (Table 2 and Figure 1 *b*). The regenerated shoots obtained from both the explants were separated and subcultured on the same best shoot regeneration medium. Shoot multiplication and elongation took place on the same medium.

During the present study, BAP and NAA used in MS medium for shoot regeneration studies were quite effective in multiple shoot induction in leaf and petiole explants compared to previous studies[11,12]. High-frequency shoot regeneration has been reported by different researchers using BAP and NAA in leaf, petiole, cotyledon and hypocotyl explants of broccoli[11,12], tomato[13] and cabbage[14]. Among these two explants, petiole (91.11%) was found better for shoot regeneration and multiplication compared to leaf (62.96%).

In vitro-developed elongated shoots (about 2–3 cm long) obtained from leaf and petiole explants were excised and cultured on MS medium containing different concentrations of NAA. Healthy and vigorous roots were formed after 10–14 days of inoculation. Maximum root regeneration response of 93.99% was obtained on MS medium supplemented with 0.20 mg/l NAA and well-developed complete plantlets were observed after 20–22 days (Table 3, Figure 1 *c* and *d*). It has been reported in a previous study[15] that high concentration of NAA is more effective for root regeneration compared to high concentration of IAA or IBA. It has also been reported that medium supplemented with 0.2 mg/l IBA is most suitable for root regeneration with maximum root regeneration response[14]. The regenerated plantlets of broccoli were successfully acclimatized in pots containing mixture of soil and organic manure 1 : 1 with 75% survival frequency and were morphologically uniform.

During the present study, the genetic fidelity of tissue culture-raised plantlets of broccoli was assessed using RAPD. The capacity to analyse the genomes (nuclear and organellar) with the number of molecular DNA marker represents one of the most recent developments in molecular biology. Genetic molecular markers are considered to be reliable in monitoring variability in the DNA sequence of regenerated plantlets. There are reports available which have which applied the RAPD technique to detect somaclonal variation[16–18] and to identify micropropagated plants and cultivar[19–21]. Genetic fidelity of *in vitro*-raised plantlets of broccoli was investigated using 21 random decamer primers. Among these, only 15 had shown scorable banding patterns and yielded 66 amplicons with an average of 2.96 bands per primer. Among the 66 amplicons, 56 showed monomorphism and 10 were observed to be polymorphic resulting in 88.45% monomorphism among all the *in vitro*-regenerated plantlets and mother plant studied. The number of bands for each primer varied from 1 to 7 and the size of the amplification product ranged from 124 to 4823 bp (Table 4 and Figure 2). The RAPD fragments obtained after amplification of genomic DNA from 20 randomly selected *in vitro*-raised plantlets and from the mother plant of broccoli were scored for presence as 1 (band present) and absence as 0 (band absent) for each plantlet. The results show that the regeneration protocol has not induced any gross genetic changes and also the RAPD approach suitable for the detection of variants.

Some variations have been observed among the *in vitro*-raised plantlets and the mother plant of broccoli that

Table 4. Total number of amplified fragments and number of monomorphic and polymorphic fragments generated by RAPD-PCR using 15 informative random decamer oligonucleotide primers.

Primer	Sequence (5′ → 3′)	Size range of amplified bands in base pairs	Total number of amplified bands	Total number of monomorphic bands	Total number of polymorphic bands	Percentage monomorphism
OPB-04	GGACTGGAGT	206 to 1387	4	4	–	100
OPB-05	TGCGCCCTTC	181 to 1250	5	3	2	60
OPB-10	CTGCTGGGAC	523 to 921	3	2	1	66.66
OPB-11	GTAGACCCGT	189 to 3552	7	5	2	71.40
OPB-12	CCTTGACGCA	655 to 885	2	2	–	100
OPB-14	TCCGCTCTGG	828 to 4823	6	6	–	100
OPB-16	TTTGCCCGGA	320 to 3676	7	7	–	100
OPC-05	GATGACCGCC	635 to 1000	3	3	–	100
OPC-06	GAACGGACTC	124 to 542	3	3	–	100
OPC-07	GTCCCGACGA	1268	1	1	–	100
OPC-09	CTCACCGTCC	192 to 2283	7	2	5	28.57
OPC-10	TGTCTGGGTG	761 to 1870	3	3	–	100
OPC-18	TGAGTGGGTG	328 to 1165	5	5	–	100
OPC-19	GTTGCCAGCC	151 to 1871	7	7	–	100
OPC-20	ACTTCGCCAC	390 to 853	3	3	–	100
Total			66	56	10	88.45

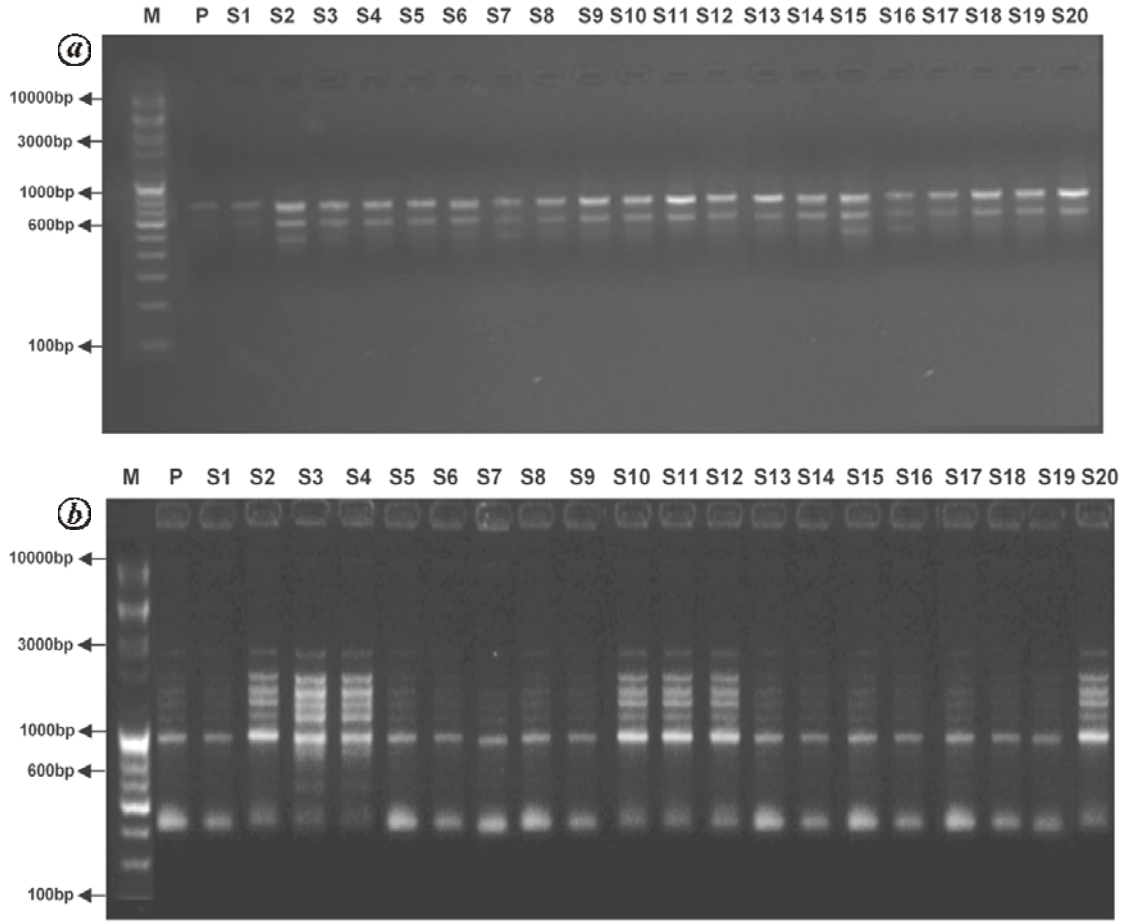

Figure 2. Genetic fidelity studies in *in vitro* regenerated plantlets of broccoli (*B. oleracea* L. var. *italica*) using RAPD markers. ***a***, RAPD profile generated by amplification of DNA of mother plant (P) along with 20 *in vitro* raised plantlets of broccoli with primer OPB-10. ***b***, RAPD profile generated by amplification of DNA of mother plant (P) along with 20 *in vitro* regenerated plantlets of broccoli with primer OPB-16. M, Marker/ladder (100–10,000 bp), P, Mother plant of broccoli. S_1–S_{20}, Twenty randomly selected *in vitro* regenerated plantlets of broccoli.

might arise during the cell division or differentiation under *in vitro* conditions[22]. The proposed mechanism is DNA methylation that may in part explain the changes that occur under *in vitro* conditions[23–25]. Qin *et al.*[26] have analysed the genetic stability in tissue culture-raised plantlets of broccoli using RAPD–PCR. A total of 62 arbitrary decamer primers were screened and a very low percentage of polymorphism was reported; the similarity ranged from 98% to 100%. Whereas in the present study 10–12% polymorphism in tissue culture-raised plantlets and mother plant of broccoli has been reported. In conclusion, analysis of genetic stability of *in vitro* regenerants using RAPD markers is an effective and rapid technique for assessing the molecular stability of and ensures the true-to-type nature of plants so obtained. It is also the utmost demand for carrying out genetic manipulations at cellular level.

A high frequency plant regeneration protocol has been developed from leaf and petiole explants of broccoli. Using RAPD for genetic fidelity, we conclude that a high-efficiency plant regeneration system for broccoli can be developed without risk of genetic instability. This protocol can be favourably exploited for genetic transformation studies of broccoli for improvement of agronomically important traits like aphid resistance, disease resistance, herbicide tolerance, salinity and drought tolerance, quality improvement, etc.

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ACKNOWLEDGEMENT. We thank Professor and Head, Department of Vegetable Science, Dr. Y. S. Parmar University of Horticulture and Forestry, Solan for providing us the germplasm of broccoli cv. Solan green head.

Received 2 March 2015; revised accepted 28 June 2015

doi: 10.18520/v109/i8/1470-1475

Aromatic fruits as baits for the management of fruit-piercing moths in pomegranate: exploiting olfaction

P. D. Kamala Jayanthi[1,*], Ravindra M. Aurade[1], Vivek Kempraj[1] and Abraham Verghese[2]

[1]Division of Entomology and Nematology,
Indian Institute of Horticultural Research, Hesseraghatta Lake PO,
Bengaluru 560 089, India
[2]National Bureau of Agriculturally Important Insects,
Bengaluru 560 024, India

'Baits' are substances that attract, trap and kill pests. In horticultural practice, baits are used to trap insect pests, thereby diverting them from cultivated crops. An important insect pest that can be managed using baits is the fruit-piercing moth (FPM), *Eudocima* species. These frugivorous moths are distributed throughout India and inflict serious damage to a wide range of cultivated fruits. In southern India, pomegranate cultivation is escalating and so is the fruit damage caused by FPM. The damage by FPM alone sums up to 40% of the production, thus causing heavy loss to farmers. However, existing control measures are ineffective in reducing damage caused by the moths. In the present communication, we study the feeding preference of FPM to banana, guava, tomato and molasses in multiple and limited-choice field experiments. The results indicate that the moths are attracted to bait fruits, viz. banana, guava compared to the main crop, pomegranate. We conclude that using these aromatic fruits as baits, we can divert the moths from the main crop, which will serve as an economically viable control measure.

Keywords: Aromatic fruits, baits, fruit-piercing moths, olfaction, pomegranate.

BAITS are luring substances that can be used to trap an organism if appropriately deployed. Baits work on the principle of exploiting olfactory response of the target organism, to lure and trap them. In insects, 'olfaction' plays an important role in locating food, mates and oviposition sites. Olfaction is the ability of insects to perceive and distinguish odours that are mediated through sensory neurons and the brain, leading to a critical and specific behaviour. Exploiting this mechanism, several trapping methods based on pheromones and kairomones are already in use for managing insect pests. Food-based baits are an effective method for insect control. They consist of an attractive feeding substance along with a toxicant that works by deceiving insects into a trap. Such baits if selective to target pests are environmentally safe.

*For correspondence. (e-mail: jaiinsect@gmail.com)

Fruit-piercing moths (FPMs) (*Eudocima* [=*Othreis*] *materna* (Linnaeus), *Eudocima fullonia* (Clerck), *Eudocima homaena* Hubner) belong to the Noctuidae family of the order Lepidoptera. They are serious pests of fruit crops in the tropical and subtropical belts spanning from Africa to the Pacific Islands[1]. Unlike other lepidopteran moths where the detrimental life stages that cause economic loss are always larvae, here the adult stage is more injurious causing huge damage to fruits, including citrus, guava, mango, papaya, banana, pomegranate, etc. FPMs are nocturnal feeders. They penetrate their proboscis into the rind of fruit and suck the juice[2] (Figure 1). The internal injury consists of a bruised area beneath the rind augmenting secondary rots[3]. Such rot leads to fermentation of the fruit and attracts secondary moth feeders that take advantage of the access hole drilled by moths of the *Eudocima* spp. A solitary moth would generally attack many fruits on a single night making them unmarketable and leading to huge economic losses. Surveys to pomegranate-growing areas of Chitradurga, Karnataka by the present authors have revealed loss ranging from 20% to 40% by FPM. Fruit loss up to 57% was reported earlier in pomegranate at Rahuri, Maharashtra with maximum damage to ripe fruits (21.06–47.62%) than unripe fruits (2.86–13.86%)[4]. Another study reported fruit damage in the range 0.00–8.67% at Bijapur and 18.45–33.9% at Raichur[5]. This is high considering the farm gate price fluctuations of Rs 60–110 per kg of harvested fruit.

The female moths oviposit on creepers of the Menispermaceae family that grow in forests and wastelands. The hatched larvae complete their life cycles on the host plants, thus making control of immature stages difficult as spraying larval hosts is not feasible[6]. Management of this pest using insecticides has not been an option because of inadequate contact of the moth with the fruit; only ripe fruits are attacked on which insecticides cannot be sprayed due to pesticide residue issues. As economic value of pomegranate is high, farmers usually deploy watch and ward with torches to swat the moths alighting on the fruit. This is far from efficacious as swarms can never be fully deterred from attacking fruits. The fact that the moths are nocturnal renders vigil impractical. Other methods of management like fruit-bagging, netting trees/orchards, hand collection of moths, light traps, advancing/delaying the cropping have their own limitations and flaws[2]. However, management of *Eudocima* species in the field through baits is less explored[2,7,8]. Several studies established the feeding preference of these moths to aromatic fruits, viz. banana, guava and tomato at laboratory level. Nevertheless, the efficacy of these bait fruits in attracting the moths in the field was not explored. Though the laboratory experiments are ideal to understand the feeding preferences of moths, the repeatability of these results in the field is fraught with several difficulties and uncertainties like huge experimental area, aroma from main crop may mask the bait odour thereby

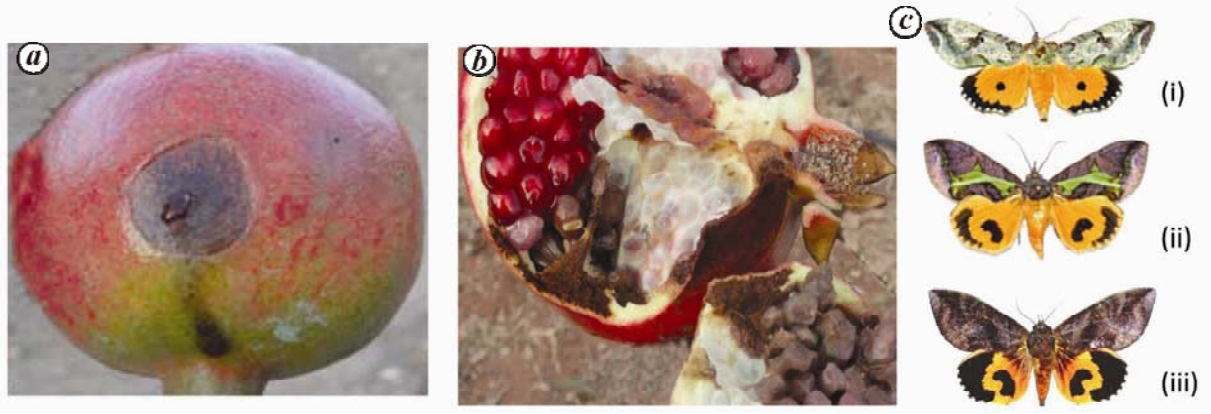

Figure 1. ***a***, Damage by fruit-piercing moth (FPM) on mature pomegranate fruit showing the pin-size feeding hole with surrounding browning. ***b***, Damaged fruit cut open showing the internal injury. ***c***, Different FPM species that attack pomegranate. (i) *Eudocima materna*; (ii) *E. homaena* and (iii) *E. fullonia*.

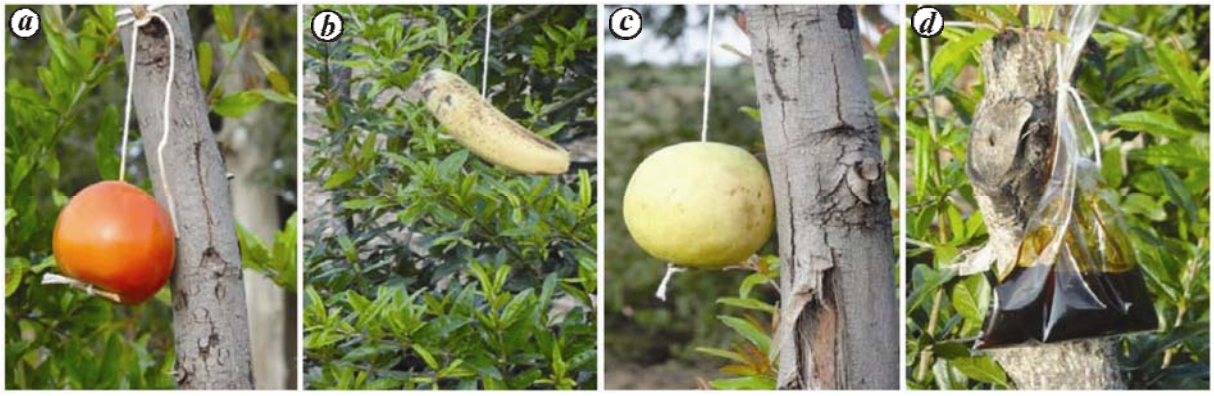

Figure 2. Different baits tied in the pomegranate field to attract FPMs. ***a***, Ripe fruits of tomato; ***b***, banana; ***c***, guava; ***d***, sachet with molasses.

influencing the orientation of target insect, etc. Therefore, the present study was taken up to explore the feasibility of bait fruits application for the management of FPMs.

In this study, we evaluated the efficiency of 'fruit baits' in trapping FPMs in multiple as well as limited-choice field experiments. The study was carried out in a pomegranate field at Hiriyur (13.95°N, 76.62°E), Chitradurga district, Karnataka, India. The trials were conducted in two different orchards located 3 km from each other. The cultivated variety of pomegranate was cv. Bhagwa. The plants were 4 years old raised by following standard agronomic practices. The bait treatments were conceived at fruit maturity. In the multiple-choice field experiment all the treatments, viz. banana, guava, tomato, molasses were screened to identify the potential attractants for FPMs. In the limited-choice field experiment only the selected treatments from the multiple-choice field experiment were further tested in an open field to establish the efficacy of the potential fruit baits in attracting moths. The experimental procedure for both multiple-choice and limited-choice field experiments is the same, but the number of treatments evaluated differs.

The baits, banana (cv. Robusta), guava (cv. Allahabad Safed), tomato (cv. Local red) and molasses were evaluated for their efficacy in attracting FPMs in the preliminary open-field choice experiment. These baits were selected based on findings from previous studies[2,5,7–10]. In the case of banana, over-ripened whole fruits having bright yellow rind with brown 'sugar spots' were selected for the study. Similarly, in the case of guava, over-ripened whole fruits with uniform yellow rind and good aroma were used. In the case of tomato, ripened whole fruits with bright uniform red colour were chosen for the study. The baits, viz. banana, guava, tomato and molasses (15 ml sealed in 50 gauge polythene sachet of 13 × 10 cm dimension) were fastened using a thread (Figure 2) to randomly erected wooden poles (placed at a distance of 10′) in the borders of the field @ 40 units per hectare in all directions. The baits were tied at the crop canopy level (~5′–6′). According to previous studies, moth visits were reported between 19:30 and 23:30 h with a peak at 23:00 h in the fields[5]. In the present study, the baits were not poisoned as there is a possibility for accidental ingestion by non-target organisms; instead, continuous scouting was carried out from 19:00 to 24:00 h to monitor the feeding activity of moths on baits. The number of moths visiting the baits and feeding punctures per fruit/sachet were recorded. Further, the moths that were feeding on

Figure 3. Feeding of FPMs on bait fruits in the pomegranate field. Active feeding postures of (***a***, ***c***) *E. materna* on guava and (***b***) *E. fullonia* on banana bait fruits during night hours.

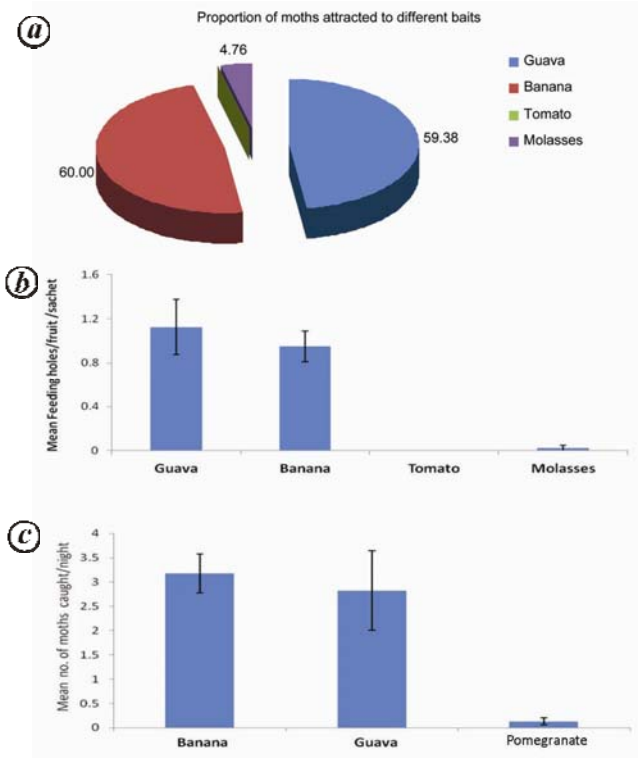

Figure 4. Food bait preferences (with error bars) of FPMs exhibited in (***a***, ***b***) multiple-choice open field experiment and (***c***) limited-choice open field experiment.

the fruits were caught using aerial insect nets and observations were taken on total moths trapped. Similarly, in the second limited-choice confirmatory trial, the selected treatments, viz. banana, guava were tried and data recording was carried out as explained above. Both the experiments were continued for two weeks without changing the baits. The data were pooled and subjected to statistical analysis using ANOVA[11].

The results of multiple-choice experiments indicated that the moths preferred certain fruits that were aromatic (Figure 2). We found *E. materna*, *E. fullonia* and *E. homaena* feeding on bait fruits (Figure 3). Guava and banana were equally attractive with 59.38% of moths visiting guava and 60% visiting banana (Figure 4 *a*). The moths did not visit tomato, revealing their non-preference. However, previous studies conducted to understand the adult feeding preferences of FPMs under laboratory conditions revealed that freshly emerged moths of *E. materna*, *E. fullonia* and *E. homaena* preferred to feed on guava followed by tomato and banana. The reported preference index was highest for guava (323.09) followed by tomato (199.29) and banana (195.51)[5]. In the present multiple-choice open field study, the moths exhibited high preference for guava and banana compared to tomato, indicating the possibility of using these fruits as baits to attract moths. In the case of molasses, only 4.76% of moths visited the sachets. Earlier studies reported molasses as the most preferred attractant, which may serve as an useful ingredient of bait formulations against *E. materna*[2]. However, molasses exhibited weak attraction compared to banana or guava fruit baits.

The mean feeding punctures among the treatments were significantly different ($F = 17.16$, $df = 156$, $P < 0.001$). The fruit baits that exhibited high attraction for FPMs (banana and guava) also recorded significantly ($P = 0.05$) more feeding punctures compared to the other treatments (Figure 4 *b*). Earlier studies reported that *E. fullonia* prefers sweet, aromatic fruit (e.g. banana or guava) to those with low sugar content (e.g. tomato or bell pepper)[12]. Cherian and Sundaram[13] observed no damage to orange fruits when ripened tomato was available in the fruit orchard. The moths attacked oranges immediately after the removal of tomato crop. The authors suggested that tomato could act as a trap crop to attract the moths. However, in the present study, FPMs did not show any preference for tomato under field conditions. Perhaps, between orange and tomato, the latter may be a more preferred host.

In the limited-choice open field experiment, maximum number of moths was found feeding on banana (36.5 ± 8.5) followed by guava (32.5 ± 0.5), confirming their potential as bait fruits (Figure 5). The number of moths feeding on the main crop, pomegranate, were comparatively less (1.5 ± 0.5) (Figure 5). Denton *et al.*[14] quantified fruit preferences of *E. fullonia* and found that the preference index was very high for banana (100) followed by guava (89), with pomegranate being the least (0). Several other laboratory studies also found that pomegranate was least preferred with cumulative preference

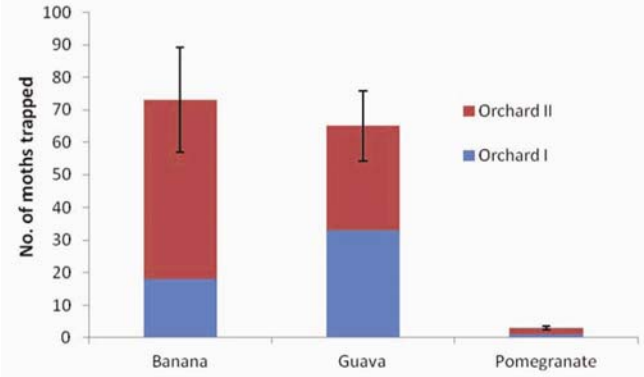

Figure 5. Total FPM catches (with error bars) on bait fruits compared to pomegranate fruits (main crop) during fortnight experimental period.

index of 3.22 as against guava and banana[2,7,9]. This clearly showed that the FPMs can be lured from the main crop to bait fruits such as banana and guava. Hanging of ripe banana fruits in orange orchards to reduce moth damage on oranges was also suggested earlier, as moths preferred banana over orange[10]. However, the present study establishes the feasibility and efficacy of bait fruit approach in the pomegranate field and validates the FPM feeding preference to over ripe banana and guava fruits.

Olfaction is the ability of an organism to detect and discriminate odours in the environment. Insects rely mainly on olfaction to locate their food[15]. The antennal structures in insects are the functional equivalent of the human nose, enabling them to detect specific olfactory information in a complicated malodorous environment[16]. In a nocturnal lepidopteran like the FPMs, olfaction is one of the major means to locate food[17]. In the present study, the olfactory preferences of FPMs towards certain preferred fruits like banana and guava[2,7,9] were manipulated using them as baits in the pomegranate orchard to lure them away from the main crop. Using bait fruits like banana and guava (40 fruits per hectare) helped in trapping 138 FPMs over a period of two weeks (Figure 5) that otherwise might have caused enormous damage to pomegranate fruits. Watch and ward easily swatted/caught moths while they are feeding on bait fruits. This obviated the need to add toxicants to bait fruits. Further, it not only makes this approach pesticide-free, but renders the baits safe to non-target organisms like bats, birds and even humans. Thus, the present study clearly establishes that the feeding preferences of FPMs to certain fruits can be exploited for their management in the field. Further, chemical identification of potential odours (that are implicated in luring the FPMs) from these bait fruits through in depth studies, viz. head space analysis, GC-MS, GC-EAD will enable formulation of synthetic baits that will disrupt the FPM olfactory orientation in the field. Such studies not only help to fine-tune the present FPM management strategies, but make them more robust and sustainable in the long run.

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ACKNOWLEDGEMENTS. We thank ICAR, New Delhi for financial support through the National Fellow project and the Director, IIHR, Bengaluru for providing the necessary facilities to carry out this work. We also thank Dr J. Poorani, NBAII, Bengaluru for adult moth photographs.

Received 19 August 2013; revised accepted 8 July 2015

doi: 10.18520/v109/i8/1476-1479

Phthalate in children's toys and childcare articles in Croatia

Lidija Barušić[1], Antonija Galić[1], Jasna Bošnir[1], Lidija Baričević[1], Ivana Mandić-Andačić[1], Adela Krivohlavek[1], Ana Mojsović Ćuić[2,*] and Domagoj Đikić[3]

[1]Institute of Public Health, Dr Andrija Štampar, Department of Ecology, Zagreb, Croatia
[2]University of Applied Health Studies, Department of Environmental Health Engineering, Zagreb, Croatia
[3]University of Zagreb, Faculty of Science, Department of Animal Physiology, Zagreb, Croatia

Children's toys are made of artificial material often softened by phthalates. These are synthetic compounds added to PVC as plasticizers for the purpose of improving its elasticity and flexibility. Phthalates can endanger the health of children exposed to their effect by inducing reproductive, hormonal and developmental disorders. The goal of the present study was to determine phthalate presence in children's toys and childcare articles from different sources sold in different areas in Croatia in 2012 and 2013. Diisononyl phthalate, di(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate, diisodecyl phthalate, di-*n*-octyl phthalate and butylbenzyl phthalate were included in the analysis. About 60% of the toys analysed contained plastic; up to 20% had detectible levels of phthalates, 94–96% containing toxic DEHP. Over 60% contained DEHP at concentrations more than 10 times than that permitted, mostly dolls and toy animals. The percentage of toys containing phthalates was higher in 2013 in comparison to 2012. We discuss the availability and similarities in composition of phthalates by comparing our results with reports from other countries.

Keywords: Childcare articles, children's toys, chromatography, phthalates.

CHILDREN'S toys are one of the most important and highly controlled products of common use in the European Union (EU). Toys in the marketplace should be in compliance with the applicable legislation in the EU for the protection of consumer health and safety, as well as the environment[1]. However, the strict requirements do not prevent the manufacture and sale of toys which do not meeting the set standards.

Children's toys are defined as products that are exclusively or non-exclusively designed or intended for use by children up to 14 years of age. Childcare articles are products intended for sleep facilitation, relaxation, hygiene, feeding or sucking by the children[1]. Toys and childcare articles made of polyvinyl chloride (PVC) usually contain softeners or plasticizers which enable flexibility. Plasticizers that are mostly used for the improvement of elasticity in PVC are phthalates (Figure 1) or diesters of ortho-phthalic acid (di-alkyl or alkyl aryl esters 1,2-benzenedicarboxylic acid)[2–5]. At a global level, 6 million tonnes of softeners are used per year, including 1 million used in Europe, 80% of which includes phthalates[6]. Phthalates are a serious threat to the environment and human health[6]. They do not form covalent bonds and thus easily migrate when in contact with lipophilic matter[6,7]. After phthalate intake, monoesters and oxidative metabolites are formed during biotransformation[8]. For example, after ingestion of DEHP, 30 metabolites are formed, namely mono-(2-ethyl-5-hexyl) phthalate (MEHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP). MEHP is primarily formed by DEHP hydrolysis in the gastrointestinal tract, which is then absorbed, while MEOHP, MEHHP and MECPP are formed by liver oxidative metabolism of MEHP and are present in concentrations 3–5 times greater than MEHP in urine[9,10]. Human exposure to phthalates occurs through food, mother's milk, by inhaling, chewing plastic and through skin contact[11]. Toys and childcare articles that can be placed in the mouth, even if not intended for that purpose, present a health risk for children if they contain phthalates[12,13]. Determination of the allowed legislative levels is based on risk estimation and exposure dose extrapolations; it is not a simple process. The guidelines for allowed estimated dose exposure to individual phthalate types are explained in detail by CSTEE (European Scientific Committee on Toxicity, Ecotoxicity and Environment)[2]. By Commission Regulation (EU) No. 143/2011, the EU has categorized phthalates as reproductive toxic compounds of category 1B and regulated the maximal allowed amounts of these toxic chemicals in toys and childcare article[12,14,15]. Toxicological reports show a connection between phthalates and breast cancer[16]. Phthalates might potentiate their adverse effects combined with other chemicals[17]. They are responsible for changes in brain function during the development of boys by influencing testosterone levels and contribute to obesity in children[18,19]. High exposure to phthalates is also associated with the use of medical products containing DEHP or DBP[20]. Animal toxicity

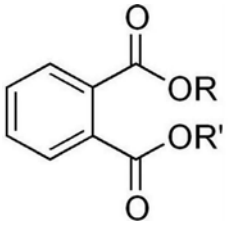

Figure 1. General structure of phthalates, *R* and *R*′ are the same or different alkyl or aryl groups.

*For correspondence. (e-mail: domagoj.djikic@biol.pmf.hr)

Table 1. Samples of toys and childcare articles sold in Republic of Croatia collected for analysis of phthalate content

Sample	Total *N* of samples	Toys		Articles (bottles etc.)	
		N Toys	Analysed for phthalate content	*N* Articles	Analysed for phthalate content*
All sampled child articles	555	518	255	37	37
2012	336	301	98	35	35
2013	219	217	157	2	2

*In childcare articles, phthalate was below detection limit.

Table 2. Products containing plastic shown by product type in joint analysis of all samples (total) and separate analysis by year of import (2012 and 2013)

Toys	Total *N*	*N* 2012	*N* 2013	Products containing phthalates above detection limit (% of *N* per product type)		
				% of Total *N*	% of *N* 2012	% of *N* 2013
Dolls	94	39	55	24	28	22
Animals	68	21	47	9	14	6
Balls	18	12	6	22	17	33
Cars	26	8	18	0	0	0
Tools, shovels buckets	21	8	13	0	0	0
Arms	9	6	3	0	0	0
Make-up set	3	1	2	0	0	0
Others	16	3	13	0	0	0
Articles						
Soothers	22	20	2	0	0	0
Bottles	5	5	0	0	0	0
Chewing soothers	10	10	0	0	0	0
Total *N* samples analysed	292	133	159			

studies indicate that DEHP causes hepatotoxicity through peroxisome proliferation, DINP causes appearance of liver neoplasms, while DEHP and BBP modify rat breeding, causing pronounced testicular and sperm damage; such adverse endocrine effects were observed even in offspring of exposed pregnant females[2,21–24]. Studies showed that DEHP is one of the most toxic phthalates with accidental poisoning recording lethal DEHP concentrations 0.3 to 1.0 mg/kg (ref. 4). Attempts have been made to regulate and replace DEHP by DINP[7]. However, the margins of safety are lower for DINP and DEHP than for DNOP, DIDP, DBP and BBP[2]. Thus, exposure to DINP is also a cause for concern. There is also a synergistic interaction between more phthalate types that can be present in children's toys and multiple sources of child exposure[2]. Thus monitoring the levels of various phthalates and the analysis of their source is important. The present study is an analysis of phthalates in child products during a period of the adaptation of the legislative framework for phthalate control prior to the accession of the Republic of Croatia to the EU. The earlier legislation was significantly different regarding the values of prohibited and/or restricted allowable phthalate concentrations and did not prescribe limits for certain phthalates. We show that the status of phthalate levels was not satisfactory, although prior to the entry of Croatia to the EU, the higher EU standards started to be applied. In particular, the study shows that despite the introduction of controls, there was an increase in the number of toys softened by prohibited phthalates recorded between two consecutive years. By analysis of particular types of phthalates we wanted to find out whether those like DEHP whose adverse effects are well known, could still appear in the even with strict laws of the EU.

Samples of toys and childcare articles ($N = 555$) were collected from the markets in the Republic of Croatia in the years 2012 and 2013. Table 1 shows the number of samples collected in 2012 and 2013, and the number of analysed samples. Samples that contained plastic materials were chosen for phthalate analysis. Tables 2 and 3 give the product type and country of origin. Analysis included DEHP, CAS No. 117-81-7/EC No. 204-211-0; DBP, CAS No. 84-74-2/EC No. 201-557-4; BBP, CAS No. 85-68-7/EC No. 201-622-7; DINP, CAS No. 28553-12-0, 68515-48-0/EC No. 249-079-5 and 271-090-9; DIDP, CAS No. 26761-40-0 and 68515-49-1/EC No. 247-977-1 and 271-091-4, and DNOP, CAS No. 117-84-0/EC No 204-214-7.

Phthalates in toys and childcare articles were determined by Gas chromatography-mass spectrometry

(GC-MS), operated in selected ion monitoring (SIM). GC-MS was chosen because it is highly specific and widely available, whereas SIM provides higher sensitivity. The most abundant ion formed in the mass spectrometer is called the base peak. In the mass spectra of phthalates (Figure 2), the base peak is indicated at the *m*/*z* value of 149. Identification and quantification of phthalates used the extracted ion *m*/*z* 149, for each of the phthalates tested, except for DNOP and DINP, because of co-elution of both. For DNOP, *m*/*z* value of 279 was used for quantification, while for DINP *m*/*z* it was 293. Confirmation of presence was monitored by the following qualifier ions: *m*/*z* 149 (DNOP), *m*/*z* 223 (DBP), *m*/*z* 149 (DINP), *m*/*z* 307(DIDP), and *m*/*z* 206 (BBP). Method for determination of phthalates in toys and childcare articles is based on extraction of sample with dichloromethane on Soxtherm, followed by GC-MS analysis of the resulting extracts. Determination of phthalates was performed on Shimadzu GC-MS QP 2010, using electron ionization (EI) mode. Separation was performed with a SPB-5 (5% diphenyl, 95% dimethyl siloxane) capillary GC column, 60 m, 30 mm ID, with film thickness of 0.25 μm. The column was held at 40°C for 5 min, ramped at 10°C/min to 280°C and finally held for 21 min. The gas chromatograph was operated in split/splitless injection mode at a temperature of 280°C. The method has been validated according to the HRN EN 14372 : 2004 norm, with some modifications regarding extraction time and quantity of analysed sample. Satisfied recovery was achieved with extraction time of 4 h instead of 6 h, and with 1 g of sample taken into the procedure instead of 2 g. This improvement, shorter extraction time and smaller sample amount lead to better efficacy, proven by proficiency testing[25,26]. In order to construct a calibration curve, regression of peak area on concentration was undertaken using the external standard calibration method. The assay linearity was studied by injection of five different standard concentrations in the range 0.5–15 μg ml^{-1} for DBP, BBP, DEHP and DNOP, and 1.0–20 μg ml^{-1} for DINP and DIDP. All correlation coefficients were > 0.990. Limit of detection (LOD) and limit of quantification (LOQ) were established by analysing samples with added analytes in different concentrations considering signal-to-noise (S/N) ratio 1 : 3 for LOD and 1 : 10 for LOQ. LOD was 0.005% for DBP, BBP, DEHP and DNOP, and 0.01% for DINP and DIDP, while LOQ was 0.01% and 0.02% respectively. Recovery was determined by analysing samples spiked at five different concentration levels. Each level has been prepared and analysed in duplicate. Recovery range was from 90% for DINP to 98% for BBP. Measurement precision was examined by injecting seven replicate injections of standard (RSD < 5%). Method precision was examined by analysing spiked sample in triplicate (RSD < 10%).

Table 3. Country of origin of analysed products

Country of origin	Total N	Products containing phthalates above detection limit (N)
China	272	47
Germany	14	0
UK	1	0
Italy	2	0
France	1	0
USA	1	0
Macedonia	1	1

In statistical analysis, the descriptive statistics is shown by percentage ratios and differences in percentage between the different years of sampling were tested with chi-square test (χ^2) with the level of significant difference of $P \leq 0.05$ between the years. Concentrations of individual types of phthalates from the total analysed sample are shown in mean values and by standard deviation, but because of the large range of data, median value is shown together with minimal and maximal values. Concentration range is shown graphically as a distribution of percentage ratio for individual percentage concentration of DEHP. A correlation between percentage concentration of DEHP and other phthalates was made. However, as no statistically significant correlation was found, the analysis is not shown.

From the total of 555 collected samples intended for use among children, around 60% contained plastic materials (Figure 3 *a*). Also 40% of the samples collected in 2012 and 80% of the samples collected in 2013 contained plastic materials (Figure 3 *a*; χ^2, $P \leq 0.05$). The products that contained plastic were chosen for phthalate analysis (Table 1). Figure 3 *b* shows that 16% of all samples contained detectable levels of phthalate. When analysing the products that contained phthalates per year, 20% of the samples collected in 2013 and only 12% of the samples collected in 2012 contained phthalates. Although in 2013 the phthalate percentage was indicatively higher, statistical analysis (χ^2) showed that there was no significant difference between the years. All of the products that contained phthalates were toys. None of the childcare articles contained phthalates (Table 2). From the total analysed toys ($N = 255$), dolls were the most abundant type (36% of analysed samples), followed by animals (26%), cars (10%) and tools (8%), whereas other types were present to a lesser extent (Table 2). Phthalates were found in 24% of dolls, 22% of balls and 9% of cars and they were not found in other types of toys (Table 2). Most of the analysed products (93.4%) were manufactured in China; therefore, most of the products with detectible levels of phthalates were from China (Table 3). Figure 3 *c* shows the percentage distribution of individual phthalate type amongst the positive samples. Forbidden DEHP was present in 95.8% of positive samples, DINP was present in 41.7% of positive samples and DBP was present in 16.7% of positive samples. DIDP and DNOP were found in only one sample (thus the n.a. mark in Table 4 for min,

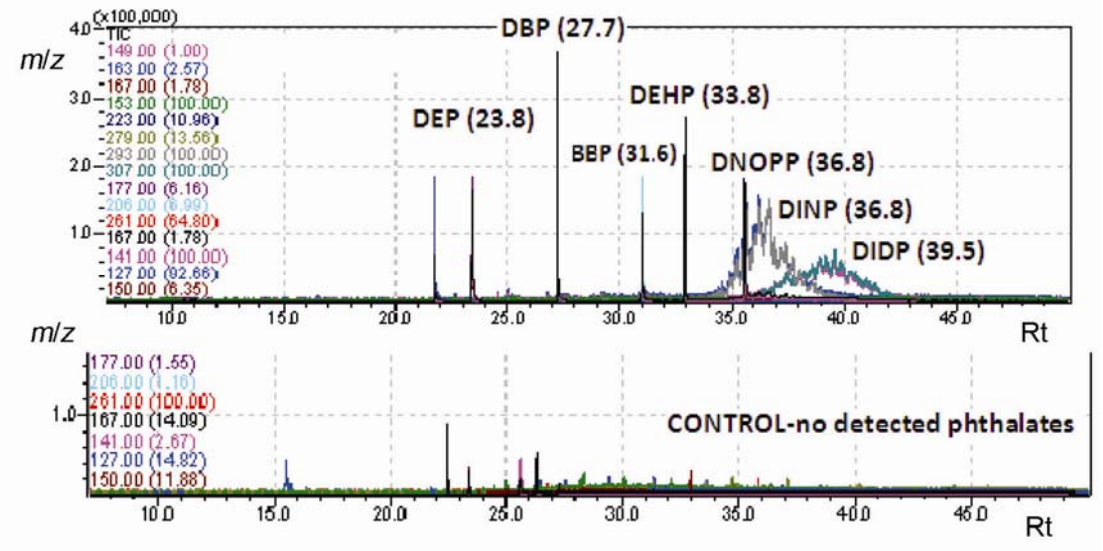

Figure 2. (Top) Chromatogram of GC-MS (sample inoculated by external standard), of six phthalate types analysed; retention time given in parenthesis. (Bottom), Sample without phthalates.

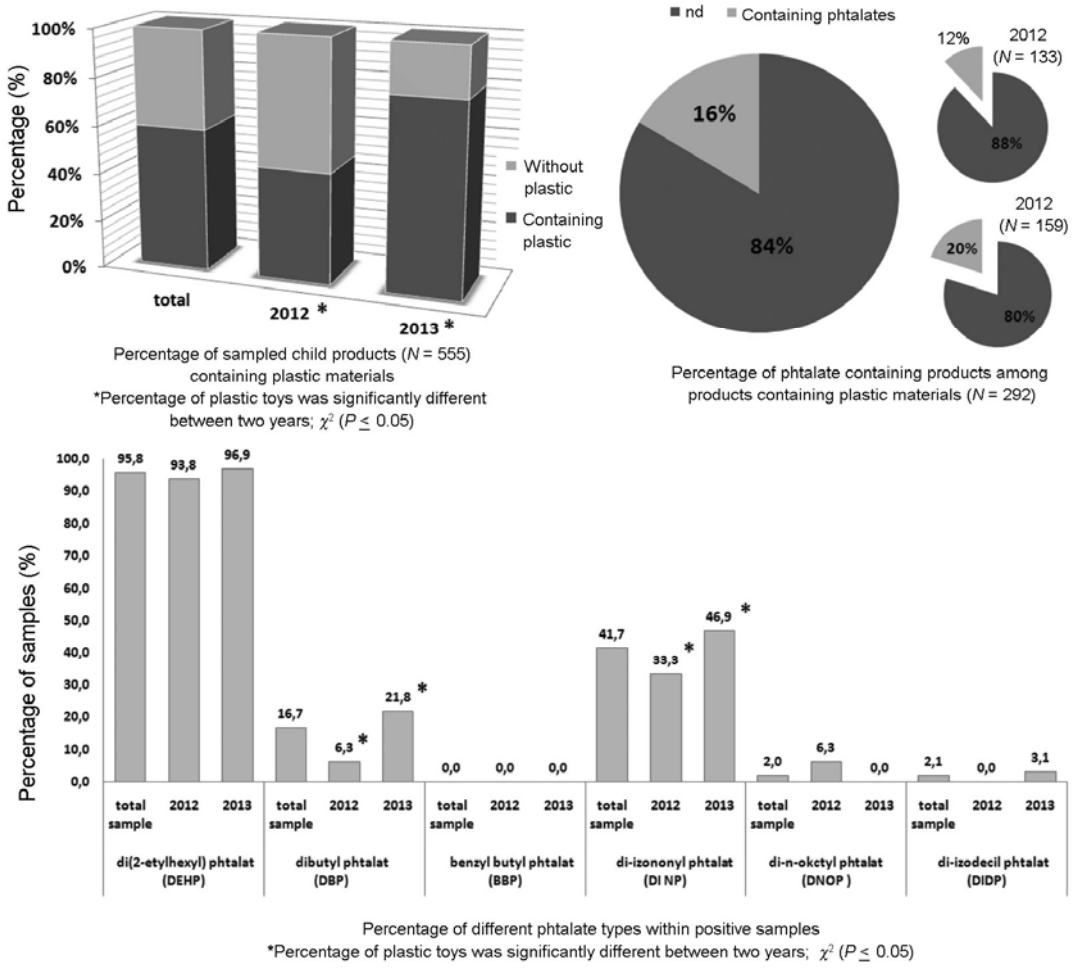

Figure 3. ***a***, Percentage of plastic toys and articles in joint analysis of all samples (total) and separate analysis by year of import (2012 and 2013). ***b***, Percentage of products positive for phthalate content above detection limit in the joint analysis of all samples (total) and separate analysis by year of import (2012 and 2013). ***c***, Individual phthalate type distribution (%) of positive child products of all samples (total) and separate analysis by year of import (2012 and 2013).

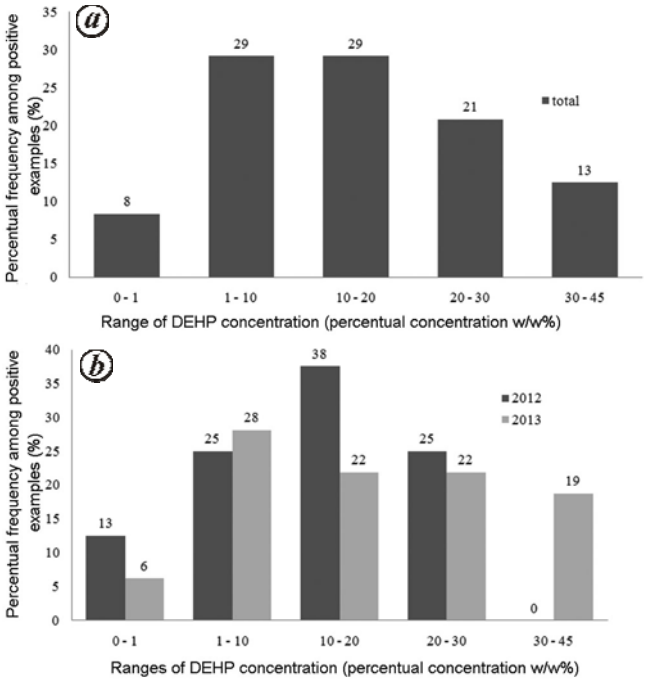

Figure 4. ***a***, Percentage of products containg different DEHP concentrations (0–40% (w/w)). ***b***, The percentage (%) of products containg different DEHP concentrations (0–40% (w/w)) analysed separately by year of import (2012 and 2013).

max and SD), and none of the samples contained BBP. Figure 4 shows products (expressed as percentage of phthalate positive products) containing DEHP expressed as concentration percentage weight (w/w) range (0–1%, 1–10%, 10–20%, 20–30% and 30–45%). Results show that over 60% of the positive samples contained more than 10% (w/w) of DEHP. Among the products collected in 2012 none contained over 30% (w/w) of DEHP, while among products collected in 2013, 19% contained high concentrations of DEHP (between 30 and 45% (w/w)). Correlation analysis did not show any statistically significant connection; thus it is not presented here.

The results of the present study show that phthalates were, present to a great extent, in the market, at the time when the Croatian legislative and control was adopting and introducing the legislative of the EU. The EU limited the use of DEHP, DBP and BBP in children's toys and childcare articles to a maximum percentage concentration of 0.1% (w/w) and restricted the use of DINP, DIDP and DNOP in toys and childcare articles which can be placed in the mouth. It was expected that the importers and vendors in Croatia would reduce the presence of toys containing the phthalates due to the strict EU regulation. However the results reveal the opposite. Out of 60% of the toys in the market that contain plastic, approximately 12–20% had detectible levels of phthalate.

In similar EU studies, conducted in Germany, Austria and Switzerland, higher presence of phthalates (27%) in the analysed samples has been reported, with 8% being articles for children[27]. However, in the case of childcare articles designed for chewing (bottles, baby soother, etc.) for children under 3 years of age, phthalate was not recorded in our study. We found that among positive samples majority contained DEHP (96%; Figure 3 *c*). Over 60% contained DEHP in concentrations more than 10 times greater than that permitted (Figure 4); mostly dolls and toy animals, with an average value more than 100 times greater than the allowed percentage concentration of 0.1% (w/w). Similar results were obtained in a study conducted in India, which also reported DEHP in 96% of products made in China, Taiwan, Thailand and India[28,29]. The maximal detected DEHP concentration was 45% (w/w), which is 450 times greater than that allowed (Table 4). Since DEHP synthesis in 1993, toxicological studies have shown its toxic effects on the liver, induction of testicular atrophy[30,31], on androgynous signalling pathway[32], premature mammary growth in females[33]; and it has also been added to a list of carcinogens according to Annex I of the Council Directive 67/548 (refs 34 and 35).

The second most commonly detected phthalate above the highest permissible value was DINP found in 41.7% of the samples with concentration ranging from 0.02% to 40% (w/w) (Figure 3 *c* and Table 4). The second most common phthalate in toys and childcare articles in the Indian market was also DINP (42% of the samples)[28], ranging from 0.1% to 16.2% (w/w). Even though Indian and Croatian studies detected DINP as the second most abundant phthalate type, other studies have shown showed DINP as the most abundant in child products, followed by DEHP, DIOP and DIDP[2,27–29]. Samples of dolls from a study conducted by Biedermann-Brem *et al.*[27] showed that DINCH (diisononyl-cyclohexane-1,2-dicarboxylate) was the most abundant phthalate in dolls and articles for children. In the present study (Figure 3 *c* and Table 4), the third most common phthalate was DBP, followed by DIDP and DNOP, with 2% of positive samples, maximal concentration being 0.53% (w/w) and 0.08% (w/w) respectively. None of the samples contained BBP. DBP, DNOP and BBP were detected in relatively low concentrations, while DBP and BBP were present in less than 1% of the samples. Therefore it is considered that they are by-products of technological procedure and not intentionally added[2,27–29]. The literature revealed that China manufactured the largest number of such toys[2,27–29,35].

From the present study we have gained important data for risk assessment of exposure of children to phthalates from toys in 2012 and 2013, prior to the introduction of legislative of the EU. Based on these data it could be estimated that there is a high possibility that among 20 toys bought in 2012 and 2013, especially plastic dolls, four could contain DEHP while 2–3 would have high probability of containing DEHP in concentrations 100–400 times greater than that allowed. Thus, we cannot

Table 4. Detected phthalates and their concentration expressed as per cent weight unit (%w/w) found in the analysed products

Phtalate type	CAS no.	LOD% (w/w)		Min % (w/w)	Max % (w/w)	Median % (w/w)	Average % (w/w)	SD
Bis(2-ethylhexyl) phthalate	117-81-7	≤0.005	Total sample	0.31	45.00	13.00	15.99	11.48
			2012	1.15	26.00	12.00	11.89	7.50
			2013	0.31	45.00	17.00	17.59	12.90
Dibutyl phthalate	84-74-2	≤0.005	Total sample	0.02	5.30	0.23	0.86	1.60
			2012	0.02	0.26	0.01	0.04	0.07
			2013	0.23	5.30	0.60	1.76	2.06
Benzyl butyl phthalate	85-68-7	≤0.005	Total sample	n.d.	n.d.	n.d.	n.d.	n.d.
			2012	n.d.	n.d.	n.d.	n.d.	n.d.
			2013	n.d.	n.d.	n.d.	n.d.	n.d.
Di-'isononyl' phthalate	68515-48-0	≤0.01	Total sample	0.02	40.00	0.60	0.00	8.97
			2012	0.02	40.00	0.01	4.16	10.79
			2013	0.19	2.41	0.60	0.76	0.63
Di-*n*-octyl phthalate	117-84-0	≤0.005	Total sample	0.08	0.08	n.a.	n.a.	n.a.
			2012	0.08	0.53	n.a.	n.a.	n.a.
			2013	n.d.	n.d.	n.d.	n.d.	n.d.
Di-'isodecyl' phthalate	26761-40-0	≤0.01	Total sample	0.53	0.53	n.a.	n.a.	n.a.
			2012	n.d.	n.d.	n.d.	n.d.	n.d.
			2013	0.53	0.53	0.53	n.a.	n.a.

LOD, Limit of detection = 0.1%; n.d., Not detected (less than LOD); n.a., Not applicable.

totally disregard or neglect the risk of exposure, especially considering that some toys are used for a number of consecutive years by children. On the basis of this information further studies on exposure are needed. Nevertheless, the percentage of 12–20% of products containing phthalates is expected to be lower since they are restricted for their toxicity.

We conclude that phthalates are still being used in the manufacture of toys and other articles endangering the health of children. Considering the fact that the global toy market is worth US$ 105 billon[28], it is clear that the issue exceeds health standards of individual countries. Hence, studies on the harmful effects of phthalates and their presence in objects of common use, as well as exposure to phthalates of risk groups should be done in all countries with and legislative control the countries of manufacture in order to minimize this global health problem, especially considering the health of children.

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Received 15 January 2015; revised accepted 13 June 2015

doi: 10.18520/v109/i8/1480-1486

Biogeochemistry of shallow lake sediments: a case study from Verlorenvlei, South Africa

Supriyo Kumar Das[1,*], Joyanto Routh[2] and Alakendra N. Roychoudhury[3]

[1]Department of Geology, Presidency University, 86/1 College Street, Kolkata 700 073, India
[2]Department of Water and Environmental Studies, Linköping University, 581 83 Linköping, Sweden
[3]Department of Earth Sciences, Stellenbosch University, Private Bag X1, Maiteland 7602, South Africa

Studying the biogeochemistry of shallow lake sediments, especially the source of sedimentary organic matter (OM), is challenging because of the low preservation of OM in shallow lake sediments. Here we report the source of sedimentary OM in a shallow freshwater lake, Verlorenvlei, in South Africa using a number of biogeochemical proxies. Elemental carbon and nitrogen ratio (C/N), and bulk stable C and N isotopes (δ^{13}C and δ^{15}N) indicate algal source of the sedimentary OM. Total organic and inorganic C, different phosphorus fractions, δ^{13}C and δ^{15}N values indicate repetitive presence of non-N-fixing cyanobacteria under moderate N-limited conditions. Cyanobacterial population in Verlorenvlei may be influenced by the availability of dissolved inorganic C. Cyanobacterial proliferation in the lake has ceased with accelerated N input as recorded at the top of the core.

Keywords: Carbon, cyanobacteria, nitrogen, organic phosphorus, shallow lakes, stable isotopes.

PRIMARY production is a major biotic process in the lakes. In-lake primary production responds quickly under stresses induced by anthropogenic activities such as external nutrients (nitrogen and phosphorus) input from

*For correspondence. (e-mail: das.supriyo.kumar@gmail.com)

catchments[1–3]. Limitation of one nutrient over another can favour certain plankton species to dominate over the rest[1]. Nitrogen limitation, for example, can create suitable conditions for cyanobacterial proliferation[1]. To understand and predict the response of lake ecosystem to various catchment activities, and for effective management of a lake, a broader understanding about the lake primary productivity is necessary. This has made primary productivity studies using lake sediments a major research focus since the past several decades[2,4,5]. However, such studies have mainly been conducted in deep and seasonally stratified lakes where prevailing anoxic conditions effectively preserve organic geochemical record[4–6]. Shallow lakes, in contrast, offer considerable challenges due to the rapid degradation of organic matter (OM) in the oxic water column and at the sediment–water interface[7]. The main objectives of the present study were to investigate the sources of sedimentary OM in a shallow lake, Verlorenvlei, South Africa with a focus on the variation in primary production using biogeochemical proxies such as total organic carbon (TOC), total inorganic carbon (TIC), N concentrations, different phosphorus (P) fractions, elemental ratios (C/N and N/P) and stable isotopes of C and N (δ^{13}C and δ^{15}N).

The coastal lake Verlorenvlei is located near Eland's Bay approximately 180 km north of Cape Town, South Africa (Figure 1). It is one of the largest (13 km long and 1.5 km wide; catchment 1890 sq. km) shallow (mean depth 2.5 m, maximum depth 5 m), natural lakes and one of the few freshwater coastal lakes in South Africa that provides valuable freshwater resource in a marginal semi-arid coastal landscape[8]. The lake encompasses a mesotrophic freshwater system, a reed swamp and a small estuary that connects it to the sea under occasional extreme storm conditions. The lake is situated in the subtropical Mediterranean winter rainfall climate zone, although the catchment area is semi-arid to sub-arid in nature (annual average rainfall <300 mm year^{-1})[9]. An unnamed river and its tributaries feed the lake during winter and early summer rainfall. The lake experiences free water circulation and water temperature varies from 11°C to 25°C (Table 1). The catchment comprises privately owned farmlands and natural grazing lands for sheep and cattle. Extensive ploughing occurs in the area, especially on the hills south of Verlorenvlei, and the lake is reportedly threatened by the over-exploitation of surface and groundwater by accelerated potato farming[10].

Unconsolidated and low-lying sand (Tertiary to Recent), which overlies shales of Malmesbury Group, dominates the regional geology of Verlorenvlei. Sandstone outcrops of the Table Mountain Group, fine-grained rocks of the Malmesbury Group and shales of the Klipheuwel Formation are also present within the catchment[11,12].

Verlorenvlei is famous for its avian diversity, and has high botanical importance due to its unique position at the transition between karroid and fynbos vegetation types[8,11]. Marshlands are present along the main river and its tributaries. Extensive beds of emergent aquatic macrophyte and dense reed are present along the margins of the lake. Large masses of filamentous green algae (*Chaetmorpha* and *Cladophora*) and spring cyanobacterial (*Microcystis aeruginosa*) bloom have been reported to occur in the lake[9].

For the present analysis, a 21.5 cm long sediment core from the deepest section of the lake was collected by pushing a plexiglass tube through the water column (<1 m) into the sediments (Figure 1). The tube was carefully pulled out without disturbing the upper layers. The core was transported on ice to the laboratory where it was sectioned at 2 cm intervals and the subsamples were freeze-dried. We did not find any visible evidence of bioturbation (e.g. burrows, chironomid mounds and/or epibenthic fauna) in the sediments.

Water was collected in austral winter (January) and summer (September) during a single year from the middle of the water column. All sampling bottles were rinsed at least three times with lake water before collection of the water sample. For dissolved inorganic carbon (DIC)

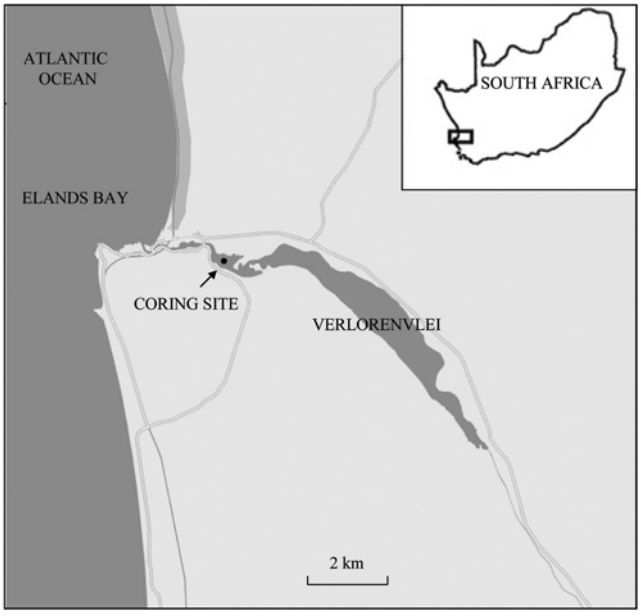

Figure 1. Location of Verlorenvlei in the Western Cape of South Africa and sampling site in the lake.

Table 1. Seasonal variations in physical and chemical characteristics in Verlorenvlei water column

	Temperature (°C)	Dissolved oxygen	pH	DIC (mg/l)
Summer	25.3	13	8.6	26
Winter	11.7	13	7.7	3

DIC, Dissolved inorganic carbon.

analysis, water samples were filtered using 0.45 μm Sarstedt nonpyrogenic sterile filters immediately after collection, and stored at 4°C until analysis. The filters were cleaned with deionized distilled water before use. The pH, dissolved oxygen and temperature were measured during sampling using a WTW® multi-parameter meter and associated electrodes. Each electrode was calibrated prior to the measurements.

All sample containers and laboratory glassware were pre-cleaned with P-free detergent, soaked for 24 h in HNO_3 (for P analysis) followed by rinsing with deionized distilled water. Acids, reagents and standard solutions used during the sampling, digestion and analyses were of suprapure grade.

DIC representing dissolved carbonate was analysed on a Shimadzu TOC-5000 analyser using the TOC–IC mode[13]. Sample reproducibility of duplicate runs was approx. ± 10%.

Total C (TC), TOC and N contents were measured using a LECO 932 CHNS elemental analyser (detection limit 100 μg C and precision ± 0.2%). Analyses of six replicates of internal standards provided coefficient of variation of 0.3 and 0.1 for C and N respectively. Sediments were treated with HCl to remove carbonates before analysing the TOC content. TIC represents the carbonate fraction in sediments and it was calculated by subtracting TOC from TC values[14].

The $\delta^{13}C_{organic}$ in the acid-treated and freeze-dried sediments, and $\delta^{15}N_{total}$ compositions of the freeze-dried sediments were determined using a Carlo Erba 2500 elemental analyser coupled to a Finnigan MAT Delta Plus mass spectrometer. Results were expressed in the conventional delta (δ) per mille notation (‰), using the international Vienna PeeDee Belemnite limestone standard (V-PDB) for organic C and atmospheric N_2 for total N. The results of the C and N isotope analysis are expressed according to the following formula

$$\delta\,(‰) = [(R_{sample}/R_{standard}) - 1] \times 1000, \quad (1)$$

where R_{sample} for C is the $^{13}C/^{12}C$ ratio of the sample and $R_{standard}$ is the $^{13}C/^{12}C$ ratio of VPDB. For N, R_{sample} represents the $^{15}N/^{14}N$ ratio of the sample and $R_{standard}$ is the $^{15}N/^{14}N$ ratio of atmospheric N_2. The precision for C and N isotope analyses was ± 0.18‰ and ± 0.06‰ respectively. Reproducibility of duplicate analyses was ± 0.1‰ and the standard deviation was <1‰.

The fractional composition of P in the freeze-dried sediments was determined by following a sequential extraction scheme that divided the sedimentary P fraction into two pools[15]: (i) HCl-P representing the chemical precipitate as well as adsorbed and detrital form of refractory P fraction bound to Ca (mainly apatite), and (ii) organic P (OP) representing the P fraction associated with OM. The process is described in detail by Ruban *et al.*[15]. The different P fractions were analysed colorimetrically on a Hitachi U-1100 spectrophotometer using a mixed reagent (5 N sulphuric acid, ammonium molybdate, 0.1 M ascorbic acid and potassium antimonyl tartrate), and following the method of Murphy and Riley[16] (absorbance measured at 880 nm). Typical precision of the duplicate run was <10%. The concentration (C in mg/g) of P (dry mass) was calculated using the following equation

$$C = \frac{SV}{1000\,M}. \quad (2)$$

where S is the concentration (mg/l) of P in the extract, V the volume of the reagent used for extraction (20 ml) and M is the mass of the test sample (200 mg dry mass).

Table 1 presents the water chemistry of Verlorenvlei. The lake water pH was relatively high and the lake remained oxic throughout the year[9]. DIC noticeably varied between the winter and summer seasons (Table 1).

TOC concentration (by dry weight) was 0.5–1.1% from the bottom of the core up to 8 cm (Figure 2). TOC was 0.3% between 8 and 4 cm, but the concentration increased above 4 cm and peaked to nearly 4% near the top of the core. TIC displayed lower values ((0.2–0.3%) at 16–18 cm and 0.3–0.4% (mean) at 10–12 cm; Figure 2) between peaks at 14 (0.8%) and 8 cm (1.1%). TIC was lowest at 6 cm (0.08%), but increased upwards to approx. 0.9% in surface sediments. Nitrogen concentration showed little variation and was approx. 0.1% from the bottom of the core up to 8 cm. It was lowest (0.02%) at 6 cm and then increased upwards to reach the highest concentration (0.5%) in surface sediments (Figure 2). Atomic $C_{organic}/N_{total}$ ratio displayed alternate high (≥ 8) and low (~4) values from the bottom of the core up to 6 cm. The C/N ratio varied from 8 to 11 between 6 cm and top of the core (Figure 2). The mean TOC, TIC and N concentrations and C/N ratio in the sediment core were 1.27%, 0.47%, 0.17% and 7.75 respectively. The $\delta^{13}C$ value increased upwards from the bottom of the core (–21.6‰) up to 4 cm (–20.3‰), with lower value (–21.0‰) at 14 and 6 cm. The $\delta^{13}C$ decreased above 4 cm and fell to –21.4‰ at the top of the core. The $\delta^{15}N$ displayed relatively higher values (3.7‰) at 18, 14 and 2 cm and lowest value (3.3‰) in surface sediments. The mean $\delta^{13}C$ and $\delta^{15}N$ values were 3.6‰ and –22.1‰ respectively.

Figure 2 depicts the results of sequential extraction of P (OP and HCl-P). The concentration of OP gently increased from the bottom of the core (1.4 μmol/kg) up to 8 cm (2.2 μmol/kg). It dropped to 0.6 μmol/kg at 6 cm followed by a sharp increase to reach the highest value (6.8 μmol/kg) near the top of the core. HCl-P concentrations were low (≤0.2 μmol/kg) between the bottom of the core and 16 cm; above this the HCl-P fraction was absent, except in surface sediments where the concentration was 1.1 μmol/kg. Total N/OP (N/P) ratio ranged from 27 to

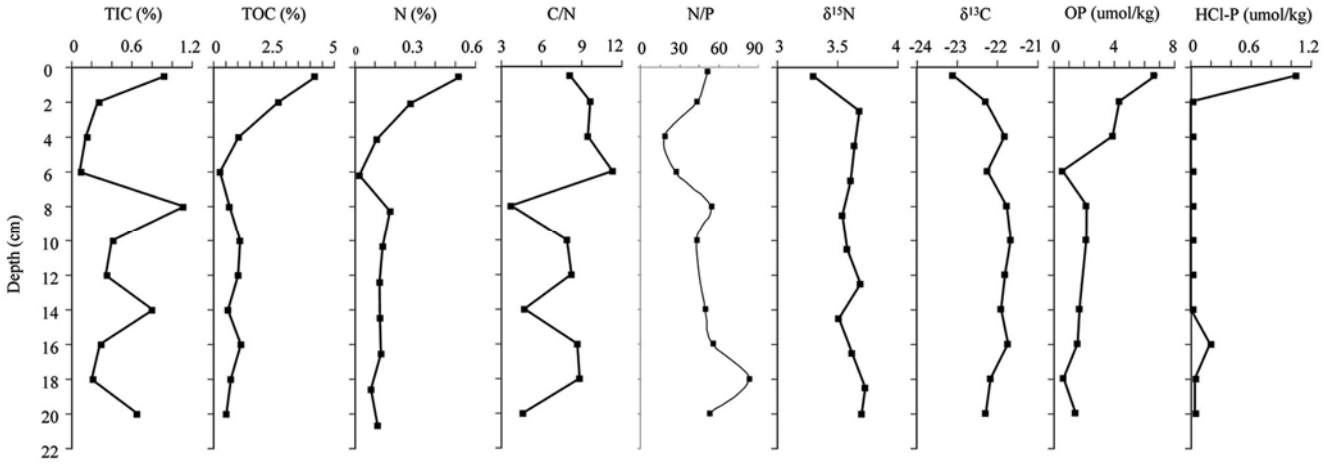

Figure 2. Depth variation of TIC, TOC, N, C/N and N/P ratios, $\delta^{15}N$, $\delta^{13}C$, OP and HCl-P fractions in Verlorenvlei sediments.

83, except at 4 cm depth where it fell to 19 (Figure 2). The mean OP, HCl-P concentrations and N/P ratio were 2.53 μmol/kg, 0.12 μmol/kg and 48 respectively.

TOC represents the fraction of OM that escapes remineralization during sedimentation[17]. The changes in sedimentary TOC concentration are widely used as a proxy to describe the abundance and preservation of sedimentary OM, and changes in lacustrine palaeoproductivity[2,5,18–20]. Similarly, atomic $C_{organic}/N_{total}$ ratio has been widely used to identify the sources of OM in lake sediments[4,21]. Organic matter derived from algae shows low C/N ratios (~4–10) due to their high protein content and absence of cellulose. In contrast, terrestrial plants are characterized by low protein and high cellulose content, and show high C/N ratios (≥20)[22,23].

In Verlorenvlei, the overall low TOC concentration from the bottom of the core up to 8 cm (Figure 2) indicates low supply of OM. This is followed by a sharp increase in TOC above 8 cm, showing an increase in OM supply. Typical $C_{organic}/N_{total}$ ratios (4–11; Figure 2) suggest algae as the dominant source of sedimentary OM. Syn- and post-depositional selective degradation of OM during early diagenesis could modify the C/N ratio of sedimentary OM[22]. However, a lack of systematic increase or decrease in the C/N ratio with depth implies that the ratio preserves the source signature of OM in Verlorenvlei. Our explanation is consistent with similar observations in sub-aqueous sediments[22]. The sedimentary OP fraction, which is higher than other P fractions (Figure 2), roughly follows the algal-derived TOC profile. This implies that the OP synthesis is controlled by in-lake primary production. Algal-derived OP is considered refractory because it consists of high-molecular-weight compounds[24]. Hence, similar trend of TOC and OP indicates that productivity record has not been affected by early diagenesis of sedimentary OM[25]. Moreover, the steep up-core increase in TOC and OP values above 5 cm depth suggests a progressively higher supply of in-lake algal-derived OM. Interestingly, the C/N ratio at 16–18, 10–12 and 2–6 cm falls in the range of moderate N-limited conditions (8.3–14.6)[26].

Algae remove dissolved CO_2 during photosynthesis. This process increases the pH and mediates $CaCO_3$ precipitation[27]. This makes the sedimentary record of TIC a useful proxy for palaeoproductivity in lakes[18,19,28]. Lowenstam[29] has reported that while all types of algae are capable of precipitating $CaCO_3$ during photosynthesis, cyanobacteria are not efficient in this process[30]. Therefore, the amount of $CaCO_3$ precipitated in sediments is not only a function of primary productivity, but also depends on the plankton species. Lowenstam's finding can be extended to Verlorenvlei, especially at depths (16–18, 10–12 and 2–6 cm), where the decline in TIC concentration (Figure 2) suggests a relative increase in cyanobacterial population over other algal species. Moderately low N/P ratios[31,32] (Figure 2) throughout the core and previously reported cyanobacterial blooms of *M. aeruginosa*[9] are consistent with our explanation[33]. HCl-P is commonly associated with aggregation of PO_4^{3-} on algal (non-cyanobacterial) $CaCO_3$ cells[18]; their absence below surface sediments indicates low precipitation of the Ca-bound P fraction due to an insignificant non-cyanobacterial algal population. This further supports our argument of cyanobacterial proliferation under N-limited conditions[30,34]. The dominance and repetition of non-N-fixing cyanobacteria can be explained in terms of gas vacuole synthesis and the availability of IC. Gas vacuoles in N-fixing cyanobacteria provide buoyancy and access to unlimited supply of atmospheric nitrogen[35,36]. However, gas vacuole synthesis depends on the availability of IC (ref. 37). Overall low DIC concentration (Table 1) in Verlorenvlei should limit the formation of gas vacuoles[36–38,39] and provide no advantage to N-fixing cyanobacteria. Our argument is supported by the reported blooms of non-N-fixing cyanobacteria, *M. aeruginosa*, in Verlorenvlei[9]. The increase in TIC and HCl-P concentrations in surface

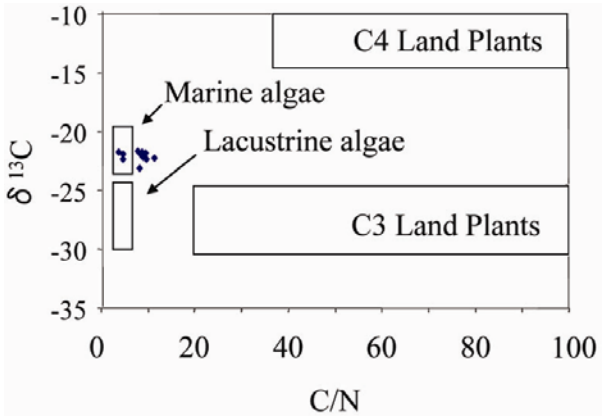

Figure 3. Relationship between C/N and $\delta^{13}C$ in Verlorenvlei sediments (after Meyers[22]).

sediments (Figure 2) most likely indicates an end in the repetitive cyanobacterial dominance in the lake.

Freshwater phytoplankton has typical $\delta^{13}C$ values between –20‰ and –30‰ (ref. 40). In contrast, OM produced by C_4 plants, growing either on land or at the lake bottom, has $\delta^{13}C$ values between –10‰ and –15‰ (refs 4 and 17). Moreover, $\delta^{13}C$ is relatively unaffected by post-photosynthetic processes[18,19]. These make $\delta^{13}C$ a popular proxy to trace OM sources and changes in the lake[18,19,41].

Stable organic carbon isotopic values (−22 to −21‰) in Verlorenvlei sediments (Figure 2) agree with the algal source character of OM as suggested by the C/N ratios, and indicate typical algal-dominated system. The relationship between C/N and $\delta^{13}C$ (Figure 3) suggesting marine algal-derived OM is justified due to the close proximity of the lake to the coast, and its occasional connection to the sea. The gradual upward increase of $\delta^{13}C$ from the bottom of the core implies a slow increase in primary productivity. Although reeds and emergent aquatic macrophytes (C_3 vascular plants) are abundant in the lake, we cannot determine their significance in the $\delta^{13}C$ budget of sedimentary OM because both macrophytes (−30‰ to −12‰)[42] and lacustrine algae use isotopically identical CO_2, present either in the atmosphere or dissolved in water[22,42,43].

Nitrate is the most common form of dissolved inorganic nitrogen (DIN) used by non-N-fixing species, whereas N-fixing cyanobacteria utilize N from atmospheric nitrogen. The typical (7–10‰) higher $\delta^{15}N$ value of dissolved NO_3^-, in comparison to atmospheric N_2, helps distinguish the N-fixing cyanobacterial source of sedimentary OM[4]. The observed $\delta^{15}N$ values (3.3–3.7‰; Figure 2) that lie outside the range of atmospheric N-fixation ($\delta^{15}N$ typically = 0 ± 2‰)[44,45] represents non-N-fixing species ($\delta^{15}N$ typically >2‰)[2,43,45,46] as a source of OM in Verlorenvlei. This finding is consistent with our earlier interpretation. The fall in $\delta^{15}N$ values above 18 cm most likely indicates the initiation of non-N-fixing cyanobacterial proliferation. This change might have been triggered by the decrease in N/P ratio at these depths (Figure 2). However, the sudden drop of $\delta^{15}N$ in surface sediments is caused by the supply of $\delta^{15}N$-depleted fertilizers from agricultural run-off[47].

Primary producers are the main source of sedimentary OM in Verlorenvlei. The lake suffers from a moderate N-limited condition where non-N-fixing cyanobacteria show a repetitive proliferation depending on the relative availability of DIC. Diminished cyanobacterial dominance recorded in surface sediments is caused by increased N input from the catchment.

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ACKNOWLEDGEMENTS. We acknowledge the financial support from SMF. We thank Helge Ax : Son Johnsons stiftelse, SIDA-NRF and VR for this study; Heather Sessions, Geof Bailey (Marine and Coastal Management, South Africa), and Heike Siegmund for help with sample analyses. Claire Schelske reviewed an earlier version of the manuscript.

Received 29 August 2014; revised accepted 20 July 2015

doi: 10.18520/v109/i8/1486-1491

Linking critical patches of sloth bear *Melursus ursinus* for their conservation in Meghamalai hills, Western Ghats, India

Santhanakrishnan Babu[1,2], Tamilarasu Karthik[2], Gopalakrishnan Srinivas[1,2] and Honnavalli Nagaraj Kumara[1,*]

[1]Sálim Ali Centre for Ornithology and Natural History, Anaikatty, Coimbatore 641 108, India
[2]Wildlife Information Liaison Development Society, 96, Kumudham Nagar, Vilankurichi Road, Coimbatore 641 035, India

The study examines the distribution and factors regulating the sloth bear in a fragmented hills of Meghamalai in Western Ghats through occupancy framework. Indirect evidences were sought over 133 grids of 4 sq. km size between December 2011 and December 2012. Indirect evidences were recorded in 58 of 133 sampled grids that estimated naïve occupancy of 0.43 ($p = 0.3180$). Understorey cover negatively affected the detection of indirect evidences. The parsimonious model contained three covariates, viz. tree height, grass cover and anthropogenic disturbance. Overall occupancy of bear was 0.54, which 25% higher than the naïve occupancy estimate. Study predicted higher proportion of evergreen forests with intermediate grass cover and less extent of disturbance determines the occupancy of bear in these hills. The 'critical link' connecting Periyar–Agastiyamalai hills and Anamalai hills is still active and supports high suitable sites for bears. It is suggested that Ammagajam–Upper Manalar Contiguity and Critical Link needs to be protected by appending with Meghamalai Wildlife Sanctuary.

Keywords: Conservation, critical patches, sloth bear, surrogate species.

SLOTH bear (*Melursus ursinus*) is one of the four species of bears found in India (the others being sun bear *Helarctos malayanus*, brown bear *Ursus arctos isabellinus* and Asiatic black bear *U. thibetanus*)[1,2]. High depiction in the country, four out of eight bears of the world, is attributed to India's geographic location at the junction of the Palaearctic and Indo-Malayan biogeographic zones[1]. The reportedly stable estimated population of 6000–11,000 adult bears is spread over in 174 Protected Areas (PAs) in India[3]. However, populations that persist outside the PAs are alleged to be highly vulnerable to human pressure[4]. In addition to the reduction in its habitat, the bear faces severe anthropogenic pressure due to poaching for its gall bladder, fat, meat and skin[5]. Crop raiding and conflict with humans by bears are attributed to loss of their habitat across their range[6]. Considering the rapid disappearance of their natural habitat, the IUCN has categorized sloth bears as 'Vulnerable'[5]. Among the large carnivores, sloth bear has relatively high density in the southern Western Ghats and occupies a wide array of natural forests. Sloth bear, being a large omnivorous animal showing a high adaptability to live in all the altitudinal gradients and vegetation types in the Western Ghats[7], is the best surrogate species to address the conservation issues.

In the southern Western Ghats, the Periyar–Agastiyamalai and Anamalai corridors have been degraded due to heavy exploitation of forests. However, both corridors are connected with Meghamalai hills through a narrow strip of forest, which has been considered as a 'critical link' by Critical Ecosystem Partnership Fund-Western Ghats profile[8]. A portion of the Meghamalai hills was declared as a PA, and the remaining area in the hills was not assessed properly for conservation. Further, high density of humans, developmental activities and croplands make this mountain range more vulnerable. In this context, the sloth bear can act as a surrogate species for the conservation of this hill and the critical link. The present study explores the factors that determine the occurrence of sloth bear in Meghamalai hills and usage of the critical link, which would help in prioritizing the area for conservation.

Meghamalai is located in the Theni Forest Division of Tamil Nadu (Figure 1). A wide range of altitudinal gradients (220–2000 m amsl) and varied rainfall pattern form an array of vegetation types ranging from dry forests (thorn, deciduous and savannah forests) to wet forests (evergreen and shola grasslands). Composition and configuration of the hills support diverse species of vertebrates, viz. 18 species of fishes[9], 35 amphibians[10], 90 reptiles[11], 254 birds[12] and 63 mammals. The mean annual rainfall of the lower and higher elevations of the study site is 1500 and 2161 mm respectively. Major rivers originating from these hills are the Vaigai and the Suruliar, on which five dams operate, viz. High Wavys, Manalar, Venniyar, Eravangalar and Shanmuganathi dams for producing electricity and for irrigation. The southern part of Meghamalai is a fragmented unit of the Periyar–Agastiyamalai corridor, connecting the PAs of Periyar Tiger Reserve in the west and Grizzled Squirrel Wildlife Sanctuary in the south, whereas the northern part of the forest division is connected to the Anamalai corridor.

Occupancy framework was followed to elucidate the factors that influence the occurrence of sloth bear in the study site. The estimated home range size of sloth bear in central India is reported to be 12 sq. km for a female and 85 sq. km for a male[7]; however, home range size for bears is not available for the Western Ghats. To accommodate the grids in the narrow patches of the forests in the critical link, we selected 4 sq. km as a minimum sampling unit to assess the occurrence of sloth bear on spatial scale. These spatial grids were overlaid on the base map

*For correspondence. (e-mail: honnavallik@gmail.com)

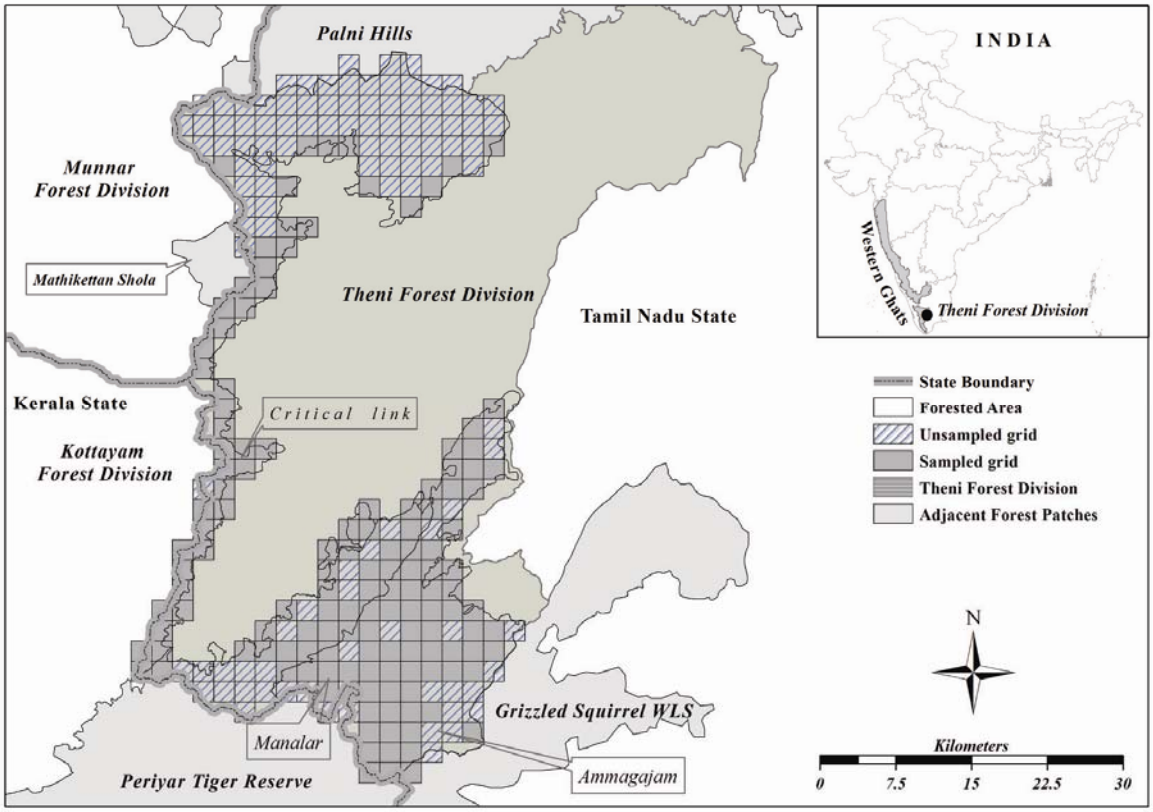

Figure 1. Map showing Meghamalai hills in the southern Western Ghats and design of sampling framework.

Table 1. Hypothetical assumption on the effect of each covariate on detection and occupancy of sloth bear

Covariates	ψ	P
Grass height	+	–
Grass cover	+	–
Weed cover	–	–
Weed height	–	–
Understorey cover	0	–
Understorey height	0	–
Tree height	+	0
CV_NDVI	–	0
Disturbance	–	0
Altitude	+	0
Slope	–	0

Positive and negative signs indicate positive and negative influences on the occupancy and detection probability respectively; 0 indicates no influence.

of Meghamalai hills. A total of 250 grid cells were established, of which we selected all 'complete' (more than 75% of the grid falls within the forest boundary) grid cells for sampling, i.e. 230 grid cells. However, due to inaccessible terrain in some of the grids, only 133 grid cells covering 532 sq. km (>70% of the total area) were sampled for the occurrence of bears. In each grid, existing trails or bearing fixed routes were used such that they represented all the habitat types of the entire grid. A minimum of 2.5 km was considered as sampling distance to cover the entire grid. Spatial replicate was followed to estimate the detection probability and occupancy[13]. Every 500 m length of walk in the grid was considered as a spatial replicate and thereby a minimum of five spatial replicates were undertaken in each grid. In each spatial replicate, every 100 m length of replicate was considered as a segment, where covariates for sampling were recorded. Occurrence of bear and other covariates was noted in all segments and replicates in a grid. Sloth bears are crepuscular/nocturnal in their activity; thus, their direct sightings are difficult during the day sampling. Therefore, the bear droppings were considered as an evidence of occurrence. To record the bear droppings, 2 m on both sides along the trail was considered as sampling area. During the walk, presence of bear droppings within the sampling area was recorded for each 100 m segment to construct the detection history. The sampling was done during December 2010–May 2011 and December 2011–May 2012.

Covariates that influence the detection and occupancy of bears were included as sampling and site covariates respectively. We hypothesized that increase in grass, weed and understorey cover would negatively influence the detection of bear droppings (Table 1). Tree density, tree height, tree cover, canopy contiguity, disturbance

Table 2. Details of habitat covariates used in modelling, occupancy, their mode of assessment, unit of measurement and scale of measure

Parameters	Mode of assessment	Unit of measurement	Scale	
			P	ψ
Altitude	GIS	Metres	–	Mean/grid
Slope	GIS	Degrees	–	Mean/grid
CV_NDVI	GIS	Variance	–	Mean/grid
Tree height	Visual	Metres	–	Mean/grid
Understorey height	Visual	Metres	Mean/replicate	Mean/grid
Understorey cover	Visual	Percentage	Mean/replicate	Mean/grid
Grass height	Visual	Metres	Mean/replicate	Mean/grid
Grass cover	Visual	Percentage	Mean/replicate	Mean/grid
Weed cover	Visual	Percentage	Mean/replicate	Mean/grid
Disturbance	Visual	Gradient	–	Mean/grid

CV_NDVI, Coefficient of variance of normalized difference vegetation index; GIS, Geographical information system.

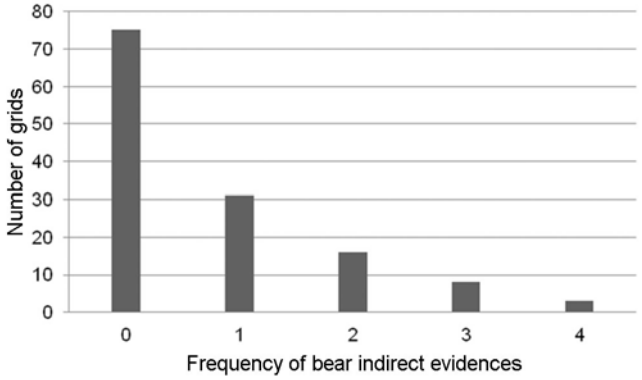

Figure 2. Frequency distribution of observed instances of indirect evidences of sloth bear in grids of Meghamalai hills.

index, altitude, slope, normalized difference in vegetation index (NDVI), and coefficient of variance of NDVI (CV_NDVI) were considered as site covariates.

A sampling point at every 100 m of replicate, thus five sampling points per replicate, was established to assess the habitat and sampling covariates. Point-centred quarter method[14] was followed to quantify tree characteristics. Distances of four nearest trees from four quarters with greater than 20 cm girth at breast height (GBH) were measured to estimate tree density. Remaining tree covariates (tree height, cover, contiguity) were measured qualitatively (Table 2). Five metre radius circular plot was established to quantify understorey characteristics. Tree height was measured in metres and tree and shrub cover as percentage. Disturbance in each replicate was coded in a gradient, ranging from 1 to 10 scoring for low to high anthropogenic disturbance. The presence of threats such as livestock grazing, illegal hunting, conflict with wild animals, non-timber forest produce (NTFP) collection, fuel wood, fodder extraction and presence of human settlements in each segment and the grids was coded as 1 and absence of the same as 0. We summed all values to get the overall anthropogenic pressure in each grid. SRTM images were used to extract altitude and slope values for each grid, while multi-dated satellite data of SPOT-VEGETATION were used for extracting NDVI and CV_NDVI. Mean NDVI was highly correlated with vegetation-related parameters and CV_NDVI, which indicate the degree of variation in NDVI, viz. low for evergreen forests and high for dry forests.

Bear droppings contributed to detection histories for each replicate, where '1' indicated detection of the animal, '0' indicated non-detection and '–' indicated a missing observation. We constructed detection histories for all the sampled grids. The two model parameters – probability that a grid is occupied by the species (ψ) and detection probability (P) were estimated using likelihood functions[15]. The program PRESENCE ver. 3.0 was used to derive maximum likelihood estimates of the model parameters. We cross-correlated these covariates to remove all the auto-correlated variables and correlation coefficient above 0.50 was set as the cut-off value for removing correlated variable. Thereby we retained ten uncorrelated covariates for further analysis (Table 1). Ground variables such as understorey, grass and weed were built-in into the model to estimate detection probability, while tree, remotely sensed covariates, understorey and disturbance variables were included for modelling occupancy. Considering previous publications regarding the covariates[4–7], 10 a priori models were developed to assess the relative influence of covariates on detection and occupancy of bear in each grid. Model selection, computation of model weights, and averaging of parameters followed the framework of Burnham and Anderson[16]. The effect of each covariate on occupancy and detection probability was evaluated using logistic model with logit link function. We calculated the model-averaged parameters using Akaike weights for proportion of sites used and detection probabilities. To infer the relative influence of each covariate on occurrence, model weights were

Table 3. Summary of factors affecting the detection of indirect evidences of sloth bear

Model	*P*	AIC	ΔAIC	w_i	K
ψ (.), *p* (USC)	0.3329	502.09	0.00	0.6068	2
ψ (.), *p* (.)	0.3180	503.05	0.96	0.3755	2
ψ (.), *p* (USH)	0.3663	509.20	7.11	0.0173	2
ψ (.), *p* (WH)	0.4051	517.06	14.97	0.0003	2
ψ (.), *p* (WC)	0.5000	525.52	23.43	0.000	2
ψ (.), *p* (GH)	0.5001	526.41	24.32	0.000	2

USC, Mean understorey cover; USH, Mean understorey height; WH, Mean weed height; WC, Mean weed cover and GH, Mean grass height.

Table 4. Summary statistics of candidate model performed and contribution of covariates for site occupancy (and SE) of sloth bear in Meghamalai hills, Western Ghats, India. Results are ranked based on AIC values of models with ΔAIC (AIC – min AIC), AIC model weight (w_i) and number of parameters (*k*)

Model	ψ	SE	AIC	ΔAIC	w_i	*k*
ψ (GC + TH + DIS), *p* (USC)	0.5478	0.0943	485.19	0.00	0.425	5
ψ (GC + GH + TH + DIS + WC + ALT + SLO), *p* (USC)	0.5464	0.1306	485.59	0.40	0.348	10
ψ (GH + GC + TH + DIS), *p* (USC)	0.5487	0.1036	487.12	1.94	0.161	6
ψ (GC + ALT + DIS), *p* (USC)	0.5536	0.0966	491.00	5.81	0.023	5
ψ (GH + TH + DIS), *p* (USC)	0.5435	0.0952	491.09	5.90	0.022	5
ψ (GC + CVN + DIS), *p* (USC)	0.5440	0.0967	492.63	7.44	0.010	5
ψ (WC + GC + DIS), *p* (USC)	0.5455	0.0970	493.18	7.99	0.008	5
ψ (GC + DIS), *p* (USC)	0.5492	0.0870	494.95	9.76	0.002	4
ψ (.), *p* (USC)	0.5370	0.0573	502.09	16.9	0.001	2
ψ (.), *p* (.)	0.5487	0.0612	503.05	17.8	0.001	2

GC, Mean grass cover; GH, Grass height; TH, Mean tree height; DIS, Cumulative anthropogenic disturbance in the grid; CVN, Coefficient of variance in NDVI; WC, Mean weed cover; ALT, Mean altitude; SLO, Mean slope and USC, Mean understorey cover.

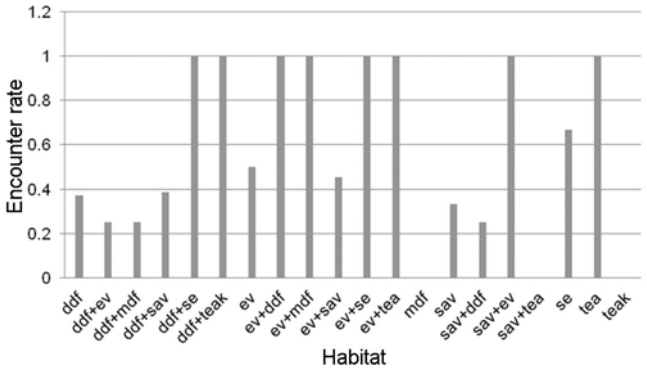

Figure 3. Naïve occupancy estimates and study efforts in different vegetation types in Meghamalai. ddf, Dry deciduous forest; mdf, Moist deciduous forest; sav, Savanna forest; se, Semi evergreen forests; ev, Evergreen forests; teak, Teak plantation and tea, Tea plantation.

summed over all models containing the particular covariate. The correlations of predicted proportion of sites occupied by species for each grid with certain tree and disturbance variables were tested using Pearson correlation. The naïve occupancy estimate was projected according to vegetation types (dry deciduous, moist deciduous, savanna, semi evergreen and evergreen forests, teak and tea plantations) and elevation.

Sloth bear was sighted during two occasions in the 586 km walk in 133 grids. The bear droppings were recorded in 58 grids (Figure 2) that provided the naïve occupancy estimate of 0.43, which varied across the grids with different vegetation types from 0.39 in open scrub forests to 0.80 in evergreen forests (Figure 3). Grids at high elevation had higher naïve occupancy estimate than those at lower elevation (high elevation – 0.8 droppings/grid; mid elevation – 0.44 droppings/grid; low elevation – 0.38 droppings/grid).

Among the six sampling covariates, detection probability (*P*) of bear droppings was influenced by understorey cover ($w_i = 0.6068$) and outperformed other candidate models (Table 3), including constant model $\psi(.)\ P(.)$. Increase in understorey cover decreased visibility of bear droppings ($\beta = -0.2530$; SE = 0.0533). The second best model was a constant model, and all subsequent models for occupancy (ψ) were performed with detection probability as a function of understorey cover. The global model (model fitted with maximum number of parameters) perfectly fitted the data (c-hat = 0.94, chi-square probability value = 0.60).

Constant model for occupancy, $\psi(.)\ P(.)$, performed poorly as evidenced by low AIC value in the summary statistics of the model (Table 4). Among other occupancy models, the most parsimonious model (ΔAIC = 0.00) included grass cover, tree height and disturbance. Grass cover ($\beta = 0.673$, SE = 0.281) and tree height ($\beta = 0.9484$, SE = 0.375) positively influenced occupancy

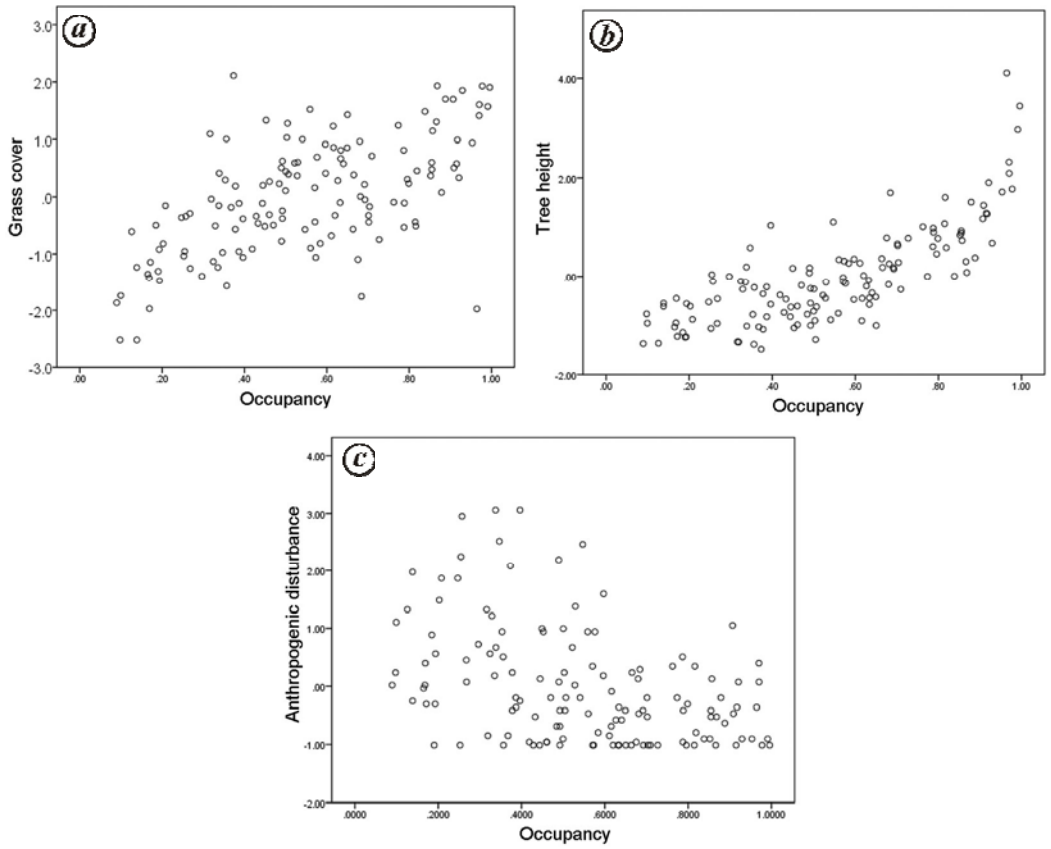

Figure 4. Relationship between estimated occupancy and site covariates: ***a***, grass cover; ***b***, tree height; ***c***, anthropogenic disturbance.

Table 5. Covariates affecting the distribution and habitat use of sloth bear in Meghamalai hills, ranked based on AIC weights (of all models) and with average β coefficient and standard errors (SE) (of top three models)

Covariate	Summed AIC weights	β coefficient	SE
Disturbance	0.998	–0.3588	0.2335
Grass cover	0.978	0.6730	0.2807
Tree height	0.956	0.9484	0.3751
Grass height	0.531	–0.0075	0.2520
CV_NDVI	0.358	–0.3834	0.2688
Altitude	0.371	0.7143	0.3458
Weed cover	0.356	0.4361	0.2592
Slope	0.348	–0.2337	0.3502

of the bears, while disturbance had a negative influence ($\beta = -0.358$, SE = 0.233; Table 5). Predicted proportion of sites occupied by the species in each grid was positively correlated with tree height ($r = 0.768$; $P = 0.000$) and grass cover ($r = 0.610$, $P = 0.000$), and negatively correlated with disturbance ($r = -0.422$, $P = 0.000$; Figure 4 *a*–*c*). Among the covariates, bear occupancy was strongly influenced by disturbance factors, which is evident from higher summed AIC model weights ($w_i = 0.998$) followed by grass cover and habitat related covariates (Table 5). The second parsimonious model (ΔAIC = 0.40) is a global model which contains all covariates considered for it. Grass height influenced the proportion of sites occupied by the bears along with other variables, but ranked third in position. None of the high-ranked models (ΔAIC ≥ 2) showed high AIC model weight; thus model averaging of occupancy and standard error was calculated[16]. The average occupancy estimate (0.5478, SE = 0.094) corresponds to a difference of 25% from the naïve estimate of occupancy (0.4361). Higher proportion of occupancy of sloth bear was estimated over critical link and the Meghamalai hills (Figure 5).

Sloth bear occupies a broad range of ecosystems, from dry plains to montane grasslands, decisively varying with temporal and spatial scales[17–19]. Principally being a lowland animal, it occurs at 2000 m altitude in the Western Ghats[1]. Though, the rate of occupancy varies across altitudinal gradients, the bear was recorded from low-altitude scrub forests to high-altitude evergreen forests of the Meghamalai hills. Estimated occupancy for sloth bear in Meghamalai hills was 0.54 with $P = 0.33$, which is comparatively lesser than Mudumalai ($P = 0.23$; $\psi = 0.83$)[20] and Daroji ($P = 0.46$; $\psi = 0.78$)[21]. Low occupancy in Meghamalai hills may be due to the inherent low density of bears.

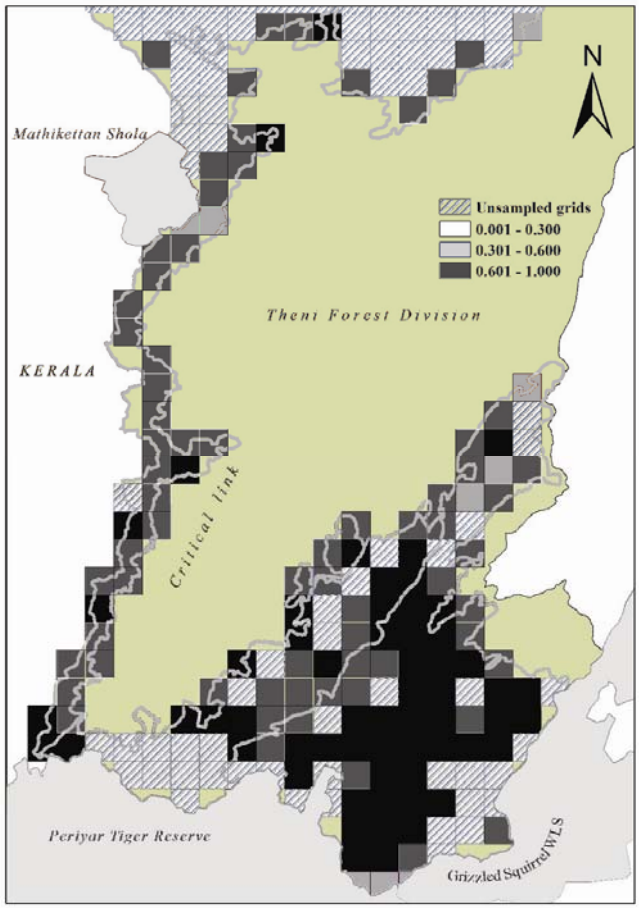

Figure 5. Predicted estimates of occupancy of sloth bear within the sampling framework of Meghamalai hills.

Among the six sampling covariates, understorey cover negatively influenced the detection of droppings. Similarly, a study in Bandipur Tiger Reserve[22] reported the negative influence of understorey in detecting the pellets of four-horned antelope, *Tetracerus quadricornis*. The habitat covariates broadly corroborate with two life-history characteristics of bears, i.e. habitat devoid of human disturbance for roosting (evergreen with less disturbance) and an imperative need for foraging ground (grass land). Grasslands being a major source of ants and termites, reported as important foraging ground for bears across India[23,24] and Chitwan NP in Nepal[25], thus positively influence the proportion of sites occupied by bears in Meghamalai hills.

The evergreen forest is one of the major determinants (higher tree height) of occupancy of bears in Meghamalai hills, which is contrary to the findings from Mudumalai[20–23] and Parambikulam[26], where bear occurrence was higher in deciduous forests with tall grasses. The sampling period of the present study coincides with other studies; thus the influence of study time can be ignored here. Higher estimates of occupancy in evergreen forests can be attributed to: (1) the availability of fruits throughout the year in evergreen forests than in dry forests; (2) dry forests in Varusanad and Meghamalai experience tremendous anthropogenic pressure that might have pushed the bears towards higher elevation; (3) *Zizyphus* spp. is reported[19] as the major food plant of bears in deciduous forests, which may have been suppressed due to heavy *Lantana camara* infestation in the deciduous forests[27].

Occupancy of sloth bear was higher in the reserve forests of Meghamalai hills, which is highly vulnerable to anthropogenic pressure. Natural forests encircled with plantations in the Ammagajam – Upper Mannalar not only show high occupancy of sloth bears, but also hold large populations of endangered lion-tailed macaque[28] and Nilgiri tahr[13], and facilitate movement of mammals between Grizzled Squirrel Wildlife Sanctuary, Periyar Tiger Reserve and Meghamalai Wildlife Sanctuary. Thus, these forest patches need to be brought under the PA network to enhance the protection of these animals.

In addition to high occupancy of sloth bears in the critical link, it also supports the occurrence and movement of many large mammals between Periyar–Agastiyamalai and Anamalai corridors. In spite of its high biodiversity value, the link also endures severe anthropogenic pressures and developmental activities such as new roads, hydro-electric projects, and existing inter-state highways. Considering the crucial biodiversity value and surging anthropogenic pressure of the link, the area requires immediate conservation attention. We suggest that further development projects should be avoided and the critical link should be included with the Meghamalai Wildlife Sanctuary.

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ACKNOWLEDGEMENTS. We thank CEPF-ATREE and Rufford Small Grant for the financial support; Tamil Nadu Forest Department for research permission and the Directors of SACON and WILDs for administrative support. We also thank B. Shanthakumar, B. M. Krishnakumar and T. Yesudas for help.

Received 31 March 2015; revised accepted 23 June 2015

doi: 10.18520/v109/i8/1492-1498